Physiological and Nutritional Features of *Corynebacterium pyogenes*

By C. ADINARAYANA REDDY* AND C. P. CORNELL

Department of Microbiology and Public Health, Michigan State University, East Lansing,
Michigan 48824, U.S.A.

(Received 12 November 1981; revised 26 May 1982)

Growth and acid metabolic products were similar when *Corynebacterium pyogenes* was grown aerobically or anaerobically in a serum-free medium (SFM). This indicated that *C. pyogenes* obtains energy for growth primarily by fermentative metabolism even under aerobic growth conditions. Growth yield was reduced by 90% in SFM minus glucose, 50% in SFM minus NaHCO_3 , 90% in SFM minus yeast extract, 100% in SFM minus Trypticase and yeast extract, and 30% in SFM minus haemin or Trypticase. Growth was not detectable when a known mixture of amino acids, vitamins, and nucleic acid bases were substituted for Trypticase and yeast extract in SFM; addition to the latter medium of a peptide source such as Trypticase or casitone supported good growth of the organism. When NaHCO_3 was omitted from SFM and dissolved CO_2 in the medium was rigorously excluded, growth was undetectable indicating that *C. pyogenes* has an obligate requirement for CO_2 for growth. Succinate, formate and acetate were the major fermentation products in SFM, whereas in SFM minus HCO_3^- or haemin, lactate was the major product and only small quantities of other acids accumulated.

INTRODUCTION

Corynebacterium pyogenes (Cummins *et al.*, 1974) is frequently isolated from a wide variety of pyogenic conditions in animals (Bruner & Gillespie, 1973; Buxton & Fraser, 1977; Smith, 1966) and humans (Chlostá *et al.*, 1970; Vega & Gavan, 1970). Previous physiological, nutritional and metabolic studies of this organism have been limited because it grew poorly on common laboratory media unless supplemented with complex nutritional sources such as blood or serum (Bruner & Gillespie, 1973; Cummins *et al.*, 1974). Haemin partially replaces the requirement for blood or serum for growth (Reddy *et al.*, 1977; Skerman, 1966), and serum-free media which support good growth of this organism have been described (Reddy *et al.*, 1980). We describe here some physiological and nutritional features of *C. pyogenes*, determined in a serum-free medium.

METHODS

Bacteria. *Corynebacterium pyogenes* strain 5 (ATCC 33157), used throughout this study, has been described previously (Reddy *et al.*, 1977). *Bacteroides fragilis* subsp. *fragilis* (ATCC 25285) was obtained from American Type Culture Collection, Rockville, Md.

Maintenance. The organism was maintained on plates of Brain Heart Infusion (BBL) agar supplemented with 5% (v/v) bovine blood as previously described (Reddy *et al.*, 1977).

Media. The serum-free medium (SFM) contained per 100 ml: glucose, 0.4 g; Trypticase (BBL), 0.5 g; yeast extract (Difco), 0.2 g; haemin, 0.0002 g; mineral solutions 1 and 2, 7.5 ml each; volatile fatty acid solution, 1.0 ml; 1 M-potassium phosphate buffer (pH 7.0), 2.0 ml; cysteine $\text{HCl} \cdot 2\text{H}_2\text{O}$, 0.05 g, and NaHCO_3 , 0.4 g. The composition of the mineral solutions 1 and 2 were as described by Caldwell & Bryant (1966). The volatile fatty acid solution contained per 100 ml: 1.6 ml acetic acid, and 1 ml each isobutyric, DL- α -methylbutyric and *n*-valeric acids. SFM was initially prepared under strict anaerobic conditions, using Hungate anaerobic techniques as modified by Holdeman & Moore (1975). This anaerobic SFM additionally contained 0.0001% resazurin as an E_h indicator and was sufficiently reduced to keep the resazurin colourless; it supported good growth ($A_{600} = 1.3$ in 24 h) of an obligate anaerobe, *B. fragilis*.

