Surface Polysaccharides in Mutants of *Xanthomonas campestris*

By C. WHITFIELD,† I. W. SUTHERLAND* and R. E. CRIPPS

1 Department of Microbiology, University of Edinburgh, College of Agriculture, West Mains Road, Edinburgh EH9 3JG
2 Shell Research Ltd, Shell Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG

(Received 12 September 1980; revised 26 November 1980)

Mutagenesis of *Xanthomonas campestris* yielded two major classes of mutant, both having cell surface polysaccharides fundamentally different from the wild-type. The wild-type bacterium produced copious amounts of extracellular slime polysaccharide containing glucose, mannose and glucuronic acid in a ratio of 2:2:1. ‘Non-mucoid’ mutants produced trace amounts of exopolysaccharide identical to the wild-type product; ‘crenated’ mutants produced material with an unusual composition containing sugars normally found in the lipopolysaccharide. Analysis of lipopolysaccharide fractions from these strains showed that the wild-type polysaccharide fraction contained predominantly glucose. Polysaccharides from the two classes of mutant bacteria were similar and contained rhamnose, galactose and smaller amounts of glucose.

INTRODUCTION

*Xanthomonas campestris* is a bacterium pathogenic for certain brassicas (Buchanan & Gibbons, 1974). Plugging of phloem occurs owing to the accumulation of acid mucopolysaccharide, pectins, melanins and wound gums (Sutton & Williams, 1969); these symptoms are important factors in the development of rot lesions. The production of exopolysaccharide by *Xanthomonas phaseoli* has been correlated with pathogenesis (Corey & Starr, 1957); the chemical structure and physical conformation of exopolysaccharides may play an important secondary role in host recognition in plant–bacterial interactions (Dazzo & Brill, 1977; Morris *et al.*, 1977).

With current interest in the economic aspects of microbial exopolysaccharides as commercial gelling agents (see Sutherland & Ellwood, 1979), analysis of exopolymer structure and precise definition of its composition have become increasingly important. It is now apparent that most exopolysaccharide preparations from *X. campestris* contain one pyruvate group per two or three repeating units (Sandford *et al.*, 1976). In a study of the biosynthesis of xanthan gum, several mutant bacteria have been isolated in which the production of exopolysaccharide or the nature of the product is significantly different from the wild-type. Alterations in surface properties suggest alterations in the lipopolysaccharide content of the outer membrane. The compositions of the exopolysaccharide and lipopolysaccharide from wild-type and mutant strains of *X. campestris* are now reported.

METHODS

Organisms and growth. *Xanthomonas campestris* ATCC 13951 (designated T646 in this study) was originally obtained from the American Type Culture Collection. This strain and mutant strains derived from it were

† Present address: University of California School of Medicine, Department of Biological Chemistry, Davis, California 95616, U.S.A.
maintained on nutrient agar slopes and subcultured at monthly intervals. Mutants were either spontaneous or isolated following exposure to γ-irradiation.

Production and purification of exopolysaccharides. Strains were grown on 500 ml portions of solid nitrogen-deficient medium (Sutherland & Wilkinson, 1965) in sterile enamel trays (35 × 25 × 2 cm). Following incubation, bacteria were resuspended in 0-85 % (w/v) NaCl containing 0-05 % formalin, using a blender, and then removed by centrifugation at 25000 g for 30 min. Exopolysaccharides were precipitated in 3 vol. ice-cold acetone and resuspended in distilled water. Purification was achieved by dialysis for 48 h against running tap water and reprecipitation with acetone. Purified exopolysaccharides were lyophilized.

Preparation of lipopolysaccharides. Lipopolysaccharides were extracted from lyophilized bacteria with aqueous phenol (Westphal & Lüderitz, 1954). Phenol was subsequently removed by dialysis and extracts were concentrated under reduced pressure. Lipopolysaccharide was isolated by centrifugation at 100000 g for 4 h and lyophilized.

Partial acid hydrolysis of lipopolysaccharides. Lipopolysaccharide (10–20 mg) was hydrolysed in 2 ml 1 % (v/v) aqueous acetic acid in sealed glass ampoules for 90 min at 100 °C. Lipid A coagulated by this treatment was removed by centrifugation and the purified polysaccharide in the supernatant was dialysed prior to lyophilization. To examine the relative amounts of O-antigen and core poly saccharide, partially hydrolysed polysaccharide samples were subjected to chromatography on a column of Sephadex G-50, superfine (40 × 2 cm) eluted with pyridinium acetate buffer (pH 5–4) at a flow rate of 20 ml h⁻¹. Fractions of 2 ml were collected and analysed for total carbohydrate.

