Cytochromes in Virulent Fixed L Forms of Agrobacterium tumefaciens

R. Beltrá, J. Rodríguez de Lecea, and D. López-Abella

Instituto Jaime Ferrán de Microbiología, del Consejo Superior de Investigaciones Científicas. Joaquín Costa 32; Madrid, Spain.

We thank L Seguido de la Fuente for technical assistance in the use of the Cary 15 spectrophotometer.

ABSTRACT

We found that the normal bacterial form of *Agrobacterium tumefaciens* was oxidase-positive, and the reduced minus oxidized difference spectrum at room temperature indicates the presence of b 559 and c 550 cytochromes. Under the same conditions, the virulent fixed L forms of the same bacterial strain were oxidase-negative, and showed the presence of only b 560 cytochrome. These experiments seem to show that oxidase-positive reaction is linked to the presence of a cytochrome c, and also that the physiological and respiratory processes in L forms of *Agrobacterium tumefaciens* are different from those of the normal form of the bacterium.

RESUMEN

Se ha encontrado que la forma bacteriana normal del *Agrobacterium tumefaciens* es oxidasa positiva y que la diferencia de espectros (reducido menos oxidado), determinados a la temperatura del laboratorio, indica la presencia de citocromos b y c. En las mismas condiciones las formas L fijas patógenas de plantas y obtenidas del *A. tumefaciens*, dieron negativa la prueba de la oxidasa, mostrando la presencia de un citocromo del tipo b. Parece demostrarse que la reacción positiva de la oxidase está relacionada con la presencia de citocromo c, así como que las formas L fijas del *A. tumefaciens* son diferentes en su fisiología y procesos respiratorios en relación con la forma bacteriana normal de donde se obtuvieron.

Phytopathology 62:933-935.

Additional key words: oxidase in virulent L forms.

Currently, it is widely believed that the reaction of oxidase is due to the presence of the enzyme cytochrome oxidase, which catalyzes oxidation of the cytochrome reduced by molecular oxygen which acts as the terminal stage in electron transfer. The oxidase-positive organisms contain c-type cytochrome, a compound which is not autoxidizable

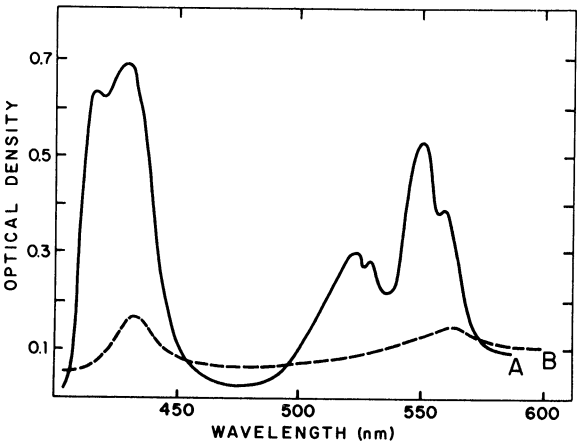

Fig. 1. Difference spectra for *Agrobacterium tumefaciens*. (Reduced with NaCN) minus (oxidized with hydrogen peroxide) at room temperature (A). The same for fixed L forms of *A. tumefaciens* (B).

but only oxidized in the presence of cytochrome c oxidase. According to Stanier et al. (10, 11), the presence of a specific cytochrome can be taken as a basic datum for characterizing and taxonomically differentiating groups of bacteria.

Sands & Schroth (9), working with bacterial phytopathogens including *Agrobacterium tumefaciens*, found that the difference of the cytochrome spectra (reduced minus oxidized) showed that the presence of a c-type cytochrome in the respiratory terminal chain of these bacteria was proof of the positive oxidase as well. Manasse & Corpe (5), working with thick suspensions of whole cells of *A. tumefaciens* prepared in glycerol:water (2:1), found that the reduced minus oxidized spectra exhibited several peaks in the visible region which were indicative of cytochrome c, b, and a_3.

In the present work, we tried to determine whether the respiratory pathway of the pathogenic L forms of *A. tumefaciens* obtained by Rubio-Huertos & Beltrá (7) had been modified, and hence if it were possible to classify them taxonomically without regard to the normal bacterial form from which they originated. It has not been possible to identify L forms with certainty on the basis of biochemical activity only, since enzymatically they are similar but not necessarily identical with the parent form.

The strain of *A. tumefaciens* (ATV of our collection) was the parent organism from which we previously had obtained the fixed L forms by means of massive transfers in culture media containing 4% glycine (7). In both fixed L and normal forms of *A. tumefaciens*, we investigated the reaction of the oxidase by the techniques of Gordon & McLeod (2), Kovacs (4), and the reaction of the cytochrome oxidase introduced by Gaby & Hadley (1). We found that the normal bacterial form of *A. tumefaciens* was oxidase-positive or cytochrome-oxidase-positive, whereas the fixed L forms of the same bacterial strain were negative.

