Confusion in the Taxonomy of a Nitrogen-fixing Bacterium Currently Classified as *Mycobacterium flavum* 301

By D. R. BIGGINS* AND J. R. POSTGATE

A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, BN1 9QJ, Sussex

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INTRODUCTION

Federov & Kalininskaya (1961*a*) described an aerobic, allegedly Gram-negative, nitrogen-fixing bacterium isolated from turf-podzol soils and classified it as *Mycobacterium flavum* 301 according to Krasil'nikov's (1959) scheme. They pointed out that it would not be so classified according to Bergey's scheme (Breed, Murray & Smith, 1957; Buchanan, Holt & Lessel, 1966). Confusion concerning the position of mycobacteria exists, and we report here some comments and experiments relevant to the taxonomy of *M. flavum* 301. A culture is lodged in the National Collection of Industrial Bacteria (NCIB), strain number 10,071. We have reported on the nitrogen-fixing system in this organism (Biggins & Postgate, 1969).

LITERATURE AND EXPERIMENTAL

The name 'Mycobacterium flavum'

Krasil'nikov used the name *Mycobacterium flavum* in a very broad sense. He distinguished several subspecies and varieties which in Bergey's scheme are placed in genera including *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Nocardia* and *Ramibacterium*. But the name *Mycobacterium flavum* was first used by Jensen (1934) for an organism which had been named *Microbacterium flavum* by Orla-Jensen (1919); see *Bergey's Manual*, sixth edition (Breed, Murray & Hitchins, 1948). The latter name became generally accepted; Jensen's name was omitted from the seventh edition of *Bergey's Manual* (Breed *et al.* 1957).

However, the name *Mycobacterium flavum* as used by Jensen (1934) is validly published and legitimate (Buchanan, Holt & Lessel, 1966). Therefore, according to the International Code of Nomenclature, any *Mycobacterium flavum* which appeared after Jensen (1934), might be expected to follow the description he gave for this name. Krasil'nikov's description of *Mycobacterium flavum* is based on *Mycobacterium flavum* Jensen, 1934 and '*Mycobacterium flavum* Orla-Jensen, 1919'. (The latter may well be an error in transliteration and may refer actually to Orla-Jensen's *Microbacterium flavum*.) It seemed reasonable, therefore, to expect *Mycobacterium flavum* 301 to resemble *Microbacterium flavum*; we therefore sought a culture of *Microbacterium flavum* conforming to the description of Orla-Jensen (1919).

* Present address: Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, U.S.A.

Type cultures of Microbacterium flavum

The American Type Culture Collection (ATCC) organism no. 10340 is a 'co-type' of *Microbacterium flavum*, reputed to be that of Orla-Jensen (1919) (Sneath & Skerman, 1966). A second culture of it is held in the NCIB as strain 8707 and was deposited by Dr Doetsch who obtained it from Orla-Jensen. This organism was described by Doetsch & Pelczar (1948) but it does not conform to the original description of *Microbacterium flavum* given by Orla-Jensen (1919). The *Microbacterium flavum* of Orla-Jensen (1919) and the *Microbacterium flavum* of Doetsch & Pelczar (1948) are two different organisms (Buchanan *et al.* 1966; personal communication from Mr A. R. MacKenzie of the NCIB). The original strain of *Microbacterium flavum* Orla-Jensen, 1919 has apparently been lost.

In the absence of any better alternative, we obtained NCIB 8707, the *Microbacterium flavum* of Doetsch & Pelczar (1948); extensive taxonomic tests of this strain have been reported by Robinson (1966*a, b*), who suggested the species should be allotted to the genus *Corynebacterium*. Jensen (1952, 1966) suggested all the microbacteria should be classified in *Corynebacterium*.

Tests on Microbacterium flavum (NCIB 8707)

Morphology. We agree with Robinson's (1966*a*) description of the cellular and colony morphology. Colony morphology on nutrient agar was indistinguishable from *Mycobacterium flavum* 301.