Abbreviations: MSFM, Modified serum-free medium; SFM, serum-free medium.

Preliminary experiments showed no difference in growth of *C. pyogenes* in anaerobic SFM and aerobic SFM (prepared aerobically and incubated statically in foam-plugged tubes). Therefore, the aerobic SFM was used in all further studies except where indicated otherwise. Aerobic SFM was prepared as follows. All components except cysteine and HCO_3^- were dissolved in water, adjusted to pH 6.5 with 5 M-NaOH, dispensed in 18 × 150 mm foam-plugged tubes (9.5 ml per tube) and sterilized by autoclaving at 121 °C for 15 min. Cysteine and HCO_3^- solutions were autoclaved separately and were added aseptically to the medium immediately prior to inoculation.

Modified SFM medium (MSFM), used for demonstrating the peptide requirement for growth of *C. pyogenes* (Expt 4, Table 1) was identical to SFM except that yeast extract and Trypticase were omitted and 1 ml each of purines and pyrimidines, trace minerals, and vitamin solutions were added. The composition of these three stock solutions was described previously (Reddy *et al.*, 1980). In addition, 10 ml stock solution of a defined mixture of free amino acids (Reddy *et al.*, 1980) were added to MSFM where indicated. Trypticase and other peptide sources added to MSFM were treated with charcoal to remove nucleotides and vitamins (Ford *et al.*, 1958). Trypticase and caseitone (Difco) were added to MSFM to give a final concentration of 0.5% (w/v) and unhydrolysed casein was added to 0.1% (w/v).

To study the CO_2 requirement of *C. pyogenes*, NaHCO_3 was omitted from SFM and dissolved CO_2 in the medium was rigorously excluded as previously described by Dehority (1971), except that the pH during the initial removal of dissolved CO_2 was 5.5. A BioFlo fermenter (model C30, New Brunswick Scientific Co.) containing 550 ml of medium at 37 °C was sparged with 100% N_2 or 90% N_2 + 10% CO_2 as indicated in the results (Expt 2, Table 1). SFM medium sparged with 100% CO_2 was used as a positive control.

Growth measurements. All media were first inoculated with one drop (about 0.05 ml) of an SFM culture ($A_{600} = 1.4$ to 1.6). To minimize carryover of nutrients, subsequent subcultures were inoculated with bacteria grown in respective nutritional media whenever possible. Tubes were incubated statically at 37 °C. Growth in all cases was measured in 18 × 150 mm tubes by determining the A_{600} with a Bausch and Lomb Spectronic-20 spectrophotometer. A_{600} of 1.0 corresponded to $1.83 \pm 0.18 \times 10^9$ cells of *C. pyogenes* ml⁻¹ or 0.45 mg dry wt ml⁻¹. Absorbance values represent the mean of maximal A_{600} values of triplicate tubes from at least two separate experiments.

Culture purity was determined periodically by examination of Gram-stained smears, examination of wet mounts by phase contrast microscopy and colony morphology on Brain Heart Infusion blood agar plates.

Gas chromatography. Cultures in the stationary phase of growth were acidified and volatile and non-volatile acids were extracted as described by Holdeman & Moore (1975). A Varian 2440 gas chromatograph with thermal conductivity detectors and a column (3 mm × 2 m) containing 15% (w/w) SP 1220/3% (w/v) H_3PO_4 on Chromosorb (Supelco, Bellefonte, Pa.) was used. Helium (25 ml min⁻¹) was the carrier gas. The temperature of the column was 135 °C. Concentrations of fermentation products were determined by comparing their retention times and peak heights to retention times and peak heights of standard concentrations of known acids under the same experimental conditions.

RESULTS AND DISCUSSION

Yields during aerobic and anaerobic growth in SFM

Corynebacterium pyogenes is generally described as aerobic to facultatively anaerobic (Cummins *et al.*, 1974), but growth of this organism under strictly anaerobic conditions has not previously been described. The yields were about the same (0.68 mg dry wt ml⁻¹) whether *C. pyogenes* was grown under strictly anaerobic conditions or aerobically. The major acidic fermentation products in both cases were acetate, succinate and formate; minor products were lactate and pyruvate.

