CORYNEBACTERIUM BARKERI, nov. spec., A PECTINOLYTIC BACTERIUM EXHIBITING A BIOTIN-FOLIC ACID INTER-RELATIONSHIP

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Received on January 17, 1962

Abstract

The isolation of a pectinolytic bacterium, *Corynebacterium barkeri*, nov. spec., is described. Its morphological, cultural, physiological and nutritional attributes are enumerated. The organism has an unusual vitamin nutrition in that its requirement for biotin is partly replaceable by folic acid. Calcium has been shown to be essential for pectin decomposition. The name *Corynebacterium barkeri*, nov. spec., in honour of Dr. H. A. Barker, has been proposed for this bacterium.

The ability to decompose pectin is shared by bacteria belonging to different genera. However, among the coryneform bacteria this property has not been conclusively demonstrated. The only species for which this attribute has ever been claimed is *Corynebacterium sepedonicum*[1], but subsequent work by Paquin *et al.*[2] has not brought forth any evidence for the presence of pectic enzymes in this species. Moreover, none of the *Corynebacterium* species screened by Smith[3] were pectinolytic. Likewise, a detailed survey of the pectinolytic flora of rets of *Malachra capitata* and *Hibiscus cannabinus*[4] and of soils[5] carried out here failed to reveal any activity among members of this group. The purpose of this communication is to report on the isolation from sewage of a pectinolytic *Corynebacterium* species that has an unusual vitamin nutrition.

Materials and Methods

The basal medium described by Khambata *et al.*[5] was employed in the isolation procedures. To this basal medium was added 0.5 % pectin (S. B. Penick & Co., Illinois), 0.05 % $(NH_4)_2SO_4$ and 1 ml. % of a 0.04% aqueous solution of bromocresol purple. The pH of the medium was adjusted to 7.4 and it was sterilized by Seitz filtration. It was then dispensed aseptically in 10 ml. amounts into sterile test tubes. Raw domestic sewage (1 ml.) was used as the inoculum. The tubes were incubated in an inclined position till growth appeared. A loopful of growth was then transferred to a fresh tube of the same medium. Growth from this second tube was plated out on the above medium fortified with 0.1 % yeast extract (as growth otherwise was poor) and solidified with agar. Colonies therefrom were picked and purified by the conventional techniques.

The pure cultures were then tested for their ability to decompose pectin by the qualitative tests described by Bilimoria and Bhat[6]. Such cultures as were positive were characterised by the employment of tests described in the 'Manual of Microbiological Methods'[7].

Nutritional studies on the pectinolytic coryneforms were carried out in the salts solution of Stokes and Gunness[8]. The absence of micronutrients had no adverse effect on the growth of our isolates. For ascertaining their carbon nutrition several compounds were tested and all but formate, oxalate and benzoate (used in 0.1% concentration) were added to the basal salts solution at 1% level. Ammonium sulphate at 0.05% level was used as the source of nitrogen. For studying the nitrogen nutrition the nitrogenous compounds were used at a concentration of 0.025% nitrogen with glucose (0.5%) serving as the source of carbon and energy. All these experiments were carried out in test tubes with 10 ml. of medium containing 1μg biotin per litre.

Vitamin requirement studies were carried out in 250 ml. Erlenmeyer flasks each containing 100 ml. of medium. Biotin was used at a concentration of 1μg per litre. The other vitamins were tested at a concentration of 0.5 μg per ml. Glucose was added as the source of carbon. In this case NH_4NO_3 (0.1%) was chosen as the nitrogen source because it could effectively suppress the formation of an uneven slimy growth which made turbidimetric readings impossible. That nitrate and nitrite can inhibit polysaccharide formation in another organism, *viz.*, *Corynebacterium laevaniformans* has been reported earlier[9]. However, it must be pointed out that for these substances to be effective in slime suppression they must remain, at least in part, unmetabolised. This observation is different from that of Linderberg[10] in that nitrate had the effect of enhancing the formation of levan sucrase in the halophilic *Achromobacter* species studied by him. The explanation offered by him was that the extra oxygen in the nitrate was conducive to the enhancement of levan sucrase synthesis. In the present instance, however, the cationic nitrogen from NH_4NO_3 was utilised leaving thus intact the nitrate ion to exert its inhibitory effect on polysaccharide synthesis.

The inoculum used at all times was a dilute suspension of aseptically washed cells harevsted from nutrient agar. The cultures were shaken on a rotary shaker at room temperature (R.T. approximately 25°C) and growth was monitored turbidimetrically in a Klett-Summerson colorimeter using the blue filter (No. 42).