Therefore, we conducted experiments to determine if the fixed L forms of *A. tumefaciens*, which were oxidase-negative, had also lost the c-type cytochrome reported by Sands & Schroth (9) in a normal strain of *A. tumefaciens*. Both the fixed L and normal forms of *A. tumefaciens* were grown in the culture medium described by Sands et al. (8) in two sets of three conical flasks each. Each flask contained 500 ml of medium. The flasks were inoculated with 10^{10} cells from 24-hr-old cultures of both forms and incubated on a reciprocating shaker (90 strokes/min, 10-cm elongation) at 27-28 C. After 24 hr of growth, cultures were centrifuged, and both the normal and the fixed L forms of *A. tumefaciens* were washed with a 0.05 M phosphate buffer solution at pH 6.8. Cells of *A. tumefaciens* and fixed L forms (0.5 g, wet weight) were suspended in 3 ml of buffer solution and stored at 0 C until difference spectra were recorded 3 hr later.

Laboratory temperature spectra were recorded using a Cary 15 spectrophotometer. Reduced minus oxidized difference spectra of cell suspensions were obtained by reducing one cuvette with 10 mg NaCN 5 min previous to storage at 0 C, and oxidizing the other one with four drops of fresh 3% H_2O_2 just before recording the spectra.

The reduced minus oxidized difference spectrum for the normal form of *A. tumefaciens* at room temperature (Fig. 1, curve A) indicates the presence of a b-type cytochrome with absorption peaks at 559, 528, and 430 nm. The presence of a c-type cytochrome with absorption peaks at 550, 521, and 415 nm has also been observed. The fixed L forms of *A. tumefaciens* (Fig. 1, curve B) showed the presence as well of a b-type cytochrome with absorption peaks at 560 and 430 nm.

Peaks corresponding to a c-type cytochrome were not observed.

Our results confirm claims made by several workers that the oxidase test is of value in distinguishing between different groups of organisms, and they support the idea (11) that the oxidase-positive reaction is linked to the presence of a cytochrome c. Keane et al. (3) studied the identification and nomenclature of different species of *Agrobacterium* and found two distinct biotypes; moreover, these workers concluded that present division of the genus into species based on pathogenicity is untenable. They found a virulent strain of *A. rizogenes* that is oxidase-negative, but these workers did not study the oxidase test; cytochrome c correlation. Our work shows that the normal form of *A. tumefaciens* can be distinguished from its fixed L forms on the basis of the oxidase test and its cytochrome complement. It also gives us no evidence for the role of cytochrome c in tumor induction.

The exact location of cytochromes in gram-negative cells has not been established, but enzymatic activity in which cytochromes participate is found in cell envelopes (6). In spite of the fact that these fixed L forms retain their cell wall as described by Rubio-Huertos & Beltrá (7), the difference we

found between the cytochromes present in the normal and the fixed L forms of *A. tumefaciens* is due to the latter having been modified in their chemical and enzymatic composition during growth on glycine. We conclude that the L forms of *A. tumefaciens* are changed, not only cytologically but also in their physiological respiratory processes.

LITERATURE CITED

1. GABY, W. L., & C. HADLEY. 1957. Practical laboratory test for the identification of Pseudomonas aeruginosa. J. Bacteriol. 74:356-358.
2. GORDON, J., & J. W. MC LEOD. 1928. The practical application of the direct oxidase reaction in bacteriology. J. Pathol. Bacteriol. 31:185.
3. KEANE, P. J., A. KERR, & P. B. NEW. 1970. Crown gall of stone fruit. II. Identification and nomenclature of Agrobacterium isolates. Australian J. Biol. Sci. 23:585-595.
4. KOVACS, N. 1956. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature, London. 178:703.
5. MANASSE, R. J., & W. A. CORPE. 1967. Chemical composition of cell envelopes from Agrobacterium tumefaciens. Can. J. Microbiol. 13:1591-1603.
6. MARR, A. G. 1960. Enzyme localization in bacteria. Annu. Rev. Microbiol. 14:241-260.
7. RUBIO-HUERTOS, M., & R. BELTRA. 1962. Fixed pathogenic L forms of Agrobacterium tumefaciens. Nature, London. 195:101.
8. SANDS, D. C., F. H. GLEASON, & D. C. HILDEBRAND. 1967. Cytochromes of Pseudomonas syringae. J. Bacteriol. 94:1785-1786.
9. SANDS, D. C., & M. N. SCHROTH. 1966. Cytochromes and the oxidase test in phytopathogenic bacteria. Phytopathology 56:898.
10. STANIER, R. Y., I. C. GUNSALUS, & C. F. GUNSALUS. 1953. The enzymatic conversion of mandelic acid to benzoic acid. II. Properties of the particulate fractions. J. Bacteriol. 66:543-547.
11. STANIER, R. Y., N. J. PALLERONI, & M. DOUDOROFF. 1966. The aerobic Pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159-271.