Journal of Industrial Microbiology, 5 (1990) 125–130
Elsevier

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Isolation and characterization of a new strain of *Cellulomonas flavigena*

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Received 1 September 1988
Revised 11 April 1989
Accepted 20 April 1989

Key words: *Cellulomonas flavigena*; Morphology; Intracellular inclusion

SUMMARY

A cellulolytic bacterium was isolated from leaf litter. Its nutritional characteristics and most of its morphological features closely resemble those of ATCC #482, which is considered to be the type species of *Cellulomonas flavigena* (Stackebrandt and Keddie in Bergey's Manual of Systematic Bacteriology). However, when stationary phase cells from cultures using a minimal medium containing an excess carbon and energy source are compared, a very prominent morphological difference is manifest. Phase contrast microscopy and transmission electron microscopy indicate the presence of large swollen polar structures in the type species. The absence of such structures from the new isolate and the production of large amounts of an extracellular polysaccharide indicate that it is a new strain of *Cellulomonas flavigena*.

INTRODUCTION

Most species of *Cellulomonas* have been represented by just a few strains and it therefore has been difficult to determine if differences among them are really species-specific or if they are just strain-specific. For this reason the 8th edition of Bergey's Manual of Determinative Bacteriology recognized only *Cellulomonas flavigena* and all other previously listed species were considered to be subjective synonyms [10]. We have isolated a cellulolytic bacterium with characteristics similar to those of the type species of *Cellulomonas flavigena*. However, because of prominent differences in several traits and because the new isolate is a prodigious producer of an unusual extracellular polyglucan, we believe it should be recognized as a new strain of *Cellulomonas flavigena*. The new isolate is the dominant organism in a patent application for the process of pro-

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ducing the polyglucan. The purpose of this communication is to characterize it taxonomically and to compare and contrast it with the type species.

MATERIALS AND METHODS

Isolation and cultivation of cellulose-degrading bacteria

Cellulose enrichment cultures were prepared by making soil suspensions in 15 × 150 mm test tubes containing 10 ml of a minimal medium supplemented with a 10 × 60 mm strip of Whatman No. 1 filter paper. The minimal salts culture medium, designated as CM9, contained (g/l): KH_2PO_4, 3.18; Na_2HPO_4, 5.20; $MgSO_4$, 0.12; NH_4Cl, 0.40; yeast extract (Difco), 0.50. Five milliliters of heavy metal solution which contained (mg per liter of H_2O): EDTA (disodium salt), 500; $FeSO_4 \cdot 7H_2O$, 200; $ZnSO_4 \cdot 7H_2O$, 10; $MnCl_2 \cdot 4H_2O$, 3; H_3BO_3, 30; $CoCl_2 \cdot H_2O$, 20; $CuCl_2 \cdot 2H_2O$, 1; $NiCl_2 \cdot 6H_2O$, 2; and $Na_2MoO_4 \cdot 2H_2O$, 3 [11], was added to the minimal medium and the pH was adjusted to 6.8. The heavy metal solution was added to insure the availability of metals which commonly are used as cofactors. All cultures were incubated at 30°C in a reciprocal shaker water bath and monitored for cellulose hydrolysis. Positive cultures were streaked for isolation on minimal agar containing 0.5% (w/v) glucose and isolated colonies were used to inoculate CM9 medium with filter paper incorporated as the principle carbon source.

Carbon and energy sources used for growth

A modified CM9 medium was used to test the utilization of various carbohydrates, organic acids and aliphatic alcohols as carbon and energy sources. In these assays the phosphate buffer concentration was reduced to 5 mM and phenol red was added to a final concentration of 17 mg/l. The culture tubes contained inverted Durham tubes. The substrates were tested at final concentrations of 0.2% (w/v or v/v). All substrates were sterilized separately: the carbohydrates and the organic acids by autoclaving, the aliphatic alcohols by filtration. All cultures were incubated for 24 h at 30°C in a reciprocal shaker incubator and then inspected for growth, acid and gas production.

