The Mode of Regulation of Bacterial Citrate Synthase as a Taxonomic Tool

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INTRODUCTION

Certain properties of bacterial citrate synthases may be taxonomically useful. Citrate synthases from Gram-negative bacteria are inhibited by NADH. This inhibition can be overcome by AMP only in strictly aerobic Gram-negative bacteria (Weitzman & Jones, 1968; Jones & Weitzman, 1971, 1974). Further, the citrate synthases of Gram-negative bacteria have been classified as 'large' (mol. wt approx. 250000) whereas those from Gram-positive bacteria are 'small' (mol. wt approx. 100000) (Weitzman & Dunmore, 1969).

We here report the properties of citrate synthase from several bacteria which either give an equivocal Gram reaction or whose taxonomic position is uncertain for other reasons.

METHODS

Strains. The bacteria used are listed in Table 1.

Gram stain. Gram stains were performed as described by Baker (1967). Acetone was used as the decolourizer and carbol fuchsin as the counter stain.

Preparation of extracts. All bacteria were grown in 500 ml nutrient broth (Oxoid) in 2 l flasks, with shaking, for 24 h at 30 °C. Bacteria were collected by centrifuging at 25000 g for 10 min, washed with buffer of composition 20 mM-tris (hydroxymethyl) aminomethane-hydrochloride (pH 8.0), 10 mM-MgCl₂ and 1 mM-ethylenediaminetetracetic acid, and disrupted by ultrasonic treatment in an MSE 100 W sonicator for 2 min at full power with cooling. After centrifuging again at 25000 g for 10 min the supernatants were used without further purification.

Enzyme studies. Measurement of citrate synthase activity and of the effects of NADH and AMP were carried out spectrophotometrically at 412 nm as previously described (Weitzman & Jones, 1968).

The molecular size of citrate synthase was determined by gel filtration on Sephadex G200 using catalase (beef liver, 2 times crystallized; Sigma) and lactate dehydrogenase (rabbit muscle, The Boehringer Corp., Ltd, London) as marker proteins (Weitzman & Dunmore, 1969). 'Large' citrate synthases were eluted before catalase and 'small' enzymes were eluted after lactate dehydrogenase.

RESULTS AND DISCUSSION

The characteristics of the citrate synthases from 9 strains of bacteria are listed in Table 1.

Cellulomonas rossica NCIB8074 and *Corynebacterium nephridii* ATCC11425 were found to have citrate synthases characteristic of Gram-negative bacteria. Clark (1951, 1952) con-

Table 1. *Characteristics of citrate synthases from different bacteria*No citrate synthase was demonstrated in *Gemella haemolysans* NCTC10244.

	Inhibition by NADH	Reactivation by AMP	Molecular size	Gram reaction of bacteria
<i>Achromobacter liquefaciens</i> ATCC15716	—	—	Small	±
<i>Aeromonas formicans</i> ATCC13137	+	—	Large	—
<i>Cellulomonas rossica</i> NCIB8074	+	+	Large	∓
<i>Corynebacterium nephridii</i> ATCC11425	+	+	Large	∓
<i>Haemophilus vaginalis</i> NCTC10287	—	—	Small	∓
<i>Pseudomonas iodinum</i> NCIB8179	—	—	Small	±
<i>Pseudomonas aeruginosa</i> * NCIB8295	+	+	Large	—
<i>Escherichia coli</i> *	+	—	Large	—
<i>Brevibacterium linens</i> * ATCC9174	—	—	Small	+

+, Gram-positive; —, Gram-negative; ±, Gram-positive with traces of Gram-negative staining; ∓, Gram-negative with traces of Gram-positive staining.

* These bacteria are included for comparison.

sidered *Cel. rossica* to be Gram-negative and suggested its removal from the genus *Cellulomonas*. Our results support this view. *Corynebacterium nephridii*, described as a Gram-positive bacterium by Büsing, Döll & Freytag (1953), was thought to be Gram-negative by Hart, Larson & McClesky (1965) and this opinion is supported by our results.