It is probable that the bottom of tubes from which oxygen was not rigorously excluded was anaerobic. The organism was therefore grown in a BioFlo fermenter aerated with 600 ml min⁻¹ of a mixture of 95% air/5% CO_2 to demonstrate conclusively that *C. pyogenes* can grow aerobically. The CO_2 in the gas mixture is needed to replenish the loss of HCO_3^- due to sparging of SFM. Growth in the fermenter was essentially identical to that seen in SFM tubes (Expt 1, Table 1). The final pH of 6.0 in the fermenter was comparable to that observed in the tubes. These results indicate that aerobic respiration is not a major source of energy for this organism and that growth is primarily fermentative even in aerated media.

Growth requirements of C. pyogenes

Omission of glucose from SFM resulted in about a 90% decrease in yield compared to that observed in SFM (Expt 3, Table 1) even though the medium is rich in peptones and *C. pyogenes*

Table 1. *Effect of air, CO₂, peptide sources and various components of SFM on growth of C. pyogenes*

Experiments 1 and 2 were performed in a BioFlo fermenter as described in Methods. The medium was continuously sparged with N₂, N₂/CO₂ or air/CO₂. In experiment 2, after 96 h incubation, the gas phase was shifted from 100% N₂ to 90% N₂/10% CO₂. Experiments 3 and 4 were performed in 18 × 150 mm foam-plugged tubes. Growth yields were calculated from absorbance values at 600 nm. (A_{600} of 1.0 = 0.45 mg dry wt ml⁻¹). Numbers in parentheses are h incubation required for maximum growth.

Expt	Medium*	Gas phase	Growth rate ($\mu_{\max}$ h ⁻¹)†	Growth yield (mg ml ⁻¹)
1	SFM	95% air/5% CO ₂	0.63	0.68 (24)
	SFM	95% N ₂ /5% CO ₂	0.63	0.72 (26)
2	SFM	100% N ₂	—	0.01
	SFM	90% N ₂ /10% CO ₂	—	0.61 (30)
3	SFM	Air	0.63	0.69 (18)
	SFM — NaHCO ₃	Air	0.12	0.32 (42)
	SFM — haemin	Air	0.24	0.45 (48)
	SFM — YE	Air	0.10	0.09 (18)
	SFM — Try	Air	0.40	0.45 (31)
	SFM — Glu	Air	0.19	0.08 (30)
	SFM — Try & Glu	Air	0.40	0.04 (20)
	SFM — Try & Cys	Air	0.50	0.14 (40)
	SFM — Glu & Cys	Air	0.50	0.08 (28)
	SFM — Try & YE	Air	NG	—
	SFM — Try, YE & Cys	Air	NG	—
4	MSFM	Air	—	0.02
	MSFM + aa	Air	—	0.11
	MSFM + Try	Air	—	0.50
	MSFM + aa + Try	Air	—	0.68
	MSFM + aa + Cas	Air	—	0.68
	MSFM + aa + casein	Air	—	0.18

* aa, Amino acids; Cas, casitone; Cys, cysteine; Glu, glucose; Try, Trypticase (BBL); YE, yeast extract.

† NG, No growth.

is proteolytic (Barksdale, 1970; Bruner & Gillespie, 1973; Cummins *et al.*, 1974). Similar yields were obtained even after eight serial transfers when glucose was omitted from SFM. When glucose was replaced by cellobiose, fructose, glycogen, lactose, maltose or ribose, normal growth yields comparable to that in SFM were obtained. However, amino acids such as alanine, arginine, aspartate, glycine, or threonine gave no more growth than that seen in SFM minus glucose. These data indicate that *C. pyogenes* requires a fermentable carbohydrate as its primary source of energy and that it obtains little or no energy from the amino acids or peptides present in SFM. The low but consistent growth in glucose-deficient SFM is probably due to low concentrations of carbohydrate present in yeast extract (Anonymous, 1973).