Motility

Motility was determined in semi-solid CM9 medium which contained 0.2% (w/v) glucose and 0.5% (w/v) Bacto-agar (Difco). Tubes containing the motility medium were inoculated with a straight needle to one-half the depth of the tube. After incubation for 24 h at 30°C the cultures were examined to determine if the organisms were motile and if they required O_2.

Vitamin requirements

The CM9 medium, without yeast extract, was used to determine the vitamin requirements. The medium contained 0.2% (w/v) glucose as the energy source. Filter-sterilized biotin and/or thiamine was added aseptically to final concentrations of 0.1, 0.5 and 2.5 mg/ml.

Electron microscopy

Bacteria were prepared for transmission electron microscopy according to the procedure of Ryter and Kellenberger [8]. Bacteria were collected by centrifugation and washed in the Ryter-Kellenberger veronal-acetate buffer. They were then prefixed by 30 min of incubation, with shaking, at room temperature, in a 0.2% (w/v) solution of osmium tetroxide (OsO_4) in Ryter-Kellenberger veronal acetate buffer. After they had been washed with cold Ryter-Kellenberger buffer the prefixed bacteria were fixed overnight by incubation in OsO_4 (1% w/v, in the buffer) at room temperature. Fixed cells were then washed with the buffer and stained for 2 h with 0.5% (w/v) uranyl acetate in the Ryter-Kellenberg buffer at room temperature. The stained cells were then collected by centrifugation, washed with distilled H_2O, and enrobed in 2% (w/v) agar. After dehydration through a graded acetone series the samples were infiltrated with Araldite 502 (Polysciences, Inc., Warrington, PA) [6] and placed in beem capsules filled with fresh resin. After polymerization of the resin the samples were sectioned by a Sorval MT 5000 ultramicrotome, using a diamond

knife. The sections were picked up on uncoated specimen screens and then stained with lead citrate [6] and uranyl acetate. Micrographs were obtained with a Philips 300 microscope operating at 60 kV.

RESULTS

Characteristics of the cellulolytic bacterial isolate

Surface colonies of the isolate subcultured onto CM9-glucose agar were circular, convex, entire, smooth, opaque, yellow, and catalase positive. The isolate was a Gram-variable, non-motile, facultative anaerobe. Cells from early- to mid-log phase broth cultures were pleomorphic and those from the stationary phase were bacilli approximately 1.5 μm in length, and were encapsulated. Endospores were not detected in cells from either liquid or solid culture.

The organism grew optimally at 30°C, producing acid, but not gas, from D-glucose, D-fructose, D-galactose, D-mannose, L-arabinose, D-xylose, D-trehalose, D-cellobiose, glycerol, sucrose, lactose, acetate, maltose, arbutin and salicin. The isolate did not utilize erythritol, adonitol, dulcitol, mannitol, *meso*-inositol, sorbitol, D-arabinose, D-ribose, L-xylose, L-sorbose, inulin, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, adipate, azelate, malonate, succinate, glutarate, pimelate, suberate, propionate, butyrate, pentoate, *p*-hydroxybenzoate, 3,4-dihydroxybenzoate, 1,4-butanediamine, or L-asparagine.

The organism required either biotin or thiamine for growth.