Hydrolysis of polysaccharides. Glycosidic linkages were hydrolysed by treatment for 18 h in 0-25 m-H₂SO₄ at 100 °C in sealed glass ampoules. The hydrolysate was neutralized with excess Amberlite IR 410 resin (HCO₃⁻ form) and dried under reduced pressure.

Gas–liquid chromatography (g.l.c.). Component sugars of polysaccharide samples were analysed as their respective peracetylated aldononitrile (PAAN) derivatives, following acid hydrolysis, according to the method of Linton & Cripps (1978). Dried hydrolysates were subjected to successive treatments with (i) 20 mg hydroxyamine hydrochloride in 0-4 ml dry pyridine at 100 °C for 40 min, and (ii) 0-5 ml acetic anhydride at 100 °C for 10 min. The solvents were evaporated, the PAAN derivatives were partitioned against chloroform/water (2 : 1, by vol.) and the chloroform layer was retained for analysis. G.l.c. analysis was carried out at 195 °C using a column (175 × 0-3 cm) of 3 % neopentyl glycol succinate on Chromosorb W-AW, with N₂ carrier gas at a flow rate of 60 ml min⁻¹. The peak areas were computed by a Hewlett Packard 3380 integrator. Components were identified by their respective retention times in comparison with authentic standards.

Chemical microanalysis. Total carbohydrate was estimated by the phenol/sulphuric acid method (Dubois et al., 1956) using D-glucose as standard. Uronic acids were quantified by the carbazole method (Bitter & Muir, 1962) using glucuronolactone as standard. Pyruvate was detected enzymically using lactic dehydrogenase (Boehringer) with analytical grade sodium pyruvate as standard. O-Acetyl groups were estimated by the hydroxylamine/ferric chloride assay (Hestrin, 1949).

Paper chromatography. Descending paper chromatography was on Whatman no. 1 paper irrigated in butan-1-ol/pyridine/water (6 : 4 : 3, by vol.) (Whistler & Conrad, 1954). Sugars were detected using the alkaline silver nitrate reagent (Trevelyan et al., 1950).

RESULTS

Isolation of mutants and strain characteristics of Xanthomonas campestris T646

Following mutagenesis and subsequent recovery by incubation overnight in nutrient broth, dilutions were made in sterile 0-85 % NaCl and bacteria were spread over the surface of solid nitrogen-deficient medium. Since suitable selective techniques for the isolation of mutations affecting the production or structure of exopolysaccharide were not available, mutants altered in gross colonial appearance were isolated. These mutations occurred at a relatively high frequency.

One class of mutants, termed non-mucoid mutants, formed smaller colonies (Fig. 1b) than the wild-type X. campestris T646 (Fig. 1a) and lacked the characteristic glossy mucoid appearance. The non-mucoid mutants tested showed similar growth rates to one another and to the parent strain. Those non-mucoid mutants studied were phenotypically similar and all were stable; no spontaneous reversion to the wild-type mucoid condition occurred and no reversion was achieved by γ-irradiation or N-methyl-N‘-nitro-N-nitrosoguanidine treatment of non-mucoid strains.
Polysaccharides of *X. campestris*

Fig. 1. Colonial appearance of wild-type and mutant strains of *X. campestris* grown on nitrogen-deficient medium at 30 °C for 96 h: (a) wild-type strain T646; (b) non-mucoid mutant T646NM2; (c) crenated mutant T646D.