Growth on various media. *Microbacterium flavum* utilized glucose and fructose (Robinson, 1966*a*); *Mycobacterium flavum* 301 did not (Federov & Kalininskaya, 1961*a, b*). *Microbacterium flavum* did not grow on Kalininskaya's low nitrogen medium (see Biggins & Postgate, 1969) with either lactate or glucose as carbon source, nor did it grow on Kalininskaya's medium with lactate + 1 g. (NH₄)₂SO₄/l. It grew well on such media supplemented with 2 g. yeast extract/l. in accord with Robinson's (1966*a*) report that *Microbacterium flavum* did not use inorganic nitrogen compounds.

Tests for acetylene reduction. Actively growing cultures of *Microbacterium flavum* in nutrient broth, dextrose peptone broth or Kalininskaya's medium (+ 2 g. yeast extract/l. with either lactate or glucose) produced no ethylene over 3 h. at 30° when tested as was *Mycobacterium flavum* 301 (Biggins & Postgate, 1969) under Ar + 0.04 atm. C₂H₂ with and without 0.05 atm. O₂.

Serology. Broken suspensions of *Microbacterium flavum* gave no reaction on Ouchterlony (1949) plates against antiserum to *Mycobacterium flavum* 301 prepared according to Coombs & Gell (1963); broken suspensions of *Mycobacterium flavum* 301 gave six precipitation lines.

Comparison of Mycobacterium flavum 301 with Corynebacterium equi

Federov & Kalininskaya (1961*a*), classifying strain 301 according to an edition of *Bergey's Manual*, considered it to be 'closely related to the species *Corynebacterium equi*'. Some strains of *C. equi* may be quite strongly acid-fast and Jensen (1952) stated that 'this species might probably with as much right be placed in *Mycobacterium*'. We obtained *C. equi* (National Collection of Type Cultures (NCTC) 1621); its cells were dumpy, almost coccoidal, rods, shorter and thicker than *Mycobacterium flavum* 301;

they did not have the swollen ends typical of the latter. *Corynebacterium equi* grew rapidly on nutrient agar (1 to 2 mm. diam. colonies in 24 h.) to produce white colonies. Suspensions of broken *C. equi* showed no reaction with antiserum to *Mycobacterium flavum* 301 on Ouchterlony plates.

Suggested relation of Mycobacterium flavum 301 to Arthrobacter

Jensen (1965) suggested that *Mycobacterium flavum* 301 'was apparently more like an Arthrobacter species', but gave no reasons for this assertion. According to Jensen (1952), mycobacteria evolved from Arthrobacter-like organisms. In Masuo & Nakagawa's (1969) survey, several Arthrobacter strains clustered at a high similarity level with species of Mycobacterium; other surveys have emphasized the mixture of organisms at present classified in Arthrobacter (Da Silva & Holt, 1965; Davies & Newton, 1969). Dr M. Tsukamura (personal communication) reported that *Mycobacterium flavum* 301 had a greater tendency to filament formation than seven representative strains of Arthrobacter.

Comparison of Mycobacterium flavum 301 with Nocardia

Morphology is a basic taxonomic character in corynebacteria though it is difficult to use because gradual transitions exist between species (e.g. the *Corynebacterium*-*Mycobacterium*-*Nocardia* complex) and because it varies with growth conditions. Morphologically, *Mycobacterium flavum* 301 appeared rather like a *Nocardia*; it tended to develop filamentous and occasionally rudimentary branched forms. However, it characteristically developed mycelial forms late in the growth phase, whereas *Nocardia* generally show mycelia early in the growth phase and fragment only later.

