The Amino Acid-fermenting Clostridia

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SUMMARY

Representative strains of 30 named species of clostridia were tested for their ability to grow in a semidefined medium containing either 1% (w/v) glucose or 3% (w/v) acid-hydrolysed casein as an energy source. Strains from 15 species were selected empirically as amino acid-fermenting types by comparing growth in the two media. After growth in 3% casein hydrolysate medium the organisms were removed and their amino acid utilization determined by examining the culture supernatants. The organisms were then divided into four main groups. The characteristic features of each group are as follows: group I, the utilization of proline with the production of 6-amino valeric acid; group II, mainly arginine and/or glycine utilized; group III, the utilization of glutamic acid, serine and histidine; group IV, serine and threonine utilized. Possible implications of these findings for the taxonomy of clostridia are discussed.

INTRODUCTION

The genus Clostridium Prazmowski includes organisms with widely differing catabolic pathways. The so-called putrefactive or proteolytic clostridia are able to use single amino acids or certain groups of amino acids as sources of energy and are able to grow anaerobically in media containing only amino acids and growth factors. (Fildes & Knight, 1933; Knight & Fildes, 1933; Stickland, 1934; Fildes, 1935; Fildes & Richardson, 1935; Gladstone, Fildes & Richardson, 1935; Woods, 1936; Barker, 1937; Woods & Clifton, 1937; Clifton, 1939, 1940, 1942; Cardon & Barker, 1947.) In the light of his studies Barker (1939) suggested that: 'In the future the anaerobic decomposition of specific nitrogenous compounds will be of the same importance for the classification of putrefactive anaerobes as is the fermentation of carbohydrates at present for the classification of saccharolytic organisms.' In the intervening 30 years no systematic comparative study of amino acid catabolism in the clostridia appears to have been made, although a few strains were included by Proom & Woiwod (1949) and Woiwod & Proom (1950) in their studies of the composition of culture filtrates of various bacterial genera.

This paper describes an investigation into the utilization of amino acids by clostridia growing anaerobically in a medium consisting of acid-hydrolysed casein supplemented with cysteine, tryptophan and growth factors. Characteristic patterns of amino acid utilization have been observed which may assist the identification and classification of putrefactive clostridia.

METHODS

Organisms. The strains used and the sources were as follows: Clostridium acetobutylicum, NCIB8049, 8052, 8653; C. acidi-urici, strain 1 (S. R. Elsden, Food Research Institute, Norwich), strain 2 (G. Hobbs, Torry Research Station, Aberdeen); C. beijerinckii, NCIB9362;
C. bifcritermants, NC1B506, 1341, 2912; s3, s17, s20, s22 (freshly isolated from soil); CR (W. M. Waite, Food Research Institute, Norwich); C. butyricum type A, NCTC887, 2012; type B, NCTC751, 3807; 178 (T. A. Roberts, Meat Research Institute, Bristol); type C, C9, C21 (T. A. Roberts); type E, NCTC8550, 4318/63 (T. A. Roberts); type F, 202F (T. A. Roberts); C. butyricum, NCB17423; C. caloritolerans, NCB19360; C. chauvoei, CC4 (C. L. Oakley, School of Medicine, Leeds); CN5231 (P. D. Walker, Wellcome Research Laboratories, Beckenham, Kent), NCTC8070; C. cochlearium, NCB16797; 4721 (M. Sebald, Institut Pasteur, Paris, France); ATCC17787; C. difficultor, 871 (L. S. McClung, Indiana Univ., Bloomington, Ind., U.S.A.); C. fallax, NCTC8380; C. histolyticum, NCB1503; 10, 4105 (M. Sebald), CN950, 3267, 3300 (P. D. Walker); C. microsporum, 22, 25, 505 (H. Beerens, Institut Pasteur, Lille, France), 61 (E. M. Barnes, Food Research Institute, Norwich); C. cedematius type A, NCTC538; CN870 (P. D. Walker), CHX, DELHOMME (M. Sebald); C. pavanutricular, 6, 11, 16 (freshly isolated from the caecum of turkey pouls); C. pasteuriwm, ATCC6031; C. pectinovorum, NCB18564; C. putrefaciens, NCTC9836; C. putrificum, NCTC4718; C. septicum, NCTC504, 547, 550; C. sordelli, NCTC6927, 6929; C. sphenoides, 507 (M. Sebald); C. sporogenes, 9 (Food Research Institute Collection), NCTC532, NCB1853, 8243, 9381, 9383; C. sticklandi (T. C. Stadman, National Heart Institute, Bethesda, Md, U.S.A.); C. subterminale, NCB19384; C. tertium, NCB1541, 9363; C. tetani, NCTC279, 5404; CN879, CN1341 (P. D. Walker); 497, 2517, 3300, 3304 (L. S. McClung); C. tetanomorphum, NCTC500, 2909, ATCC3606; 252, 299 (L. S. McClung); C. tyrobutyricum, 3CL, B2-15 (M. E. Sharpe, National Institute of Research in Dairying, Reading, Berkshire); C. welchii, type A, NCTC6719, 8237; type B, NCTC3110; type C, NCTC3181; type D, NCTC8503; type E, NCTC8084; type F, NCTC8081.