Omission of yeast extract, which is a source of vitamins, nucleotides and amino acids resulted in a decrease in growth yield of approximately 90% with decrease in the rate of growth (Expt 3, Table 1).

The growth yield in SFM minus Trypticase was about 65 to 70% of that in the complete SFM and was undetectable when both Trypticase and yeast extract were omitted (Expt 3, Table 1). This indicated that yeast extract and Trypticase can partially substitute for each other in satisfying the nitrogen and other requirements for growth of *C. pyogenes*, and that cysteine and (NH₄)₂SO₄ in the medium cannot, by themselves, satisfy the nitrogen requirements for the growth of this organism.

The growth yield decreased by 60% when cysteine was omitted (data not shown), comparable to the decrease when Trypticase was omitted, but by 80% when both Trypticase and cysteine were omitted. In the latter case, the incubation time required to attain maximum growth was 40 h, about twice that in SFM (Table 1).

Omission of haemin or HCO_3^- resulted in approximately 30% and 50% decrease in growth yields, respectively (Expt 3, Table 1) with considerable decrease in the growth rate. Compared to a doubling time of 1.6 h in complete SFM, the doubling times in SFM minus haemin and in SFM minus HCO_3^- were, respectively, 4.1 and 8.4 h. Little growth occurred when both haemin and HCO_3^- were omitted from SFM and the doubling time was 12.8 h. Further results showed that no growth occurred in CO_2 -free SFM under a gas phase of 100% N_2 (Expt 2, Table 1). When after 96 h the gas phase was changed to 90% N_2 /10% CO_2 (v/v), an immediate spurt in growth was observed and the final yield was comparable to that in SFM sparged with 100% CO_2 . These results clearly indicate that *C. pyogenes* has an obligate requirement for CO_2 for growth.

Peptide requirement for growth

Decreased growth in SFM minus Trypticase suggested that peptide(s) might be stimulatory or required for the growth of *C. pyogenes*. To investigate this further, Trypticase and yeast extract in SFM were replaced by a defined mixture of purines and pyrimidines, trace minerals and vitamins. Growth in this modified serum-free medium (MSFM) was negligible ($A_{600} = 0.05$). Only a slight increase in yield was seen on addition of a defined mixture of 18 free amino acids to MSFM. In contrast, single addition of Trypticase to MSFM gave growth yield equivalent to 75% of that observed in SFM. Addition of both Trypticase and amino acids resulted in growth equivalent to that obtained in SFM, but only after incubation for 44 h. Substitution of Bacto-Casitone (Difco) for Trypticase yielded similar growth. However, many commercially available peptides (Sigma) including Gly-Gly-Gly, His-His, Ala-Met, Ala-Phe, Gly-Val, Leu-Gly-Gly, Gly-Asp, Gly-Try, glutathione, oxytocin, eledoisin-related peptide and polyproline could not substitute for Trypticase. The requirement for Trypticase or Casitone, even in the presence of a full complement of amino acids, suggested that *C. pyogenes* has a peptide(s) requirement for growth. Peptide requirement for growth even in the presence of a full complement of amino acids has been shown for other bacteria (Kihara *et al.*, 1952; Pittman & Bryant, 1964). A tryptic digest of casein was previously shown to be stimulatory or required for the growth of other strains of *C. pyogenes* (Skerman, 1966).

*Effect of haemin and HCO_3^- on glucose fermentation by *C. pyogenes**

The objective of this experiment was to determine the effect of omitting haemin or HCO_3^- from SFM on metabolic products produced.

