The Presence of N₂-fixing Bacteria in the Intestines of Man and Animals

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(Accepted for publication 3 October 1969)

SUMMARY

Cultures of N₂-fixing bacteria have been isolated from the intestinal contents of humans, pigs and guinea-pigs. Acetylene-reduction tests indicate that N₂-fixation may be occurring in the intestines. Best N₂-fixation was by cultures provisionally identified as *Klebsiella aerogenes*, but other genera were also involved. All cultures fixed more N₂ anaerobically than aerobically but some fixation occurred when 20 % O₂ was present.

INTRODUCTION

Recent studies have shown consistent negative nitrogen balances in sweet potato-eating people in the highlands of New Guinea (Luyken, Luyken-Koning, Pikaar & Blom, 1964; Oomen & Corden, 1969). The apparent deficit in nitrogen was about equal to the daily dietary intake of N (about 2.0 g.). It seemed possible that fixation of atmospheric N₂ in the intestines of these people living on a diet of very high C:N ratio, could result in high levels of faecal-N and so give spurious nitrogen balance results. This communication establishes that N₂-fixing bacteria can occur in the intestines of man and animals.

METHODS

Nutrient agar (Oxoid or Difco) and Burk N-free agar (Burk & Lineweaver, 1930) were used for maintenance of cultures. N-free broth cultures were grown on Burk medium under an atmosphere of N₂. Yeast extract (Oxoid), 150 mg./l., was used to supplement Burk medium for some cultures. Media for biochemical tests were those listed by Carpenter, Lapage & Steel (1966). N₂-fixation was measured by increase in culture total-N, by ¹⁵N₂ incorporation and by the acetylene-reduction method (Hardy, Holsten, Jackson & Burns, 1968). ¹⁵N was analysed as previously described (Bergersen, 1962) using Kjeldahl digestion of cultures, distillation of the NH₃ and oxidation to N₂ by NaOBr; mass 29:28 ratios were measured with an Atlas M 86 mass spectrometer. The reduction of acetylene to ethylene was measured in rubber-capped bottles of 13.5 ml. capacity with a gas phase containing 20 % (v/v) acetylene, or on cultures on agar in McCartney bottles fitted with perforated caps and thick rubber seals to enable gassing and sampling through hypodermic needles. Ethylene production was measured in 200 µl. gas samples using a Philips 4000 gas chromatograph equipped with 2 m. × 2 mm. columns of Poropak R at 50°. Control tests without acetylene did not give any detectable ethylene production with faecal material or cultures. In the guinea-pig experiment there was no ethylene production before the introduction of acetylene into the chamber.

RESULTS AND DISCUSSION

N₂-fixation in human faecal material

Samples of about 1 g. (fresh weight) of freshly voided faeces from five Europeans in Canberra were homogenized in 2 ml. 1.0% (w/v) glucose and incubated for 24 hr at 37° under N₂. The gas was then replaced with acetylene-argon mixture and incubated for 2 hr. One sample (H) showed a small but significant production of ethylene amounting to about 1.5 mμmole. Later, a further sample was obtained from the same donor and examined in the same way together with samples from four other European donors. Again H was the only positive reaction observed. Five serial sub-cultures of H faeces in Burk liquid medium under N₂, followed by plating on Burk agar, yielded a pure culture of a Gram-negative, lactose-fermenting, non-motile bacterium, which grew well anaerobically with N₂ as the sole nitrogen source (culture H 1). A similar culture was obtained from the same donor by the same procedure several months later (culture H 2).

On two occasions, samples of faecal material from sweet potato-eaters were flown to Canberra, packed in ice, from the Morobe district, Territory of Papua-New Guinea (T.P.N.G.). These were examined for acetylene reduction, and on both occasions all specimens showed slight but significant activity. Cultures were obtained from all of the second set of samples, which were able to grow well on Burk medium supplemented with traces of yeast extract. These cultures were obtained from high dilutions of the original faeces (10⁻⁶ to 10⁻⁸), indicating that the organisms were present in high numbers (cultures H 3 to H 6). On another occasion cultures were isolated from diluted fresh human faeces at Baiyer River, T.P.N.G. Data are presented for two of these (H 46, H 55).