Cultures were checked for purity on receipt and then subjected to a series of standard differential tests based on that used by Moore, Cato & Holdeman (1966). In most respects the majority of the organisms conformed with the characteristics of the designated species.

**Growth media.** The basal medium contained (per 1. distilled water): adenine, 100 μg.; guanine, 100 μg.; uracil, 100 μg.; thiamine, 500 μg.; riboflavin, 500 μg.; pyridoxal HCl, 250 μg.; pyridoxamine, 250 μg.; nicotinic acid, 5 mg.; calcium pantothenate, 1 mg.; biotin, 50 μg.; folic acid, 250 μg.; vitamin B12, 100 μg.; inositol, 16 mg.; p-aminobenzoic acid, 50 μg.; choline Cl, 200 mg.; nitritotriacetic acid, i-5 g.; MgSO₄.7H₂O, 100 mg.; MnSO₄.H₂O, 2-4 mg.; FeSO₄.7H₂O, 9-6 mg.; CaCl₂, 2-0 mg.; CoCl₂, 160 μg.; ZnSO₄.7H₂O, 1-6 mg.; CuSO₄.5H₂O, 0-9 mg.; Na₂MoO₄, 10 μg.; KH₂PO₄, 4-5 g.; tryptophan, 0-4 g.; cysteine HCl, 0-3 g.; Oxoid casein hydrolysate (acid), 2 g.; pH 7-2.

Amino acid utilization was studied using the basal medium supplemented with casein hydrolysate at a final level of 3% (w/v). The medium then contained 16 mg. total N (Kjeldahl method)/ml. and the level of amino N in different batches was checked routinely by the method of Pope & Stevens (1939) as modified by Schroeder, Kay & Mills (1950). Using the Volhard method a 3% solution of casein hydrolysate was found to contain 0-51% of Cl⁻. The medium was distributed in 15 ml. amounts in 19 x 150 mm. plastic-capped test tubes and autoclaved at 121° for 15 min.

For the glucose medium filter-sterilized glucose was added to the autoclaved basal medium to a level of 1% (w/v).

Just before use 0-03% sodium thioglycollate was added to all media. The tubes were then inoculated with 0-2 ml. of a fresh culture grown in either cooked-meat medium containing digest broth (Mackie & McCartney, 1960) and twice the usual quantity of meat, or the VL medium of Beerens, Schaffner, Guillaume & Castel (1963) as modified by Barnes & Impey (1968). Clostridium acidi-urici was grown in the medium of Barker & Beck (1942) with 0-5%
Difco yeast extract substituted for yeast autolysate. Inoculated tubes were incubated for up to 6 days at 25°, 30° or 37° under hydrogen plus 10% CO₂ in an anaerobic jar.

**Measurement of growth.** Growth was measured with an EEL nephelometer against an arbitrary standard opacity tube. Daily measurements were made until no further increases in turbidity were detected.

**Preparation of extracts.** Cultures grown in 3% casein hydrolysate medium were treated with 1 ml. of 20% (v/v) perchloric acid/10 ml. of culture. Each culture was centrifuged at 3000 g for 20 min. and the supernatant neutralized with 4 N-KOH to precipitate potassium perchlorate which was removed by centrifuging again.

