A Comparative Study of Chemical Fractions Isolated from *Agrobacterium tumefaciens* and from its Stable L-Form

By R. BELTRÁ, J. RODRIGUEZ DE LECEA AND C. DE LA ROSA

*Instituto 'Jaime Ferrán' de Microbiología,
del Consejo Superior de Investigaciones Científicas,
Joaquín Costa 32, Madrid-6, Spain*

(Received 3 December 1971; revised 23 May 1972)

Rubio-Huertos & Beltrá (1962) were the first to obtain from a phytopathogenic bacterium a stable L-form having pathogenic characteristics similar to the parent. This L-form could induce tumours on different plant species and be recovered from the tumour, showing that it had not reverted *in vivo* to the parental type. The pathogenicity, chemical composition, etc. of a variety of stable L-forms of micro-organisms pathogenic to man have been studied fairly extensively (e.g. González & Rubio-Huertos, 1959; Nesbitt & Lennarz, 1965). Less attention has been paid to these forms of plant pathogenic bacteria, although Santaolalla (1963, 1965, 1966) studied *Agrobacterium tumefaciens*. This communication describes a comparative chemical study of the carbohydrates, amino acids, trichloroacetic acid (TCA)-insoluble fraction and nucleic acids from *A. tumefaciens* and from its fixed L-form.

Throughout this work we used strain no. 13 (from our collection) of *Agrobacterium tumefaciens*. The fixed L-form was obtained by Rubio-Huertos & Beltrá (1962) by several successive transfers on glucose nutrient agar containing 4 % (w/v) glycine and incubation at 26 °C for 2 to 3 days. Chemicals used throughout this work were of analytical grade from Sigma Chemical Co., St Louis, Missouri, U.S.A., from Probus S.A., Barcelona, Spain, or from Fluka A.G., Buchs S.G., Switzerland.

Bacteria were grown at 26 °C for 5 days on agar-glucose broth of the following composition (g/l): peptone, 10; Difco meat extract, 3; NaCl, 5; glucose, 20; agar, 20; pH 7.2, in Roux bottles. They were suspended in 0.85 % NaCl solution and then repeatedly centrifuged (3000 g, 20 min) and washed. The sediment was dried *in vacuo* over P₂O₅ and then weighed. For analysis, the dry bacteria were suspended in distilled water and TCA added to a final concentration of 10 % (v/v). After storage at 1 °C overnight, the TCA-insoluble fraction was obtained by centrifugation as above. The precipitate was washed several times with ethanol, ethanol-ether (1:1, v/v), and finally ether, and amounted to 56.05 g and 41.40 g/100 g dry bacteria of the normal and L-forms respectively.

The TCA-insoluble fraction was subjected to an acid (6 N-HCl, 100 °C, 16 h) or alkaline (Ba(OH)₂ (4 %, w/v), 125 °C, 18 to 20 h) hydrolysis and the amino acids identified and estimated by the chromatographic method of Kay, Harris & Entenman (1956). The same amino acids were present in the acid hydrolysate of both the normal and the fixed L-form: tryptophan was present in the alkaline hydrolysates of both forms. The concentrations of these amino acids are listed in Table 1. These results are in accord with the view of Bolton (1962) that the capacity of sphaeroplasts to form their amino acid pool is the same as that of the parent organism. A lower content of amino acids was found for the L-form compared with the normal form (cf. Britten & McClure, 1962, working with *Escherichia coli*); this could have been due to damage to the integrity of the normal cell by the action of glycine, permitting a partial loss of the amino acid pool.

Table 1. Amount of amino acids present in the normal and in the stable L-forms of *Agrobacterium tumefaciens*

Results are expressed as mg amino acid/g trichloroacetic acid-insoluble fraction (columns A and B) and as mg amino acid/g of dry wt bacteria (columns C and D).

Amino acid	(A) Normal form (mg/g)	(B) Stable L-form (mg/g)	(C) Normal form (mg/g)	(D) Stable L-form (mg/g)
Unknown	Trace	1.92	Trace	0.50
Leucine	37.57	33.88	16.90	8.91
Phenylalanine	7.22	11.70	3.25	3.08
Valine	28.03	23.00	12.61	6.05
Tryptophan*	6.61	2.68	4.05	1.03
Tyrosine	6.90	11.31	3.10	2.97
Alanine	46.95	42.70	21.13	11.20
Threonine + glutamic acid	54.07	47.10	24.33	12.40
Glycine	30.04	31.90	13.52	8.40
Aspartic acid	24.57	17.53	11.05	4.61
Arginine	20.05	17.20	9.02	4.50
Lysine	21.86	24.70	9.83	6.50
Ornithine	1.93	5.17	0.86	1.30
Diaminopimelic acid	2.16	3.70	0.97	0.98

* Estimated after alkaline hydrolysis; other amino acids estimated after acid hydrolysis.

