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Taxonomic Studies on Some Gram-Positive Coryneform Hydrogen Bacteria

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Abstract. Recently isolated coryneform hydrogen bacteria were investigated under taxonomical aspects. Strains *7 C*, *RH 10*, and *14 g* are characterized by the snapping type of cell division, 68.5 to 69.7% GC content, DL-diaminopimelic acid in the cell wall, content of metachromatic granules, weak utilization of sugars and inhibitory effect of citrate. The strains are placed to the group 1—genus *Corynebacterium*—of the classification of coryneform bacteria of Yamada and Komagata (1972) and the name *Corynebacterium autotrophicum* sp.nov. is proposed.

Strains *11 X* and *RH 12* are characterized by the bending type of cell division, a GC content of 70.2 and 70.5%, LL-diaminopimelic acid in the cell wall, absence of metachromatic granules, utilization of several sugars and no changes in cell morphology by citrate. The strains have to be placed to group 6 of coryneform bacteria.

Key words: Coryneform Hydrogen Bacteria — Taxonomical Classification — *Corynebacterium autotrophicum.*

Originally the hydrogen bacteria were considered to be a rather homogeneous group of Gram-negative bacteria. The taxonomic study by Davis *et al.* (1969, 1970) clearly indicated, however, that the Gram-negative strains known so far can be placed to several genera, *Pseudomonas*, *Alcaligenes* and *Paracoccus*. Among the Gram-positive hydrogen bacteria only strains of *Nocardia* and *Mycobacterium* were investigated from a taxonomic point of view (Hirsch, 1961; Lukins and Foster, 1963). Several new strains of Gram-positive hydrogen bacteria have been isolated recently and were investigated under physiological aspects. Strain *11X* (Eberhardt, 1969), strain *14 g* (Schneider *et al.*, 1973), strain *7 C* (Siebert, 1969; Tunail, 1973; Tunail and Schlegel, 1972) and *Nocardia opaca 1 b* (Siebert, 1969; Probst and Schlegel, 1972; Aggag and Schlegel, 1973). The present study aimed at the classification of these new coryneform hydrogen bacteria in addition to some other strains which have been isolated recently.

In Bergey's Manual of Determinative Bacteriology the coryneform bacteria were characterized mainly on a morphological basis and taxonomically classified into the genera *Corynebacterium*, *Arthrobacter*,

Microbacterium, *Cellulomonas*, *Listeria*, and *Erysipelothrix*. From the studies of Jensen (1966), Bousfield (1969), and Veldkamp (1970) it became evident that the data given were not sufficient for a classification of coryneform bacteria. Since then new characters were studied usefully for classification. The chemical structure and composition of the cell wall peptidoglycan was found to be a useful tool for further differentiation (Yamada and Komagata, 1970; Fiedler, 1971; Schleifer and Kandler, 1972). Considering the type of cell fission, GC content, peptidoglycan composition and other characters, Yamada and Komagata (1972) subdivided the coryneform bacteria into seven taxonomical groups. The data presented in this study allow to place some of the coryneform hydrogen bacteria to two of these groups. Furthermore, a species name is proposed for the strains placed to the genus *Corynebacterium*: On the basis of their ability to grow chemolithoautotrophically as hydrogen bacteria three strains are named *Corynebacterium autotrophicum* sp.nov. with strain *7 C* as the type strain.

Material and Methods

Strains Examined. The coryneform strains *7 C* (DSM 432; Siebert, 1969; Tunail, 1973), *11 X* (DSM 577; Eberhardt, 1965, 1969) and *14 g* (DSM 431; Rudolph, 1968; Schneider *et al.*, 1973) were obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen. The strains *RH 10* and *RH 12* were recently isolated by the authors. *Arthrobacter globiformis* ATCC 8010, *A. simplex* ATCC 6946 and *A. atrocyaneus* CCM 1645 were obtained for this study from the ATCC and used for comparison.