Dramatic changes in metabolism of this organism were noted in media from which different constituents were omitted as compared to that observed in SFM. Acetate, succinate and formate were the major fermentation products in SFM and accounted for about 87% of the products of glucose fermentation; lactate accounted for 13% of the product carbon. A trace amount of pyruvate was produced. Lactate was the major product (88% of the total carbon produced) when HCO_3^- was omitted. Succinate, acetate, pyruvate and formate together accounted for 12% of the carbon produced. These results, together with the previous observation that in Tryptose broth (Difco) or Brain Heart Infusion broth (BBL) *C. pyogenes* produced major amounts of lactate and minor amounts of acetate (Reddy *et al.*, 1977; Reddy & Kao, 1978), indicate that lactate and succinate are alternate fermentation products under different growth conditions. Increased acetate production in SFM, in comparison to that in SFM minus HCO_3^- , would be expected to yield additional ATP by substrate phosphorylation and this may explain in part the higher yield observed in this medium. The results also suggest that in SFM *C. pyogenes* apparently fixes CO_2 (or HCO_3^-) to produce succinate. This succinate pathway, demonstrated previously in other bacteria, is known to yield additional ATP coupled to fumarate reduction to succinate (Buchanan & Pine, 1967; Kröger, 1974; Macy *et al.*, 1973; Reddy & Peck, 1978; Jacobs & Wolin, 1963; deVries *et al.*, 1974; White *et al.*, 1962). If energy is conserved during fumarate reduction by *C. pyogenes*, this would explain the higher yields associated with increased succinate production in SFM (Table 1). The above results also indicate that *C. pyogenes* is metabolically more similar to *Actinomyces* (Buchanan & Pine, 1967) than to lactic acid bacteria as previously suggested (Barksdale, 1970; Cummins *et al.*, 1974).

Lactate was the major product in the absence of haemin and accounted for 60% of the product carbon; acetate, formate and succinate together accounted for 13% of the product carbon. Haemin is required for the synthesis of a *b*- or *c*-type cytochrome which mediates the energy-conserving reduction of fumarate to succinate. As previously reported, haemin also appears to be required for *b*-type cytochrome synthesis in *C. pyogenes* (Reddy *et al.*, 1977; Cornell & Reddy, 1979).

Journal article no. 9200 from the Michigan Agriculture Experiment Station.