The concentrations of leucine, valine, threonine, glutamic acid, glycine and arginine in the normal form of *Agrobacterium tumefaciens* were practically double (and three times for aspartic acid) those in the L-form, whereas the concentrations of phenylalanine, tyrosine and lysine were approximately the same. Only ornithine was found in a higher concentration in the L-form than in the normal. Santaolalla (1963) did not find tyrosine, glycine, proline, arginine and ornithine in the 'walls' of the fixed L-form of *A. tumefaciens*, suggesting that these compounds could be of protoplasmic origin.

Diaminopimelic acid (DPA) was present in the fixed L-form of *Agrobacterium tumefaciens* at the same concentration (on a dry wt basis) as in the normal form and in a higher concentration, on the basis of the TCA-insoluble fraction.

One or both of two explanations could account for this result. Firstly, mucopeptides or their constituents could be present in the 'walls' of the L-form: amino sugars, alanine and glutamic acid were also detected, and Santaolalla (1963) found a low concentration of DPA in the 'wall' of the L-form. Secondly, the DPA could also be of cytoplasmic origin, as a constituent of nucleotide precursors of mucopeptides (cf. Sharp, 1963; Panos, 1964).

These results are similar to those of Fleck (1965) and of Weibull *et al.* (1967), who found the greatest concentration of DPA in the cytoplasmic fraction of the stable L-form of *Proteus mirabilis* and of the L strain VI and LD strain of *P. vulgaris*. However, Kandler & Zehender (1957) considered that DPA was present only in the unstable L-form of *P. vulgaris*, and not in the stable one.

The sugars present in weighed amounts of dried cells of *Agrobacterium tumefaciens* and of its L-form were identified and estimated after hydrolysis (2 N-H₂SO₄, 100 °C, 2 h). The hydrolysate after neutralization with Ba (OH)₂ and pyridine extraction was chromatographed using butan-1-ol + acetic acid + water (40 + 10 + 22, by vol.) by the method of Partridge (1947) with conventional modifications for quantitative determination of the separated sugars. Concentrations (mg/g dry wt) of the sugars found in the normal and L-form of *Agrobacterium tumefaciens* were respectively: rhamnose, 3.54 and 3.49; fucose plus ribose, 3.61 and

0.24; unknown I (R_F 0.96), 1.38 and 0.54; xylose, 0.83 and 0.12; arabinose, 2.35 and 0.11; glucose plus galactose, 14.14 and 4.11; unknown II (R_F 0.90), 0.74 and 0.46; sucrose, 0.65 and 0.09; raffinose, 0.73 and 0.03; maltose, nil and 0.03; unknown III (R_F 0.19), 0.72 and nil; glucosamine, nil and 0.14. Maltose and glucosamine were thus present in the fixed L-form but not in the normal form. The concentration of the sugars found in the L-form was generally smaller than in the normal bacterial form: rhamnose was the only methylpentose in approximately the same amount in both forms. These results are generally similar to those of Weibull (1958), who found that the conversion of *Proteus vulgaris* to its L-form involved a decrease in reducing sugar content.

For nucleic acid estimation, *Agrobacterium tumefaciens* and its fixed L-form were grown in two sets of shake cultures on the glucose-nutrient broth at 26 °C for 48 h. From each bacterial form, nucleic acid was isolated by the method of Marmur (1961) and estimated by the method of Byvoet (1966). DNA concentrations (mg/g dry wt) were: normal, 4.63; L-form, 13.43. Corresponding values for RNA were 13.40 and 161.91. Thus, contents of both DNA and RNA were much higher in the stable L-form than in the normal form. These results contrast with those of Panos (1965), who found, working with other micro-organisms, that the total content of cellular DNA in the stable L-form of a *Streptococcus* sp. was practically the same as that of the parent and that the concentration of RNA in the L-form was lower than in the normal form.

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