Table 1. Lipopolysaccharide yields from *X. campestris* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth temp. (°C)</th>
<th>Yield of crude lipopolysaccharide from lyophilized cells (% by wt, of dry cells)</th>
<th>Yield of lipid-free polysaccharide (% by wt, of lipopolysaccharide)</th>
<th>Ratio of O-antigen + core : core</th>
</tr>
</thead>
<tbody>
<tr>
<td>T646</td>
<td>30</td>
<td>1.8</td>
<td>16.7</td>
<td>1:0:22</td>
</tr>
<tr>
<td>T646NM2</td>
<td>30</td>
<td>8.9</td>
<td>51.4</td>
<td>1:0:26</td>
</tr>
<tr>
<td>T646D</td>
<td>37</td>
<td>4.9</td>
<td>38.6</td>
<td>1:0:37</td>
</tr>
<tr>
<td>T646KR</td>
<td>30</td>
<td>4.9</td>
<td>27.6</td>
<td>1:0:61</td>
</tr>
<tr>
<td>T646E</td>
<td>37</td>
<td>8.3</td>
<td>39.4</td>
<td>1:0:36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.6</td>
<td>40.9</td>
<td>1:0:40</td>
</tr>
<tr>
<td>T646E soluble*</td>
<td>30</td>
<td>3.9</td>
<td>37.8</td>
<td>1:0:53</td>
</tr>
<tr>
<td>T646E soluble*</td>
<td>37</td>
<td>Not tested</td>
<td>89.0</td>
<td>1:0:03</td>
</tr>
</tbody>
</table>

* See text.

A further class of mutants of unusual colonial morphology were collectively termed crenated mutants (Fig. 1c). These strains grew faster than the parent strain, produced extracellular polysaccharides at 30 °C and further resembled the wild-type in that no discernible capsule was present. Crenated strains could grow at 37 °C, whereas the maximum growth temperature for the parent strain was 30–32 °C, but no exopolysaccharide was produced at the higher growth temperature. A comparison of culture supernatants following 48 h incubation at 30 °C in nitrogen-limited batch culture showed that the wild-type produced about 18 mg exopolysaccharide ml⁻¹ whereas crenated mutants T646D, T646KR and T646E produced 10–12 mg ml⁻¹ and the non-mucoid mutant T646NM2 produced only trace quantities (1–3 mg ml⁻¹) under similar conditions. The pigmentation of the mutants and their cellular morphology were essentially the same as for the wild-type. Confirmation of their identity has been obtained by inserting a streptomycin-resistance marker in the wild-type and re-isolating marked mutants that conform in all respects tested to the mutants described here (I. W. Sutherland, unpublished work).

**Lipopolysaccharide analysis**

The lipopolysaccharides of *X. campestris* strains were studied to determine whether the changes in mutant characteristics could be attributed to alterations in the outer membrane. The lipopolysaccharide yields from the wild-type and mutants studied are given in Table 1.
The apparent yields of crude lipopolysaccharide were unusually high. Contamination with nucleic acids was confirmed by the absorbance at 260 nm; the $A_{260}$ correlated with the overall yield. Aqueous phenol extracts of the crenated mutants contained substantial quantities of low molecular weight polysaccharide not sedimented by centrifuging at 100,000 g for 4 h (this material from T646E is designated T646E soluble in Table 1). Yields of lipopolysaccharide from the wild-type were lower than those from the non-mucoid mutant T646NM2. Yields of lipopolysaccharide from the crenated mutants were similar whether they were grown at 30 °C or 37 °C.

The yield of polysaccharide obtained by partial hydrolysis of crude lipopolysaccharide showed that the soluble material from T646E was predominantly polysaccharide with little or no lipid A.

Chromatography of partially hydrolysed lipopolysaccharide samples on Sephadex G-50 typically revealed two peaks of carbohydrate material (Fig. 2). High molecular weight material, presumably O-antigen plus core (see, for example, Koval & Meadow, 1977), was excluded from the gel; the composition of this fraction was similar to that for the corresponding strains (Table 2). The second, minor peak contained material assumed to be core polysaccharide; it contained glucose, galactose and small amounts of amino sugar. Fractions corresponding to each peak were pooled and assayed for total carbohydrate; the ratios of peaks for polysaccharide from each strain are listed in Table 1. The results show that strains T646 and T646NM2 contained more O-antigen than the crenated strains. The soluble material from T646E contained no significant amounts of core polysaccharide.

The sugars in the polysaccharide fraction of the lipopolysaccharides were analysed as their respective PAAN derivatives. It was assumed that the relative proportions of the PAAN derivatives indicated the respective proportions of underivatized sugars in the original material and that the detector response was the same for each PAAN derivative. Seymour et al. (1979) found that the response for hexose derivatives was the same, but responses for L-rhamnose and D-ribose were 93% and 95% of the glucose value, respectively.