Animal experiments

A guinea-pig which had been fed on a low-N diet for several days was placed in a large desiccator of about 6 l. equipped with an O₂ metering device and a CO₂-absorbing by-pass. The atmosphere was slowly replaced by one containing 20% (v/v) O₂, 10% acetylene, and the balance was argon. A cooked potato diet and drinking water were supplied. The gas in the chamber was analysed at intervals for ethylene. Slight but progressive ethylene production was observed over a period of 22 hr, finally amounting to about 75 mμmoles. After removal of the animal, some ethylene production continued but when the faeces were removed, this ceased. Diluted caecal contents of a guinea-pig on low-N diet yielded discrete grey colonies when incubated anaerobically under N₂ on Burk medium (culture P 5 B). Attempts to demonstrate nitrogen fixation in the intestine of these animals by means of the acetylene method failed because of the difficulty of maintaining the acetylene concentration in a gas bubble injected into the lumen of the gut, for more than a few minutes.

A pig was fed on a diet of raw potato for a week before slaughter. Very watery colonies of a motile bacterium were obtained on Burk medium, supplemented with yeast extract, from diluted caecal contents obtained after slaughter (culture P 11). On subculture the nature of the colonies changed to a small, dense, raised type.

Properties of the N₂-fixing cultures

The biochemical tests done on the cultures are summarized in Table 1. Cultures H4, H5 and H6 were Gram-negative rods but they failed to grow in most of the simple media used. This may have been due to a growth factor requirement, since all three cultures required traces of yeast extract for growth on Burk medium. They were also yellow-pigmented on nutrient agar. Six other cultures were members of the Enterobacteriaceae, being Gram-negative, facultative, anaerobic rods producing acid and gas from glucose and growing on media containing bile salts. Their provisional identification is shown on the basis of the criteria given by Carpenter, Lapage & Steel (1966). Those provisionally identified as *Klebsiella aerogenes*, H1, H2 and P5B, were differentiated from *K. pneumoniae* by their methyl red Voges-Proskauer reactions but their properties are similar to those of N₂-fixing strains of *K. pneumoniae* isolated from sugar cane by Nunez & Colmer (1968) and identified from the classification of Edwards & Ewing (1962). Isolate H46 may be a non-citrate-utilizing strain of *K. aerogenes*. The cultures H3, H55 and P11 were distinct from the *Klebsiella* cultures in being motile. If their identification as *Enterobacter cloacae* and *Escherichia coli* is confirmed, they represent new examples of organisms able to fix N₂.

The ability of these cultures to fix N₂ has been confirmed in a number of separate tests using ¹⁵N₂ and in the C₂H₂ reduction test. Table 1 gives the result of one experiment in which cultures were grown on Burk agar, supplemented with yeast extract, in McCartney bottles with atmospheres containing ¹⁵N₂. After 24 hr the growth was washed off the agar in 5 ml. water and digested for nitrogen analysis. Cultures H1, H2, H46, H55 and P5B grew better and fixed more N₂ anaerobically than aerobically. Culture P11 grew equally well under aerobic and anaerobic conditions but fixed more N₂ anaerobically. Cultures H4, H5 and H6 appeared to grow as well aerobically as the best N₂-fixing cultures grown anaerobically, but they fixed only small amounts of N₂. Culture H1 produced H₂ and CO₂ in the ratio of 1:2 when grown anaerobically with glucose as energy source in a shaken liquid culture in which 11.7 µg.N/ml. culture was fixed in 48 hr.

The results presented show that N₂-fixing bacteria can be isolated from the faeces of man and the intestinal contents of pigs and guinea-pigs. The best nitrogen-fixers appeared to be *Klebsiella aerogenes* but at least three other genera also occur. It has not been established that N₂-fixation occurs *in situ*, but the fact that several cultures have been obtained from high dilutions of intestinal contents and faeces indicates that substantial populations of these organisms may be present. Positive acetylene reduction tests have been obtained with fresh faeces and with the guinea-pig chamber experiment. This provides some evidence which supports the possibility of N₂-fixation occurring in the intestines. Further work is being done in identifying the bacteria which occur and in determining the parts of the intestine in which they are most numerous.

We thank Dr L. A. Malcolm, Regional Medical Officer, Lae, and Dr J. Crotty, Commonwealth Health Laboratory, Canberra, for active co-operation in this work. Mr G. L. Turner did the ¹⁵N analyses and Mrs L. Hush provided technical assistance.

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