**Chromatography.** The culture extract (40 μl.) was applied to a strip of Whatman no. 3 MM paper, 57 x 15 cm., moistened with formic acid/acetic acid/water buffer, pH 2.0 (Efron, 1960), and subjected to electrophoresis for 40 min. at 5 kV in a Locarte high voltage electrophoresis apparatus (Locarte Co., London S.W. 7). The paper was then dried, stitched to a wick and a sheet of 3 MM paper 46-5 x 41 cm., and developed by descending chromatography using n-butanol/n-butyl acetate/acetic acid/water, in the ratio 19: 1:5:25 (Richmond & Hartley, 1959). Chromatograms were run for 16 h. at 32°, dried and sprayed with ninhydrin to reveal the amino acids. Unknown spots from Clostridium sporogenes 532 were eluted and identified provisionally by chromatography; their identity was confirmed with a mass spectrometer.

**Amino acid analysis.** Quantitative analysis of extracts was made with a Beckman 120C amino acid analyser.

**RESULTS**

Amino acid-fermenting strains were selected by comparing growth in the basal medium containing 1% glucose with that in the medium containing 3% casein hydrolysate. The concentration of glucose was approximately 56 μmoles/ml.; that of the total amino acids in the casein hydrolysate medium was 101 μmoles/ml. The maximum amount of any one amino acid was, with the exception of glutamic acid, aspartic acid and proline, less than 10 μmoles/ml. Since individual strains would be unlikely to utilize all of the amino acids present it was decided to select for further study those organisms which attained a growth level in casein hydrolysate equivalent to at least one-third of that in the glucose medium. The organisms which were considered to be predominantly saccharolytic all grew well in the glucose medium but made little or no growth in 3% casein hydrolysate unless glucose was added. Furthermore, when glucose was added the amount of growth obtained was approximately proportional to the concentration of glucose. Of those examined the saccharolytic organisms were: Clostridium acetobutylicum; C. beijerinckii; C. botulinum 17B (type B), type E, type F; C. butyricum; C. chauvoei; C. fallax; C. microsporum 22, 505, 61; C. oedematiens; C. paraputrificum; C. pasteurianum; C. pectinovorum; C. septicum; C. sphenoides; C. tertium; C. tyrobutyricum, and C. welchii.

The two strains of Clostridium acidi-urici failed to grow in either of the test media; nor was growth obtained when the 3% casein hydrolysate medium was supplemented with 5 mg. L-serine/ml. (Serine is an intermediate in the degradation of purines by this organism and is deaminated and converted to pyruvate by intact cells; Sagers & Beck, 1956.) Both strains grew readily, however, when 0.3% uric acid was added to the medium.

Other organisms such as Clostridium kluyveri and C. propionicum with special growth requirements which were not provided by the test media were excluded from the present survey.

The remaining organisms fulfilled the proposed requirement for amino acid-fermenting...
types and were: Clostridium bifermentans; C. botulinum type A, type B, 751, 3807, type C; C. caloritolerans; C. cochlearium; C. difficile; C. histolyticum; C. microsporum 25; C. putrefaciens; C. putrificum; C. sordellii; C. sporogenes; C. sticklandii; C. subterminale; C. tetani and C. tetanomorphum. Levels of growth of some of these organisms in the test media and also in VL broth are shown in Table 1. In the case of C. bifermentans and C. sordellii the measurement of growth was hindered by the viscous, stringy nature of the organisms, especially in the 3% casein hydrolysate medium.

Table 1. Growth of representative strains of amino acid-fermenting clostridia in VL broth and media containing either glucose or casein hydrolysate as the source of energy

<table>
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<td>64</td>
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* 0.1% yeast extract added.
† Growth in Trypticase.
‡ 6% acid-hydrolysed casein.
§ 5 mg./ml. arginine added.