Morphological characteristics such as cell form, cell size, pleomorphism, and Gram-stain were observed at cells grown on liquid R-medium consisting of 7.0 g yeast extract (Difco), 5.0 g casamino acids (Difco), 2.0 g beef extract (Difco), 5.0 g gluconic acid, and 1000 ml distilled water (pH 7.2) after 6 hrs, 24 hrs, 4 days, and 10 days incubation at 27°C. The citrate effect as described by Komagata *et al.* (1969) was examined using cells grown on nutrient broth (Difco) supplemented by 2% sodium-citrate.

Staining-Methods. Gram-stain (Huckers' modification) metachromatic granules (Albert's diphtheria-stain), and acid-fast staining (Ziel-Neelson-method) were tested according to the Manual of Microbiological Methods (Society of American Bacteriologists, 1957) after 24 hrs, 4 days, and 10 days growth on R-agar-medium.

Colony form (*e.g.* surface structure, pigmentation etc.) on agar plate culture was inspected after 1 and 10 days incubation on nutrient broth (Difco) and R-medium.

Physiological Characteristics. Voges-Proskauer reaction, H_2S-production from cysteine (paper-strip-method), indole production, reduction of nitrate, gas formation from nitrate and catalase reaction were tested according to the Manual of Microbiological Methods. The cleavage of carbohydrates was observed on a solid medium containing 3.0 g peptone (Difco), 2.4 g NaCl, 5.0 g carbohydrates, 20 g Bacto agar, and 1000 ml distilled water (pH 7.2). Bromcresol purple was used as a pH-indicator. Assimilation of organic acids was tested on the medium consisting of 2.0 g organic acids, 0.1 g yeast extract (Difco), 0.1 g peptone (Difco), 1.0 g K_2HPO_4, 5 g NaCl, 0.012 g phenol red, 20 g Bacto agar, and 1000 ml distilled water. Assimilation of

organic acids was detected by the change of the color after 5 days of incubation. Hydrolysis of gelatine, casein and starch was tested using brain-heart-infusion broth (Difco) as a basal medium containing 0.4% of each. Inspection for hydrolysis occurred after 1, 3 and 5 days incubation at 27° C. Urease and extracellular DNase were detected by using the Difco medium supplemented by 0.2% brain-heart-infusion medium (Difco). The relationship to NaCl concentration and temperature were tested using brain-heart-infusion broth as basal medium.

Determination of DNA Base Composition. The bacteria tested were grown in the basal medium described by Schlegel *et al.* (1961) supplemented by 0.5% Na-gluconate, 0.1% tryptose (Difco), and 0.1% yeast extract (Difco). To achieve lysozyme sensitivity, strains *7 C*, *RH 10* and *14 g* were transferred to a medium containing glycine at a final concentration of 0.25%. Strains *11 X* and *RH 12* were grown in the same medium containing 4% glycine. The cells were harvested in the logarithmic growth phase, and washed twice with 0.15 M NaCl and 0.1 M EDTA, pH 8.0. The isolation and purification of DNA was carried out according to the method of Marmur (1961). The guanine-cytosine (GC) content was measured by the thermal denaturation method of Mandel and Marmur (1968) using a tenfold dilution of the standard saline citrate buffer. As a control DNA (% GC value 62) was prepared from *Arthrobacter globiformis* ATCC 8010. The determination was carried out using a Pye Unicam SP 1800 spectrometer with automatic cuvette changer. The temperature of the cuvette holder was raised at a rate of 30° C/hr with a Lauda U3-S15 thermostat coupled to a Lauda P 120 linear temperature programmer. During heating the temperature was measured directly in the DNA solution with Pt 100 resistance sensors inserted in the sample cuvettes and a Doric digital thermometer DS 100-T5 (Doric Scientific Corp., U.S.A.). Absorbance and temperature values of each sample were read each minute and printed by a PA BCD-Moduprint. T_m was determined graphically after correction of absorbance values for thermal solvent expansion.

Cell walls were prepared from bacteria grown in the medium mentioned above, however, without glycine. Cells reaching the stationary growth phase were harvested and washed twice with distilled water. The preparation of the cell walls and the determination of cell wall components were carried out according to the procedure of Fiedler *et al.* (1973).