RESULTS AND DISCUSSION

In all 52 cultures were isolated from 20 samples of sewage. Of these 24 were observed to be able to degrade pectin and 6 therefrom were clearly coryneforms and are the subject of this report. The remaining isolates were found to belong to different genera as detailed elsewhere.[11] The morphological, cultural and physiological characteristics itemised below are representative of all the 6 coryneform cultures studied. All of them were also examined for their

general nutrition but the elucidation in detail of the vitamin requirements was limited to the observations made on one isolate (No. 7).

Morphological attributes:—Young cultures on nutrient agar were Gram positive short rods with no marked tendency towards either pleomorphism or metachromatism (Plate I, 1). The so called "Chinese letter" arrangement was, however, evident and a few cells occasionally exhibited a club-shaped appearance. Old cultures on nutrient agar were also rod shaped. Neither spore nor capsule was present. When young, the cells were motile by virtue of a single, easily stainable polar flagellum somewhat characteristic in the sense that it was straight and not wavy.

It was of considerable interest to observe that the cytological pattern of cells grown in a liquid synthetic medium (glucose, NH_4NO_3) was a function of the vitamin status of the medium. By and large, cells from media with an adequate supply of biotin were weakly Gram positive rods with a few strongly Gram positive inclusions. Staining by Neisser's method[7] rarely showed any inclusions (Plate I, 2). The cells were not acid fast (3% HCl-alcohol[7]). On the other hand, cells from biotin deficient medium or from media containing folic acid instead of biotin (*vide* nutritional attributes) were markedly beaded and intensively Gram positive. Neisser's stain revealed profuse "metachromatic" granules (Plate I, 3) and, what is more, the cells were acid fast. That this change in structure was not due to the differences in pH attained by the two cultures was verified by keeping the medium at a uniform pH level by the incorporation of $CaCO_3$ in the medium. The morphological differences observed are therefore indicative of the influence of vitamins in the medium and find a parallel in the role of vitamin B_6 on the morphology of *Lactobacillus arabinosus*[12] and of vitamin B_{12} on that of an *Arthrobacter* species.[13]

Cultural attributes:—The colonies on nutrient agar were yellow (lemon), opaque, round, convex, moist, with an entire margin and they attained a size of about 3 mm on incubation for 3 days at R.T. The yellow pigment did not diffuse into the surrounding medium. In nutrient broth, a uniform and general turbidity accompanied by some sediment was produced. All the isolates grew luxuriantly on potato stubs producing an intense lemon yellow pigment. The potato did not change in consistency as might have been expected if proto-pectinase was produced. The isolates grew in nutrient broth containing 0.02% K-tellurite, the medium assuming a grey to black colour. On nutrient agar containing tellurite black colonies were produced.

In liquid synthetic medium containing glucose and $(NH_4)_2SO_4$ a slimy, stringy growth that did not emulsify to yield a uniform suspension even on vigorous shaking was formed. This property was quite characteristic of the species.

All the six cultures grew equally well at R.T. and at 37°C on nutrient agar. No growth took place at 42°C. Aeration by shaking had a beneficial effect on growth in liquid culture.

Physiological attributes:—None of the cultures produced indole or acetylmethylcarbinol; the M.R. test gave negative results. The utilisation of citrate (with biotin) was a variable feature. None of the cultures hydrolysed arginine, reduced nitrate in nutrient broth or utilised cellulose as the sole source of carbon and energy. No urease activity could be detected by the Levine test[11]. Hydrogen sulphide was produced, sometimes weakly so (tested by the lead acetate paper method). Both starch and gelatin were hydrolysed (tested by the plate technique). An acid reaction, sometimes very slight, was produced in peptone water containing either glucose, sucrose, lactose, maltose or mannitol. The action on glycerol was variable. In no instance was gas produced. In Hugh and Leifson's[15] medium containing glucose an acid reaction was observed under both aerobic and anaerobic conditions, the reaction being more intense in the former.

Pectinolytic attribute:—The results are recorded in Table I. One + sign under the column "pectin decomposition" indicates that the added pectin was not detectable after a week's incubation on a rotary shaker at room temperature while a – indicates that the pectin could be detected in the medium even after 15 days' incubation. It is pertinent to mention that at no stage of incubation was any material that formed a gel with $CaCl_2$ detected. The results indicate that our isolates have a pectin polygalacturonase though the concomitant presence of a demethylating enzyme cannot be excluded. They also reveal that for pectinolysis, calcium is an essential element.

This observation is in accord with past observations on bacterial polygalacturonases[16, 17, 18] and tends to support the view that they are distinct from fungal and plant polygalacturonases. In this context it is pertinent to refer that Paquin *et al*[2], whilst ascertaining the pectin hydrolysing ability of *Corynebacterium sepedonicum* did not add calcium to their growth medium and hence their observations call for reinvestigation notwithstanding the fact that one of the substrates they had employed was calcium pectate.