With some organisms a low level of growth was increased by modifying the medium. Increasing the concentration of casein hydrolysate to 6% was effective for Clostridium cochlearium 6797 and C. putrificum, but C. sticklandii required an unidentified peptide factor present in yeast extract for optimal growth (Stadtman, 1954), and 0.1% Difco yeast extract plus an additional 5 mg. arginine/ml. was necessary to obtain good growth with 3% casein hydrolysate. Clostridium histolyticum did not produce heavy growth in any of the test media, and attempts to increase growth significantly were unsuccessful when 3% casein hydrolysate was supplemented with additional quantities of amino acids either singly or in combination. Growth was markedly improved, however, by substituting 3% (w/v) BBL Trypticase for acid-hydrolysed casein. Trypticase at a level of 2% (w/v) supported better growth of C. botulinum type C than did acid-hydrolysed casein, and growth was further increased by the addition of 0.1% Difco yeast extract.

Amino acid utilization. Paper chromatograms of extracts showed highly characteristic
Table 2. Quantitative utilization of amino acids by representative strains of groups I to IV

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* Organism grown in 6% casein hydrolysate medium.

The amino acid-fermenting clostridia

51
patterns due to the specific utilization of certain amino acids and the production of new ninhydrin-positive compounds. The organisms were divided initially into two main groups depending upon the presence or absence of δ-amino valeric acid, and these groups were further subdivided on the basis of the patterns of amino acid utilization obtained. The results were confirmed and supplemented by quantitative data of which some examples are shown in Table 2.

The main distinguishing features of the groups were as follows:

**Group I.** δ-Amino valerate produced; proline utilized.

**Group IA.** Clostridium bifermantans, C. sordellii, C. sticklandii. All strains utilized serine and threonine. Valine and tyrosine were either produced or not utilized; most strains produced α-amino butyrate and γ-amino butyrate. Clostridium bifermantans (8 strains) utilized arginine and aspartate; utilization of tryptophan, histidine, methionine, leucine, lysine and phenylalanine varied from strain to strain. Alanine was either produced or not utilized. Clostridium sordellii (3 strains) differed slightly from C. bifermantans in that arginine and aspartate were less readily utilized while utilization of alanine was slight. Clostridium sticklandii (1 strain) utilized arginine and methionine.

Although no quantitative analysis of the growth medium was made, the examination of chromatograms indicated some utilization of lysine, histidine and phenylalanine. Alanine, α-amino butyrate and γ-amino butyrate were produced.

**Group IB.** Clostridium botulinum type A (2 strains) and type B (2 strains); C. calortolerans (1 strain); C. sporogenes (6 strains). These organisms utilized arginine, phenylalanine, serine and tyrosine. Tryptophan, methionine and glycine utilization varied from strain to strain. Valine, lysine and histidine were either produced or not utilized; α-amino butyrate and γ-amino butyrate were produced.

**Group IC.** Clostridium cochlearium 4721. This strain utilized serine, methionine, threonine and leucine. Alanine, α-amino butyrate and γ-amino butyrate were produced.

**Group ID.** Clostridium difficile. A distinctive feature of this strain was the utilization of valine. Serine, methionine, leucine, isoleucine, threonine, alanine, aspartate and phenylalanine were also utilized. α-Amino butyrate was produced.

**Group IE.** Clostridium putrefaciens. This organism utilized mainly lysine; some utilization of proline and alanine occurred; α-amino butyrate was absent.

**Group II.** δ-Amino valerate not produced; arginine and/or glycine utilized.

**Group IIA.** Clostridium botulinum type C (2 strains); C. histolyticum (6 strains). Arginine was utilized by all strains and usually glycine (except for one strain of C. botulinum); no utilization of tyrosine occurred. Most strains of C. histolyticum also utilized serine.

**Group IIB.** Clostridium cochlearium 6797. Only glycine was utilized completely, but significant utilization of proline and glutamate also occurred. The disappearance of proline was not paralleled by the production of δ-amino valerate in this instance.

**Group IIC.** Clostridium subterminale (1 strain). Glycine and lysine were utilized.

**Group III.** δ-Amino valerate not produced; glutamate, serine and histidine utilized.

**Group IIIA.** Clostridium cochlearium 17787; C. tetani (8 strains); C. tetanomorphum (5 strains). Clostridium cochlearium 17787 utilized aspartate, threonine and tyrosine. Clostridium tetani utilized aspartate; threonine, methionine and tyrosine utilization varied from strain to strain. Clostridium tetanomorphum varied from strain to strain in the utilization of aspartate, threonine and tyrosine.