Achromobacter liquefaciens ATCC15716, *Haemophilus vaginalis* NCTC10287 and *Pseudomonas iodinum* NCIB8179 possessed citrate synthases characteristic of Gram-positive bacteria. These results are in agreement with the views of many other workers. Thornley (1967) and Jones (1975) reported that the Gram staining reaction of *Ach. liquefaciens* was more nearly Gram-positive than Gram-negative and a numerical taxonomic study by Jones (1975) indicated a close relationship between this bacterium and certain Gram-positive bacteria.

Haemophilus vaginalis usually gives a Gram-negative staining reaction and Criswell *et al.* (1972) interpreted electron micrographs of thin sections of the wall as indicating a structure typical of Gram-negative bacteria. However, other workers are of the opinion that it is Gram-positive (Zinneman & Turner, 1963; Reyn, Birch-Andersen & Lapage, 1966; Dunkelberg, Skaggs & Kellogg, 1970; Vice & Smaron, 1973). Our results support the latter view.

Pseudomonas iodinum NCIB8179 is a species *incertae sedis* in the latest edition of *Bergey's Manual of Determinative Bacteriology* (1974). Described by Davis (1939) as invariably Gram-negative and named *Chromobacterium iodinum*, it was later transferred to the genus *Pseudomonas* (Tobie, 1945). Sneath (1956) described it as undoubtedly Gram-positive and this was later confirmed by Colwell *et al.* (1969) who concluded it was probably better classified in the Gram-positive genus *Arthrobacter*.

There has been controversy over the taxonomic position of *Aeromonas formicans* ATCC-13137 since it was described by Crawford (1954) because, although its metabolism resembles that of *Escherichia coli*, it is a polarly-flagellated rod. Colwell & Liston (1961) thought it was closely related to *Pseudomonas* spp, but later biochemical studies showed it to contain

a β -galactosidase similar to that of *E. coli* (Rohlfing & Crawford, 1966) and that the mode of tryptophan synthesis in *Aer. formicans* was similar to that in the enterobacteria (Crawford, Sikes & Melhorn, 1967). Our results are in agreement with the latter findings.

Gemella haemolysans NCTC10244, first described as a Gram-negative coccus *Neisseria haemolysans* (Thjøtta & Bøe, 1938), is now considered to be a Gram-positive coccus in the genus *Gemella* in the family Streptococcaceae (Reyn *et al.* 1966; Reyn, 1970; Reyn, Birch-Andersen & Berger, 1970). The lack of demonstrable citrate synthase in this bacterium supports this classification (Weitzman & Jones, 1968).

The reaction of bacteria to treatment with the Gram stain has long been considered a character of fundamental importance in bacterial classification. This difference has been reinforced by electron microscopy studies of cell wall structure (Glauert & Thornley, 1969) and the lack of genetic exchange between the two groups (Jones & Sneath, 1970).

In most cases the Gram reaction of bacteria is unequivocal but there are some bacteria whose reactions to the Gram stain are not clear-cut. This difficulty can generally be clarified by examination of thin sections of the cell wall by electron microscopy. However, this procedure does not always resolve the problem as, for example, in the case of *H. vaginalis* discussed above. Furthermore, the technique is not always available to bacteriologists and is a laborious procedure best performed and interpreted by specialists. In such cases the different regulatory and molecular properties of citrate synthase may provide the extra evidence required.

In other cases examination of citrate synthase may offer a first clue to misclassification. Such an example is provided by our study of the enzyme from *Brevibacterium leucinophagum* ATCC13809 (Jones & Weitzman, 1974). The citrate synthase of this supposedly Gram-positive bacterium was found to be of the Gram-negative type, suggesting an error in classification, and a thorough examination, including electron microscopy studies, confirmed that this bacterium is indeed Gram-negative. These results emphasize the value of studies on citrate synthase in establishing taxonomic relationships amongst bacteria.

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