Nocardia calcarrea NCIB 8863 and *Nocardia cellulans* NCIB 8868 were chosen for comparison because nitrogen-fixing ability had been claimed for these species (Metcalfe & Brown, 1957) though in this laboratory the type cultures failed to fix nitrogen (Hill & Postgate, 1969). They also showed smooth, wet-type colony morphology, unlike most nocardias (Tsukamura, 1969) but like *Mycobacterium flavum* 301. They had a similar morphology to *M. flavum* 301, including a tendency to form terminal granules and swollen ends, and were of similar dimensions in shake-flask cultures on nutrient broth except that *N. cellulans* was usually about 50 % longer. Broken suspensions and crude supernatant extracts (38,000 g for 30 min.) of each *Nocardia* species did not produce any precipitation lines with antiserum to *M. flavum* 301 on Ouchterlony plates. Hill & Postgate (1969) reported that they did not reduce acetylene, even in media appropriate to *M. flavum* 301.

Mycobacterium flavum 301 showed some arylsulphatase activity which most *Nocardia* do not (Tsukamura, 1969), but only after prolonged (2 weeks) incubation. Unlike most rapidly growing mycobacteria (Tsukamura, 1966), strain 301 did not tolerate 0.2 % picric acid on Sauton's (1912) agar; it did not utilize mannose as sole carbon source, nor did it form acid from it. *Mycobacterium flavum* 301 was only partially acid-fast. Mycobacteria are usually more strongly acid-fast than *Nocardia* but acid-fastness is not a reliable taxonomic character (Jensen, 1952, 1966; Gordon, 1966); it varies with medium and staining treatment (Harrington, 1966).

Some relevant properties of Mycobacterium flavum 301

Gram reaction. We could not confirm the report of the discoverers that the organism was Gram-negative. In our hands it was Gram-positive from nutrient agar; organisms from Kalininskaya's nitrogen-free medium with lactate stained weakly but Gram-positive with only occasional Gram-negative organisms.

DNA base composition. Measurements of the melting point (T_m) of purified DNA, kindly performed at the National Collection of Industrial Bacteria, indicated 69 % guanine + cytosine. This falls within the range quoted by Hill (1966) for *Mycobacterium* (64 to 70 %) or *Nocardia* (64 to 72 %) but outside *Arthrobacter* (60 to 64 %) and *Corynebacterium* (48; 51.5 to 59 %; *Corynebacterium equi* = 58.5 %).

Mycobactin formation. No mycobactin (see Snow, 1970) was detected by Dr C. Ratledge in 0.69 g. dry wt organism grown in an iron-deficient variant of Kalininskaya's medium with lactate + 1 g. NH_4Cl and 0.2 g. yeast extract/l.

Heat resistance. Microbacteria are notably heat-resistant, though *Microbacterium flavum* is less resistant than other species in the genus, surviving 60° but not 65° for 10 min. (Robinson, 1966a). *Mycobacterium flavum* 301 grown on Kalininskaya's medium with lactate, tested for heat resistance using Robinson's conditions, survived 55° but not 60° for 10 min.

DISCUSSION

The taxonomic status of *Mycobacterium flavum* 301 is of importance when considering the distribution of nitrogen fixation among bacteria. In Bergey's schemes it might belong in the genera *Microbacterium*, *Corynebacterium*, *Nocardia*, *Arthrobacter* or *Mycobacterium*. Our experiments do not provide an unequivocal placing for the organism, chiefly because of the generally unsatisfactory nature of the taxonomy of the coryneform bacteria (Jensen, 1952, 1966), but also because *Microbacterium flavum* (NCIB 8707) appears not to be a co-type of Orla-Jensen's organism. However, *Mycobacterium flavum* 301 corresponds neither to NCIB 8707 nor to Orla-Jensen's original description. The DNA base ratio tends to restrict it to *Nocardia* or *Mycobacterium*; the absence of mycobactin in conditions in which it would readily be detected in normal mycobacteria argues against it being in the latter genus. Our experiments suggest that it is closest to *Nocardia*, which would be consistent with Metcalfe & Brown's (1957) report of nitrogen fixation in that genus, even though the type strains no longer fix nitrogen. Our findings emphasize the need to reclassify *Mycobacterium flavum* 301 according to Bergey's taxonomy and we recommend that it be included in any future taxonomic surveys of the coryneform bacteria.

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