**Group IIIB.** Clostridium microsporum 25. This strain utilized arginine.

**Group IV.** δ-Amino valerate not produced; serine and threonine utilized.

**Group IVA.** Clostridium putrefaciens (1 strain).
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DISCUSSION

Most of the 30 named species examined made good growth in the basal medium containing either glucose or acid hydrolysed casein as an energy source, and on this basis they were separated into two initial groups. A few strains did not grow well without additional growth factors and/or increased levels of limiting amino acids, but increasing the concentration of casein hydrolysate above 3% was generally avoided because of the Cl⁻ content of the commercial product. Ideally a complete protein hydrolysate is required which does not have this disadvantage.

Organisms found to be saccharolytic under the test conditions were not investigated further in the present study. These included Clostridium acetobutylicum and C. butyricum, listed by Nisman (1954) as species capable of carrying out the ‘Stickland’ reaction when studied as washed cell suspensions. The apparent discrepancy would seem to emphasize the difference between the compounds used by a given organism as sources of energy and those utilized by a washed cell suspension of that organism.

Strains which were considered to be amino acid-fermenting could be divided into easily recognizable groups on the basis of the patterns of amino acid utilization shown by chromatography. Interpretation of the chromatograms has been based largely upon the known mechanisms of amino acid utilization in anaerobic bacteria (see review by Barker, 1961). Four major groups could be recognized and these have the following metabolic characteristics.

Group I includes organisms which carry out the Stickland reaction between certain pairs of amino acids. A consistent feature of the reaction under the growth conditions used is the reduction of proline to δ-amino valeric acid (Stickland, 1935a), with serine, phenylalanine and possibly other amino acids serving as electron donors. In preliminary experiments with Clostridium sporogenes 532 and C. bifermentans 506 it was found that growth was supported by the basal medium with only proline and phenylalanine added. In this medium the amount of δ-amino valerate produced was about equivalent to the amount of proline utilized in each case. With 3% casein hydrolysate (or a synthetic mixture of the constituent amino acids) the apparent production of δ-amino valerate was considerably greater in most cases than that attributable to the utilization of proline and/or arginine alone (Table 2). Either the analyser was unable to separate δ-amino valerate from some unidentified substance, or δ-amino valerate was being produced from an additional source. An unsuccessful attempt was made to effect a further separation of the δ-amino valerate peak by using an ion exchange column containing Zeo Karb 225 (8 to 10 μm. mesh; buffer pH 5-25); investigation of this problem is continuing.

Most of the organisms in group I produced α-amino butyric acid. Stadtman (1954) found that dried cells or cell-free extracts of Clostridium sticklandii produced α-amino butyrate by the reduction of threonine. When organisms representative of group I were grown in the basal medium containing appropriate donor and acceptor pairs of amino acids plus threonine, α-amino butyrate appeared on the chromatograms obtained but was absent when threonine was omitted. This was shown for C. bifermentans 506, C. caloritolerans, C. difficile and C. sporogenes 532. In addition C. bifermentans and C. caloritolerans produced α-amino butyrate from methionine when tested in the same way. In general the amounts of α-amino butyrate produced in the casein hydrolysate medium were in accordance with the amounts of threonine and/or methionine utilized (Table 2).

Apart from Clostridium difficile and C. putrificum the strains in group I also produced γ-amino butyrate, the decarboxylation product of glutamic acid, but the test conditions
would not be expected to be optimal for decarboxylation (Gale, 1941) since the pH of the medium did not fall below 6.3 during growth. In the quantitative analysis γ-amino butyrate was not separated from lysine, and the apparent production of lysine by organisms in group I may well reflect the production of γ-amino butyrate.

The organisms in groups II, III and IV produced neither δ-amino valerate, α-amino butyrate nor γ-amino butyrate. Group II includes strains which utilized mainly arginine and/or glycine but most of these organisms did not grow well in the 3 % casein hydrolysate medium, and therefore their full capabilities for amino acid utilization may not be expressed under such conditions.