Results

Morphological Characteristics

The hydrogen bacteria included in this investigation were immotile non-flagellated coryneform rods. All strains were Gram-positive or Gram-variable and non-acidfast. The largest cells were found after 6 hrs growth on nutrient broth (Difco) medium. The yellow pigmented strains *7 C*, *RH 10* and *14 g* did not exhibit a distinctive pleomorphism; the cell shape was rather uniform; they formed rods with a diameter of about 0.4 to 0.7 μm and length of 0.8 to 3.0 μm (Fig. 1). A snapping type of cell division was observed. In contrast, the cell shape of the strains *11 X* and *RH 12* changed from rod-shaped to coccoid with increasing age of culture (Fig. 2).

The yellow pigmented strains grew faintly in the medium containing 2% citrate as carbon source, and no branching or remarkable elongation of the cells was observed in this medium. Slight branching and elongation

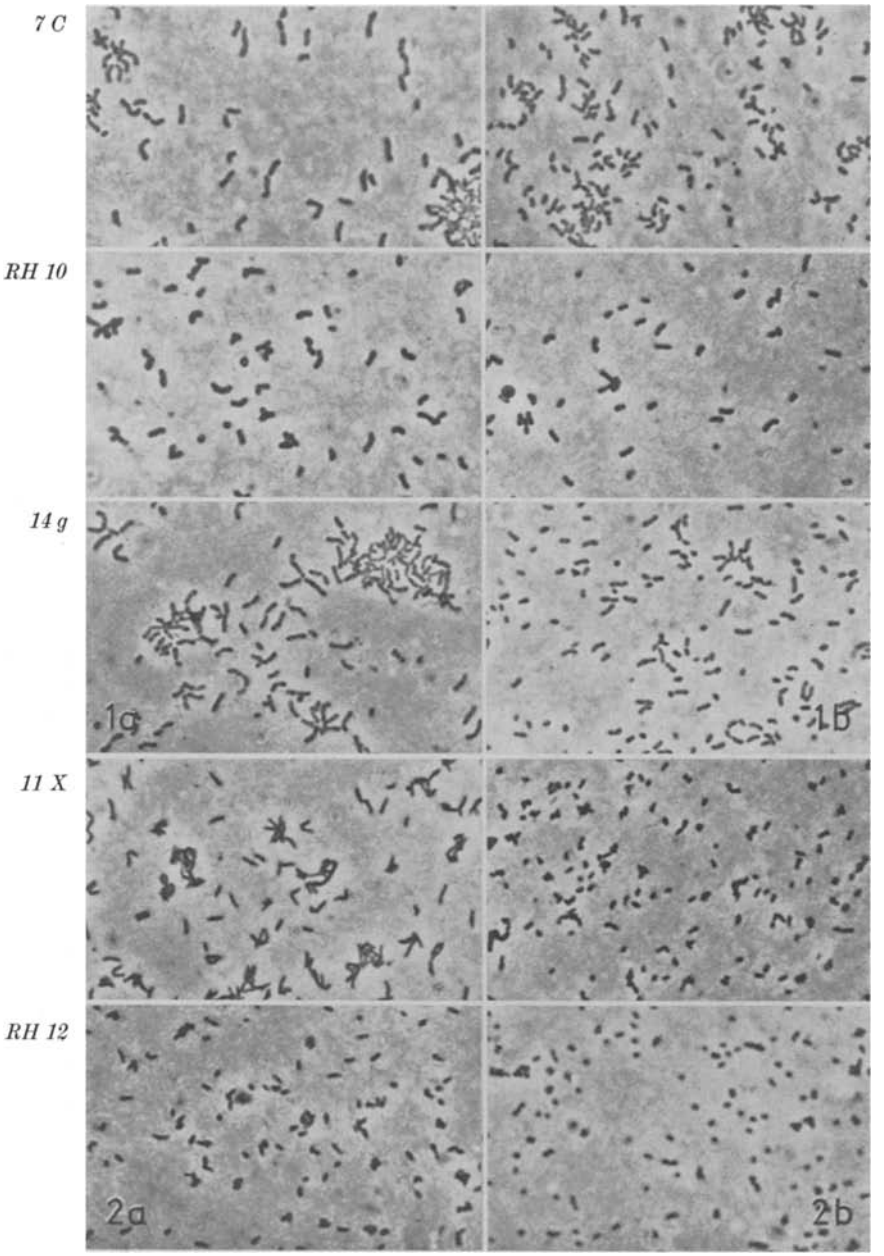

Fig. 1 a and b. Morphological changes in cells of strains *7 C*, *RH 10* and *14 g* after growth in R-medium at 27° C (a) for 6 and (b) for 24 hrs. Phase-contrast micrographs, magnification 850-fold

Fig. 2 a and b. Morphological changes in cells of strains *11 X* and *RH 12* after growth in nutrient broth (Difco) medium at 27° C (a) for 6 and (b) for 24 hrs. Phase-contrast micrographs, magnification 850-fold

was observed in a medium containing succinate. Large spherical bodies, cystites, were never observed. Metachromatic granules were formed in R-medium after 24 hrs. Strain *14 g* could be easily differentiated from the other yellow pigmented strains by the formation of rosettes in liquid media containing nutrient broth.

On solid media colonies were formed which are round, yellow, slimy, opaque, and with an entire edge. The yellow pigmentation was caused by carotenoids (*14 g*: Schneider *et al.*, 1973; Nybraaten and Liaaen-Jensen, 1974; *7 C*: Tunail and Schlegel, 1972).