REFERENCES

- ANONYMOUS (1973). *BBL Manual of Products and Laboratory Procedures*, p. 163. Maryland, U.S.A.: BBL Microbiology Systems.
- BARKSDALE, L. (1970). *Corynebacterium diphtheriae* and its relatives. *Bacteriological Reviews* **34**, 348–422.
- BRUNER, D. W. & GILLESPIE, J. H. (1973). In *Hagan's Infectious Diseases of Domestic Animals*, 6th edn, pp. 308–323. Ithaca: Cornell University.
- BUCHANAN, B. B. & PINE, L. (1967). Path of glucose breakdown and cell yields of a facultative anaerobe, *Actinomyces naeslundii*. *Journal of General Microbiology* **46**, 225–236.
- BUXTON, A. & FRASER, G. (1977). In *Animal Microbiology, Vol. 1: Immunology, Bacteriology, Mycology, Diseases of Fish and Laboratory Animals*, pp. 178–179. Oxford: Blackwell Scientific.
- CALDWELL, D. R. & BRYANT, M. P. (1966). Medium without rumenfluid for nonselective enumeration and isolation of rumen bacteria. *Applied Microbiology* **14**, 794–801.
- CHLOSTA, E. M., RICHARDS, G. K., WAGNER, E. & HOLLAND, J. F. (1970). An opportunistic infection with *Corynebacterium pyogenes* producing empyema. *American Journal of Clinical Pathology* **53**, 167–170.
- CORNELL, C. P. & REDDY, C. A. (1979). Relationship between growth yields and fermentation products of *Corynebacterium pyogenes* in media with and without HCO₃⁻ and/or hemin. *Abstracts of the Annual Meetings of the American Society for Microbiology* p. 41.
- CUMMINS, C. S., LELLIOTT, R. A. & ROGOSA, M. (1974). Genus *Corynebacterium*. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 602–617. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.
- DEHORITY, B. A. (1971). Carbon dioxide requirement of various species of rumen bacteria. *Journal of Bacteriology* **105**, 70–76.
- FORD, J. E., PERRY, K. D. & BRIGGS, C. A. E. (1958). Nutrition of lactic acid bacteria isolated from the rumen. *Journal of General Microbiology* **18**, 273–284.
- HOLDEMAN, L. V. & MOORE, W. E. C. (1975). *Anaerobe Laboratory Manual*. Blacksburg, Virginia, U.S.A.: V.P.I. Anaerobe Laboratory.
- JACOBS, N. J. & WOLIN, M. J. (1963). Electron transport system of *Vibrio succinogenes*. I. Enzymes and cytochromes of the electron transport system. *Biochimica et biophysica acta* **69**, 18–28.
- KIHARA, H., MCCULLOUGH, W. G. & SNELL, E. E. (1952). Peptides and bacterial growth. I. L-Alanine peptides and growth of *Lactobacillus casei*. *Journal of Biological Chemistry* **197**, 791–800.
- KRÖGER, A. (1974). Electron-transport phosphorylation coupled to fumarate reduction in anaerobically grown *Proteus rettgeri*. *Biochimica et biophysica acta* **347**, 273–289.
- MACY, J., PROBST, I. & GOTTSCHALK, G. (1973). Evidence for cytochrome involvement in fumarate reduction and adenosine 5'-triphosphate synthesis by *Bacteroides fragilis* grown in the presence of hemin. *Journal of Bacteriology* **123**, 436–442.
- PITTMAN, K. A. & BRYANT, M. P. (1964). Peptides and other nitrogen sources for the growth of *Bacteroides rumenicola*. *Journal of Bacteriology* **88**, 401–410.
- REDDY, C. A. & KAO, M. (1978). Value of acid metabolic products in identification of certain corynebacteria. *Journal of Clinical Microbiology* **7**, 428–433.
- REDDY, C. A. & PECK, H. D., JR (1978). Electron transport phosphorylation coupled to fumarate reduction by H₂, and Mg²⁺-dependent adenosine triphosphatase activity in extracts of the rumen anaerobe *Vibrio succinogenes*. *Journal of Bacteriology* **134**, 982–991.
- REDDY, C. A., CORNELL, C. P. & KAO, M. (1977). Hemin-dependent growth stimulation and cytochrome synthesis in *Corynebacterium pyogenes*. *Journal of Bacteriology* **130**, 965–967.
- REDDY, C. A., CORNELL, C. P. & FRAGA, A. M. (1980). Chemically defined growth medium for *Corynebacterium pyogenes*. *American Journal of Veterinary Research* **40**, 843–845.
- SKERMAN, T. M. (1966). *Comparative studies of coryneform bacteria of animal origin, with special reference to their nutrition*. Ph.D. thesis, University of Reading, U.K.
- SMITH, J. E. (1966). *Corynebacterium* species as animal pathogens. *Journal of Applied Bacteriology* **29**, 119–130.
- VEGA, L. E. & GAVAN, T. L. (1970). *Corynebacterium pyogenes*—a pathogen in man. Report of a case. *Cleveland Clinical Quarterly* **37**, 207–214.
- DEVRIES, W., VANWIJCK-KAPTEYN, W. M. C. & OOSTERHUIS, S. K. H. (1974). The presence and function of cytochromes in *Selenomonas ruminantium*, *Anaerovibrio lipolytica* and *Veillonella alcalescens*. *Journal of General Microbiology* **81**, 69–78.
- WHITE, D. C., BRYANT, M. P. & CALDWELL, D. R. (1962). Cytochrome-linked fermentation in *Bacteroides rumenicola*. *Journal of Bacteriology* **84**, 822–828.