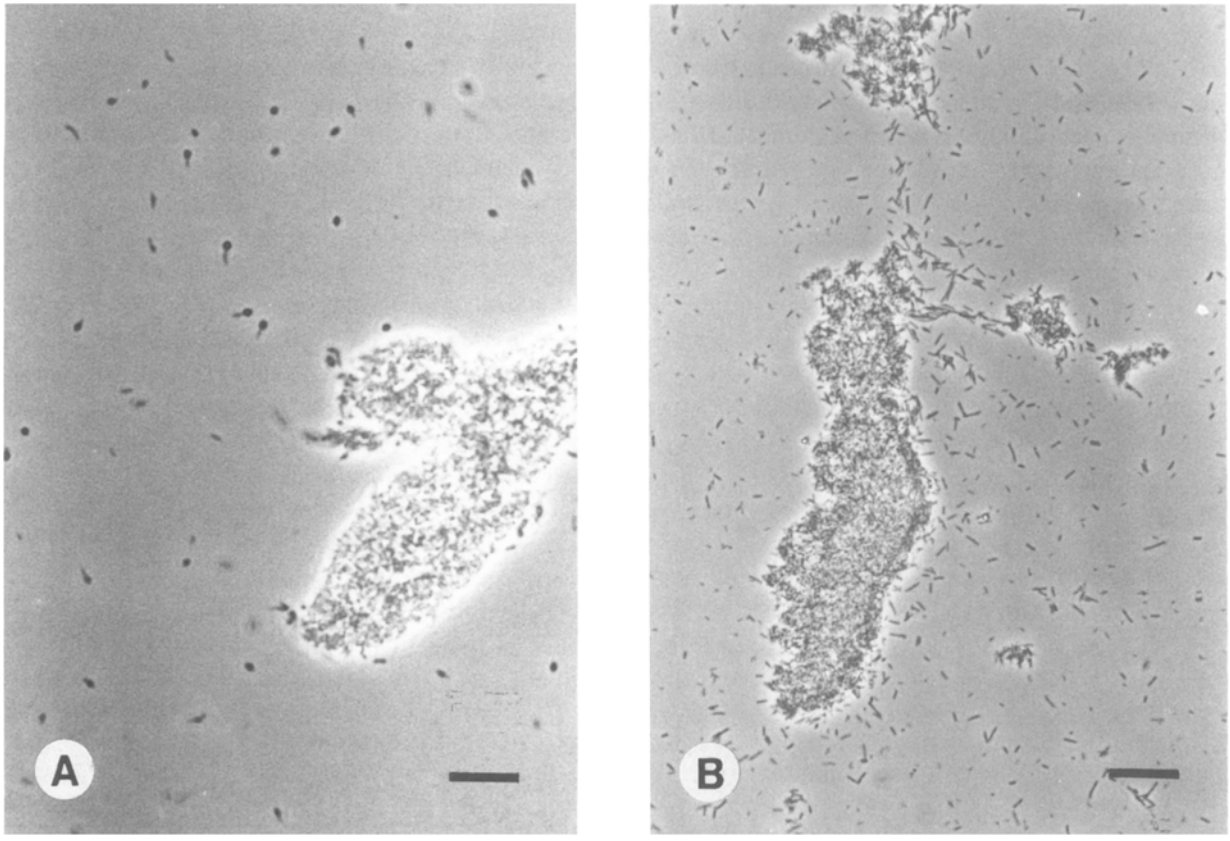

Fig. 1. Phase contrast micrographs of: A, aggregated *C. flavigena* ATCC #482 (type species) and B, aggregated *C. flavigena* strain KU. Bar = 12.5 μm.