The non-pigmented strains *11 X* and *RH 12* differed from the strains described in several characters. They exhibited a very distinctive pleomorphism. They multiplied by the bending type of cell division. Cystites and elongations were often observed. After 72 hrs growth the cells became short rods or cocci with an average size of about 1 μm, however, growth in 2% sodium citrate medium did not result in irregular cell shapes. Metachromatic granules were never encountered. The colonies on agar media were circular, undulate, smooth, gray to white, and opalescent.

Physiological and Biochemical Properties

All 5 coryneform strains were able to utilize ammonium as a nitrogen source. Their ability to use carbohydrates as sole source of carbon was rather distinctive. Whereas the non-pigmented strains *11 X* and *RH 12* grew with a fairly wide range of carbohydrates, the pigmented strains *7 C*, *RH 10* and *14 g* were more fastidious (Table 1). Growth of all strains was good on formate, acetate, citrate, succinate, and fumarate. The strains *7 C*, *RH 10* and *14 g* were able to grow on DL-lactate, pyruvate and some other organic acids in addition (Table 2). The strains *RH 10* and *RH 12* required thiamine for growth. Neither acid nor gas was produced from carbohydrates by any strain.

The following physiological properties were common to all strains: Hydrolysis of starch: negative; Voges-Proskauer reaction: negative; hydrogen sulfide production from cysteine: negative; indole production: negative; urease formation: negative; nitrate reduction: nitrite produced but no gas formation; optimum temperature for growth: 30—37°C; no growth at 52°C; catalase: positive; anaerobic growth (with or without nitrate): negative.

The following characters allow a subdivision of these five strains into two groups: the first group formed by strains *7 C*, *RH 10* and *14 g* was neither able to hydrolyze gelatine or casein nor showed activity of extracellular DNase. The GC-content determined by the thermal denaturation method of Mandel and Marmur (1968) was within a range from 68.5 to 69.7%. The characteristic amino acid of the peptidoglycan was DL-DAP.

Table 1
Assimilation of carbohydrates and organic acids by coryneform hydrogen bacteria