Nutritional attributes:—All the isolates utilised arabinose, glucose, sucrose, lactose, maltose, dextrin, glycerol, mannitol and inositol as the sole source of carbon and energy in a mineral medium containing an inorganic source of nitrogen and biotin. Succinate, malate, fumarate and α-ketoglutarate were likewise utilised. The utilisation of citrate was a variable feature. On the other hand, formate, oxalate, acetate, tartrate, benzoate and ethanol were not suitable as substrates. Acetate was metabolised, however, in the presence of yeast extract as was evidenced by the production of an alkaline reaction (as compared to the control without acetate). Ammonium sulphate, ammonium phosphate, ammonium nitrate, ammonium acetate, sodium nitrate, urea, glycine, glutamate and casein hydrolysate served as good sources of nitrogen. Sodium nitrite allowed only slight growth. That the poor growth was due to the bacteriostatic action of nitrite was ascertained by (1) lowering the concentration

TABLE I

Decomposition of pectin by *C. barkeri*

Addition to the salts solution of Stokes and Gunness[8] + NH_4NO_3 (0.1 %) + biotin (1 μg/1000 ml.)	Method of sterilisation of medium	Growth response[1]	Pectin decomposition[2]
Pectin (0.5 %)	Filtration	–[3]	–
Pectin (0.5 %) + $CaCO_3$ (0.5 %)	Filtration	+ +	+
Pectin (0.5 %) + $CaCl_2$ (0.01 %)	Filtration	+ +	+
Pectin (0.5 %)	120°C for 10 min.	+	–
Pectin (0.5 % + $CaCl_2$ (0.01 %)	120°C for 10 min.	+ +	+
Pectin (0.5 %) + Glucose (0.5 %)	120°C for 10 min.	+ +	–
Pectin (0.5 %) + Glucose (0.5 %) + $CaCO_3$ (0.5 %)	120°C for 10 min.	+ +	+
Peptone (0.5 %) Pectin (0.5 %)	120°C for 10 min.	+ +	+

1. ++ indicates response comparable to that obtained in a medium containing glucose, NH_4NO_3 and biotin

   \+ indicates growth less than ++

   – indicates no growth

2. See text "Pectinolytic attribute" for details

3. The pectin was purified by washing with ethanol prior to use. Pectin that was not so purified allowed slight growth.

of nitrite and noticing the beneficial effect, and (2) the observation that nitrite could inhibit growth when present in an otherwise suitable medium. The strains were unable to use elemental nitrogen as nitrogen source. Glutamate, aspartate and asparagine, but not glycine, hippurate, urate and urea, served as suitable sources of both carbon and nitrogen.

As can be seen from Table II all the isolates grew well in a mineral medium containing glucose and NH_4NO_3 and biotin as the only vitamin. The requirement for biotin could, however, be partly met by folic acid. That this was not due to a carryover of biotin with the inoculum was concluded from the

TABLE II

Growth of *C. barkeri* with either biotin, folic acid or p-aminobenzoic acid incorporated in a mineral medium containing glucose and NH_4NO_3. Growth expressed as Klett-Summerson units

Supplements in medium	Culture No.					
	7	8	9	10	11	12
No vitamin	0	0	0	0	0	0
Biotin	115	115	126	117	121	117
Folic acid	45	39	18	37	15	46
p-Aminobenzoic acid	0	0	0	0	0	0

observation that cells grown in folic acid medium were able to grow for several generations in this same medium. Moreover, media devoid of both folic acid and biotin failed to show growth. Contamination of the sample of folic acid with biotin was ruled out by the observation that increasing the concentration of folic acid in the growth medium did not result in any increase in the growth.

This is the first instance, as far as the authors are aware, in which folic acid has been found to replace biotin for any microorganism and brings to mind the observation of Mitbander and Sreenivasan[19] on *Lactobacillus arabinosus 17-5* in that for this organism biotin (as well as Tween 80) was shown to enhance the synthesis of compounds with folic acid activity from p-aminobenzoic acid. The explanation offered by these authors, however, for their findings does not appear adequate particularly in view of the present observations. Nor do the present results allow any definite conclusions, though it is pertinent and interesting to recall that in germ-free rats, at least, feeding with biotin has been shown to result in an increase in the synthesis of folic acid[20] thus suggesting a role for biotin in the synthesis of folic acid.