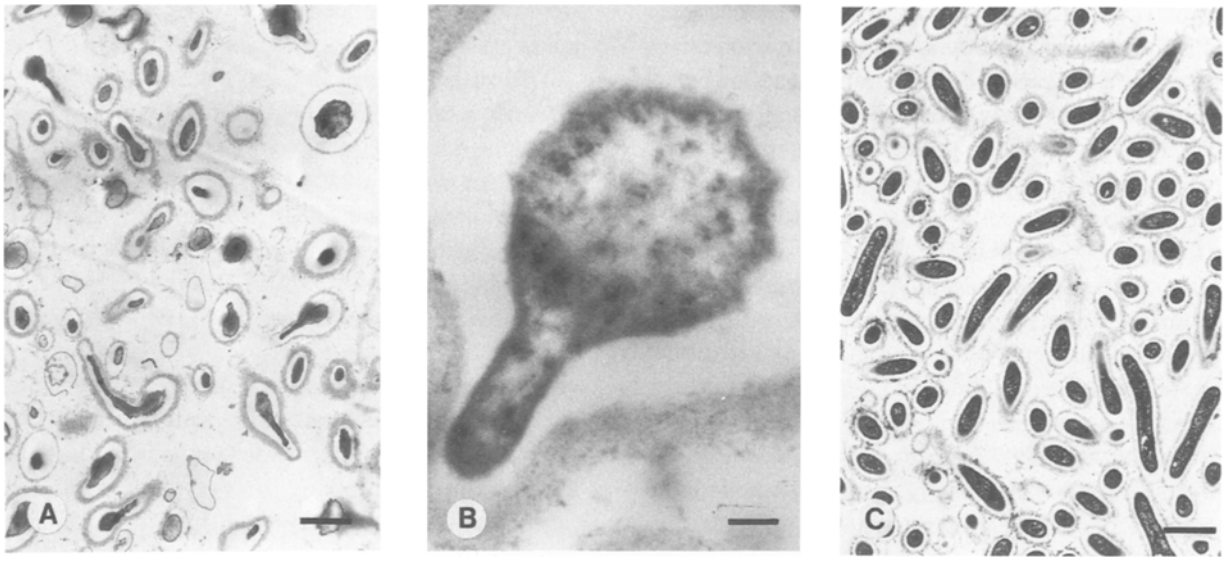

Fig. 2. Electron micrographs of aggregated *C. flavigena* ATCC 482 (type species) and *C. flavigena* strain KU (new isolate). A, type species, low magnification (bar = 1 μm); B, type species, higher magnification (bar = 0.1 μm); and C, new isolate, low magnification (bar = 1 μm).

On the basis of the above characteristics the isolate was considered to be *Cellulomonas flavigena* as described in the eighth edition of Bergey's Manual of Determinative Bacteriology [3]. However, when both the type species and the isolate were grown in a CM9 media containing 0.005 M NH_4Cl, a concentration which is growth limiting, and glucose was in excess, a prominent morphological difference became apparent. Fig. 1 is a comparison, by phase contrast microscopy, of samples taken during the stationary phase of such cultures. The ends of the type species cells, which are rod shaped during earlier stages of growth, had developed into large, swollen structures. Similar structures did not develop in the new isolate. Fig. 2 is an electron micrograph of sections of ATCC #482, the type species of *C. flavigena*, grown under conditions leading to the development of swollen cells (Fig. 2A and B) and of the new isolate (Fig. 2c).

A further difference in the two strains, again apparent after growth in CM9 glucose medium, is in the rate of their biomass synthesis. Because of technical problems attendant to aggregation of the cells, we could not determine accurately the generation time of either. However, the amount of time required by cultures of *C. flavigena* #482 to develop a given mass of cells (eg. 10 mg/ml, wet weight) was at least 3 times that required by the new isolate.

DISCUSSION

A cellulolytic bacterium isolated from soil suspensions was identified as *Cellulomonas flavigena* and is herein designated as *C. flavigena* strain KU. Apart from its vitamin requirements, the phenotypic characteristics of the isolate agreed with those in previous descriptions of *C. flavigena* [2–4]. The isolate differed from the type species in that either biotin or thiamine, but not both, was required when cultivated in a medium not containing yeast extract. The genus description of *Cellulomonas* [3] indicates that both biotin and thiamine are essential growth factors. A strain specific variation in vitamin requirements has previously been reported by Kim and Wimpenny [5] who found that *C. flavigena* KIST 321 required only thiamine as an exogenous growth factor although addition of biotin to the medium further enhanced growth of the organism.

The newly isolated *C. flavigena* strain KU de-

scribed here differs further from the type species of *C. flavigena* ATCC #482. A prominent morphological difference became manifest during the growth of the isolate and that of the type species in CM9 medium containing an excess of glucose and growth limiting amounts of inorganic nitrogen. Although morphological features alone usually are of little importance in classification [9], the difference in this case may be related to the production of an unusual extracellular polyglucan which is produced by *C. flavigena* strain KU [1] which may be used as an energy reserve [12].

ACKNOWLEDGEMENTS

This research was in part supported by grants 3379-0038 and 3978-0038 from the University of Kansas. We thank Lorraine Hammer for assistance with the electron microscopy.

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