Substrates tested	Strains				
	7 C	*RH 10*	*14 g*	*11 X*	*RH 12*
Glucose	−	−	−	+	+
Fructose	+	+	−	+	+
Mannose	−	−	−	−	−
Galactose	−	−	−	−	−
Cellobiose	−	−	−	−	−
Arabinose	−	−	−	−	−
Maltose	−	−	−	+	+
Sorbose	−	−	−	−	−
Sucrose	−	−	−	+	+
Xylose	−	−	−	+	+
Trehalose	−	−	−	+	+
Rhamnose	−	−	−	−	−
Acetic acid	+	+	+	+	+
Pyruvic acid	+	+	+	−	−
D, L-Lactic acid	+	+	+	−	−
Succinic acid	+	+	+	+	+
Fumaric acid	+	+	+	+	+
Malic acid	+	+	+	+	+
Citric acid	+	+	+	+	+
Formic acid	+	+	+	+	+
Gluconic acid	+	+	+	+	+
Hippuric acid	−	−	−	−	−
Uric acid	+	+	+	−	−
α-Oxo-glutaric acid	+	+	+	−	−
Glutaric acid	+	+	+	−	−
Malonic acid	−	−	−	−	−
Glyoxylic acid	+	+	+	−	−

Table 2. Characteristics for grouping the coryneform hydrogen bacteria

	Strains tested	
	7 C, RH 10, 14 g	*11 X, RH 12*
Principal amino acid in the cell wall	DL-DAP	LL-DAP; Glycine
Type of cell division	snapping	bending
GC content (%)	69.7 69.2 68.5	70.2 70.5
Gram-stain	positive	weakly positive
Motility	non-motile	non-motile
Metachromatic granules	existent	non-existent
Pleomorphism	non-distinctive	distinctive
Citrate effect	inhibited	not inhibited
Growth in 5% NaCl	negative	positive
Utilization of sugars	weakly or absent; no acid production	positive no acid production
Utilization of organic acids	+	+
Hydrolysis of casein and gelatine	−	+
DNase activity	−	+
Urease activity	−	−

Strains *11 X* and *RH 12* form a second group; they are characterized by the ability to hydrolyze gelatine and casein and by extracellular DNase activity. The GC-content ranged from 70.2 to 70.5%. The characteristic amino acid of the peptidoglycan was LL-DAP.

Comparing these morphological and physiological data to the classification of the coryneform bacteria given by Yamada and Komagata (1972) the yellow pigmented strains *7 C*, *RH 10*, and *14 g* have to be placed to the group 1-coryneform bacteria, *i.e.* genus *Corynebacterium*. The strains *11 X* and *RH 12* have to be placed into the group 6 (LL-DAP type of coryneform bacteria). This group comprizes some of the well known *Arthrobacter* strains like *A. simplex*, *A. tumescens*, *A. variabilis*, and *A. atrocyaneus*.

Discussion

The taxonomical classification of coryneform bacteria in the system of Bergey's Manual of Determinative Bacteriology has been very difficult. The difficulties became evident in the relationship of *Brevibacterium* to the other genera of coryneform bacteria. DaSilva and Holt (1965) found by numerical analysis that the type species *Brevibacterium linens* was quite similar to *Arthrobacter globiformis*. In contrast, Bousfield (1969) and Mulder *et al.* (1966) emphasized that *B. linens* differs in several respects from *A. globiformis* and both should not be placed in the same group. On the other hand, Bousfield (1972) accomodated *B. helvolum*, *B. liquefaciens* and other species of *Brevibacterium* in the group represented by *Arthrobacter globiformis*.

These different interpretations of features indicate that the generic concept for the classification of coryneform bacteria has not been clearly established and that the features commonly used were not sufficient for classification. For their taxonomical studies Yamada and Komagata (1972) introduced some new characteristics of coryneform bacteria such as mode of cell division, GC-content of DNA, presence of metachromatic granules, and composition of the cell wall. They pointed out, that for taxonomy the type of cell wall is one of the most important characteristics because of the close relationship to other features of the coryneform bacteria. Strains containing DL-DAP in the cell wall multiplied by the snapping type of division and did not show pleomorphism. In contrast those bacteria containing LL-DAP or lysine exhibited a distinctive pleomorphism and the bending type of cell division. Furthermore, the strains characterized by DL-DAP contained metachromatic granules, whereas the LL-DAP containing bacteria did not.