Tables III and IV bring out the effect on various vitamins on the growth of a strain (No. 7) of the organism under study. It is clear that none of the vitamins tested had any effect on growth, individually or in combination, with either biotin or folic acid. It may also be mentioned that neither pimelic acid, desthiobiotin, succinic acid, glutamic acid, aspartic acid nor asparagine were

TABLE III

Growth of *C. barkeri* (7) as affected by different vitamins

	Growth response (Klett-Summerson units)
Vitamin mixture[1]	117
Biotin	115
Biotin + folic acid	113
Biotin + p-aminobenzoic acid	120
Biotin + pantothenate	120
Biotin + pyridoxine	122
Biotin + nicotinic acid	115

[1] Vitamin mixture : Biotin, thiamine, pantothenate, pyridoxine, nicotinic acid, p-aminobenzoic acid and folic acid.

TABLE IV

Growth of *C. barkeri* (7) as affected by different vitamins in the absence of biotin

Vitamin subtracted from mixture	Growth response (Klett-Summerson units)
Biotin	52
Biotin, thiamine	50
Biotin, pantothenate	50
Biotin, pyridoxine	46
Biotin, nicotinic acid	48
Biotin, p-aminobenzoic acid	50
Biotin, folic acid	0
All except biotin	115
All except folic acid	46

able to replace biotin. Thus, the observation that neither p-aminobenzoic acid nor glutamic acid can replace biotin is suggestive of the role biotin has in either the synthesis of the pteridine moiety or the formation of some compound with folic acid activity by our isolates from these (*viz.*, either pteroic acid, p-aminobenzoylglutamic acid or folic acid).

Taxonomic considerations:—The morphological and physiological attributes of the isolates under study are consistent with those described for the genus *Corynebacterium*. This genus, as described in "Bergey's Manual of Determinative Bacteriology"[21], is made up of species derived from plant and animal sources and does not allow inclusion of saprophytic forms from other environs like soil, for example. Even though the possibility of our isolates being phytopathogens has not been ruled out by proper experiments, in view of the previous observations that bacteria which tally in description to the well defined plant pathogens[22, 23] do exist in soil and can be isolated by appropriate methods, is suggestive of the need to revise the system of classifictaion obtaining for these forms and make it more accommodative. In the mean time we propose the inclusion of our isolates in this genus notwithstanding the fact that they were isolated from sewage. It may be stated that we have not placed our isolates in the genus *Arthrobacter* because unlike the species of this genus our isolates do not fragment into coccoidal forms in old cultures Inclusion of isolates such as ours in this genus would go against the very reasons which led to its creation. We suggest, therefore, that irrespective of their habitat all the nonmotile and the flagellated coryneforms which show no marked "life-cycles" be brought together into a single genus *Corynebacterium*, as such a system would aid in a more effective cataloguing (this is all that should be aimed at in the present state of our knowledge of these bacteria) of this group of bacteria. It is pertinent to record in this connection that recently[24] polar flagellation has been demonstrated in *Corynebacterium vesiculare*, an organism derived from an animal source and previously believed to be nonmotile. This property, it may be pointed out, has been hitherto associated with the phytopathogenic and saprophytic coryneforms only. There is thus enough justification for re-defining the genus *Corynebacterium* unbiased by ecological considerations as more recent reports have pointed out the existence of similar bacteria in environs other than plant and animal tissue[5, 25].

At the species level, however, our isolates do not resemble any of the described species. We feel justified, therefore, in creating a new species and propose the name *Corynebacterium barkeri*, in honour of Dr. H. A. Barker, University of California, Berkeley.

ACKNOWLEDGMENT

The authors wish to thank Dr. S. C. Pillai for his kind suggestions, Dr. M. K. Subramaniam for the photomicrographs and Dr. S. Bhagavantam for his keen interest.

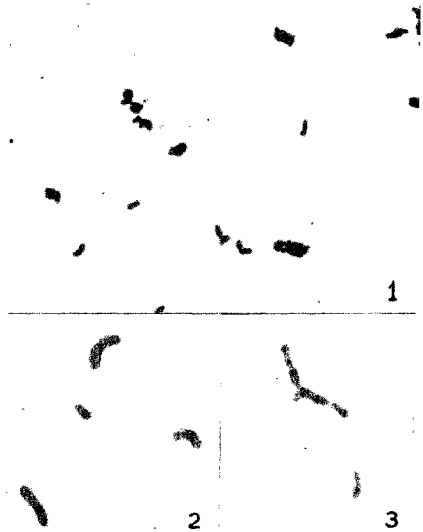

PLATE I

1. Cells from a 48 hr. old nutrient agar culture; crystal violet × 2,500 approx
2. Cells from a 48 hr. old glucose–NH_4NO_3–biotin aerated culture; Neisser's stain × 4,500 approx.
3. Cells from a 48 hr. old glucose–NH_4NO_3–folic acid aerated culture; Neisser's stain × 4,500 approx.

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