Growth of *Clostridium histolyticum* and *C. botulinum* type C was markedly improved by the substitution of a partial casein hydrolysate for the acid-hydrolysed form; by contrast, increasing the levels of amino acids had little effect.

From the limited number of strains of *Clostridium botulinum* examined in the present study three different physiological types can be recognized: saccharolytic strains of types B, E and F, the proline-reducing type A and B strains, and the type C strains with apparently more complex growth requirements, as indicated by the nutritional study of Kindler, Mager & Grossowicz (1956). However, c9 and c21 were atypical in being proteolytic.

The organisms included in group III are characterized by the fermentation of glutamic acid which has been studied in *Clostridium tetani* and *C. tetanomorphum* by a number of workers and reviewed by Barker (1961). The fermentation products are known to be principally a mixture of acetic and butyric acids. A preliminary examination of volatile fatty acids present in culture extracts used for amino acid analysis was kindly made by Mr B. J. H. Stevens of this laboratory, using a representative strain of each species. The results showed that for *Clostridium cochlearium* 17787, *C. microsporum* 25, *C. tetani* 5404 and *C. tetanomorphum* 2909 acetic and butyric acids were the major fatty acids present. Apart from *C. microsporum* a small amount of propionic acid was also detected in each case, and this appears to be related to the utilization of threonine (Cardon & Barker, 1947). Propionic acid was not detected in the culture extract of *C. microsporum*, and threonine utilization by this strain was negligible (Table 2).

Other amino acids utilized by *Clostridium tetani* and *C. tetanomorphum* are in agreement with the findings of Woods & Clifton (1937), Clifton (1942) and Pickett (1943) using washed cell-suspensions of the organisms.

*Clostridium cochlearium* 17787 is indistinguishable from the strains of *C. tetani* and *C. tetanomorphum* on the basis of amino acid utilization. The three strains named as *C. cochlearium* were clearly different from each other in the principal features of amino acid utilization. Only ATCC17787 utilized glutamic acid to any significant extent and thus conformed with an essential property of the species according to Barker (1939).

*Clostridium microsporum*, 25 also differed from the other strains assigned to this species in that growth occurred in the absence of glucose. The strain was originally regarded as a non-sporing organism (*Fusiformis bia-cutus*) and was included in a computer study carried out by Barnes & Goldberg (1968) in which it showed a low similarity to other strains of *F. bia-cutus* now known to produce spores. All these strains were renamed as *C. microsporum* by Reinhold, Barnes & Beerens (1967).

The sole organism placed in group IV is *Clostridium putrefaciens*, which utilized all of the threonine and most of the serine in the medium and therefore differs from organisms in the other groups. The psychrophilic habit of *C. putrefaciens* is a unique property among the species included in this study.

Following the earlier work of Prévot and his colleagues, recent studies on the identifica-
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fermentation of clostridia have been concerned mainly with metabolic end-products, especially volatile fatty acids (Moore, Cato & Holdeman, 1966; Lewis, Moss & Jones, 1967; Moss et al. 1970). Despite the usefulness of end-products for purely identification purposes they are not necessarily of comparable taxonomic value in the absence of information on the mechanism of their formation. With anaerobic bacteria there is considerable diversity in the catabolic systems which could be concerned in the formation of some fatty acids, as can be seen from the following examples. Acetic acid is a common end-product which can be formed from glycine via the Stickland reaction or via serine, from serine via pyruvate and from glutamic acid via citramalate. Acetate is produced by yet other routes in the fermentation of ornithine, lysine and γ-aminobutyrate. Propionic acid may be obtained from pyruvate via succinyl-CoA, from alanine via acryl-CoA and from threonine via α-ketobutyrate. Ideally for taxonomic purposes the formation of end-products should be related to the utilization of particular substrates by specific catabolic pathways. Patterns of amino acid utilization have been shown here to indicate the principal types of fermentation which are characteristic of certain groups of clostridia, and they could readily be incorporated in routine identification procedures.

The author is indebted to Professor S. R. Elsdon for his advice and encouragement throughout this study. Thanks are also due to Miss A. M. Chamberlain and Mr M. G. Hilton for their technical assistance.

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