From the results obtained and summarized in Table 2 it can be concluded that the strains *11 X* and *RH 12* have to be placed to group 6 of coryneform bacteria. There is full agreement with respect to all feat-

ures described for this group by Yamada and Komagata (1972). The cells were weakly Gram-positive and showed elongation, sometimes branching. Irregular variation of the cell form in a medium containing 2% sodium citrate was not distinctive. LL-DAP and glycine were found in the cell wall. The GC-content ranged from 70.2 to 70.5%. All these characteristics are in agreement with those postulated by Yamada and Komagata (1972) for group 6 (LL-DAP type coryneform bacteria). Strains of *Arthrobacter simplex* and *Arthrobacter tumescens* were placed to this group of coryneform bacteria. The proposal to classify such coryneform bacteria containing LL-DAP in their cell wall within a distinct group of *Arthrobacter* is in agreement with that of Schleifer and Kandler (1972). It seems very likely that this group of coryneform bacteria is closely related to *Streptomyces*. The GC content of both genera is quite similar. Both are characterized by complicate cell forms such as elongated and branching cells. Furthermore, LL-DAP and glycine were found in the cell walls of both genera of bacteria.

The yellow pigmented strains *7 C*, *RH 10* and *14 g* belong to the group 1—genus *Corynebacterium*—of the classification of coryneform bacteria by Yamada and Komagata (1972). The characteristics of these strains agree well with the morphological and physiological properties of bacteria of this group; especially with respect to the snapping type of cell division, the presence of metachromatic granules, the type of cell wall, and GC content. They were, however, different with respect to their tolerance to higher salt concentrations and to the citrate effect. It has to be mentioned that DL-DAP containing coryneform bacteria growing strictly aerobically have been placed into a new group by Schleifer and Kandler (1972). Most of these organisms were included into the genus *Brevibacterium*.

With respect to the most outstanding physiological properties of these bacteria, to grow chemolithoautotrophically as hydrogen bacteria, a species name is proposed: *Corynebacterium autotrophicum* sp. nov. Strain *7 C* (Siebert, 1969) and strain *14 g* (Rudolph, 1968) were the first coryneform bacteria which were isolated and characterized as hydrogen bacteria. Phenetically the three strains resemble each other; they are, however, different with respect to the ability to utilize fructose, to produce slime and to star formation. The type species is strain *7 C*.

The description of *Corynebacterium autotrophicum* is as follows: *Corynebacterium autotrophicum*, Baumgarten, Reh, Schlegel. sp. nov. auto.tro'.phi.cum. Gr.adj. *autos* by oneself, unaided; Gr.noun *trophe* nutrition (*autotrophic* = able to grow with carbon dioxide as sole carbon source).

Morphology: Cells rod-shaped with a diameter of about 0.4 to 0.7 μm and length of 0.8 to 3.0 μm. No distinctive pleomorphism, binary fission

by the snapping type of cell division. Non-motile, Gram-positive. Metachromatic granules are present. *Culture*: Obligately aerobic, chemolithoautotrophic (with molecular hydrogen) or facultatively chemoorganotrophic. Growth of all strains good on citrate, succinate, fumarate, malate, gluconate and lactate. The ability to use carbohydrates is not distinctive; only two from three strains are able to use fructose for growth. Did not hydrolyze gelatine, casein or starch nor showed extracellular DNase activity. Nitrite produced from nitrate, however, no gas formation and no anaerobic growth with nitrate. Able to use ammonium as nitrogen source or to fix molecular nitrogen.

Optimum temperature for growth 30—37°C; no growth at 52°C. No growth in the presence of 5% NaCl.

Pigments: Yellow pigmentation with zeaxanthin diglycoside as the main carotenoid.

Physiology: Inducible hydrogenase, particle-bound and not reducing NAD. Catalase is present, urease absent. No production of indole, hydrogen sulfide and carbinols. If utilized at all, fructose is degraded via the Entner-Doudoroff pathway.

DNA base composition: 68.5—69.7 moles percent guanine plus cytosine. The value for the type strain is 69.7.

Cell wall: The characteristic amino acid of the peptidoglycan is DL-diaminopimelic acid.

Habitat: Soil and mud.

Type: Strain *Corynebacterium autotrophicum 7 C* is deposited with the Deutsche Sammlung von Mikroorganismen as DSM 432.

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