The polysaccharide moiety from lipopolysaccharides of mutant strains consisted predominantly of rhamnose (50–60%) with glucose (5–10%) and galactose (20–30%) also present (Table 2). No substantial differences were apparent in the polysaccharides obtained from the crenated strains grown at different temperatures. Significantly, the soluble material from T646E resembled the authentic lipopolysaccharide in composition.
Table 2. Composition of partially hydrolysed lipopolysaccharide from X. campestris strains

Values for each PAAN derivative are expressed as a percentage of the total carbohydrate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth temp. (°C)</th>
<th>Mannose</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Rhamnose</th>
<th>Ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td>T646</td>
<td>30</td>
<td>0</td>
<td>94.6</td>
<td>3.9</td>
<td>1.6</td>
<td>trace</td>
</tr>
<tr>
<td>T646NM2</td>
<td>30</td>
<td>5.5</td>
<td>9.8</td>
<td>20.0</td>
<td>61.3</td>
<td>2.8</td>
</tr>
<tr>
<td>T646D</td>
<td>30</td>
<td>trace</td>
<td>9.8</td>
<td>28.2</td>
<td>53.4</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0</td>
<td>7.5</td>
<td>30.6</td>
<td>51.0</td>
<td>3.1</td>
</tr>
<tr>
<td>T646KR</td>
<td>30</td>
<td>trace</td>
<td>8.3</td>
<td>29.6</td>
<td>53.5</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>trace</td>
<td>5.3</td>
<td>31.4</td>
<td>52.2</td>
<td>0.9</td>
</tr>
<tr>
<td>T646E</td>
<td>30</td>
<td>0</td>
<td>8.3</td>
<td>29.6</td>
<td>53.5</td>
<td>3.2</td>
</tr>
<tr>
<td>T646E soluble*</td>
<td>30</td>
<td>4.7</td>
<td>8.9</td>
<td>27.7</td>
<td>53.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* See text.

Table 3. Analysis of exopolysaccharide from X. campestris strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose (%)</th>
<th>Mannose (%)</th>
<th>Uronic acid (%)</th>
<th>Galactose (%)</th>
<th>Rhamnose (%)</th>
<th>Pyruvate [mmol (g polymer)^{-1}]</th>
<th>Acetate [mmol (g polymer)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>T646</td>
<td>42</td>
<td>38</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>0.7</td>
</tr>
<tr>
<td>T646NM2</td>
<td>40</td>
<td>38</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>0.7</td>
</tr>
<tr>
<td>T646D</td>
<td>22</td>
<td>19</td>
<td>13</td>
<td>24</td>
<td>22</td>
<td>&lt;0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>T646KR</td>
<td>29</td>
<td>21</td>
<td>11</td>
<td>27</td>
<td>12</td>
<td>&lt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>T646E</td>
<td>37</td>
<td>12</td>
<td>9</td>
<td>28</td>
<td>14</td>
<td>&lt;0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Wild-type lipopolysaccharide showed a different composition in that glucose (95%) was the major constituent with small amounts of galactose (4%) and rhamnose (1.6%).

Significant amounts of ribose were detected in each sample studied, but the presence of ribose may have been the result of extensive contamination of lipopolysaccharide extracts with nucleic acids, as indicated above.

**Exopolysaccharide analysis**

Exopolysaccharide was harvested from cultures following incubation for 60 h at 30 °C. No difference was seen in the composition of exopolysaccharide produced earlier in growth from that harvested at 60 h.

Analysis of the product from strain T646 (Table 3) revealed glucose, mannose and glucuronic acid forming the monomeric units in an approximate ratio of 2:2:1. Acetate and pyruvate groups were detected. Trace amounts of extracellular polysaccharide produced during growth of the non-mucoid mutant T646NM2 were analysed. The composition was similar to that of the wild-type polysaccharide, but smaller amounts of pyruvate were detected.

The extracellular polysaccharide from the crenated mutants was difficult to precipitate from the culture supernatant with ice-cold acetone. Polymer from wild-type cultures precipitated as a thick gel which could be wound around a stirring rod. In contrast, material from the culture supernatants of crenated strains formed an almost granular precipitate which could only be removed after allowing 1–2 h for settling.

Paper chromatography of hydrolysed polysaccharide from the crenated mutants revealed glucose, mannose and glucuronic acid together with significant amounts of galactose and rhamnose, sugars thought to be specific to the lipopolysaccharide. Chemical and g.l.c. analysis of the component sugars was confused by the finding that batches of exopoly-
saccharide from the same strain grown under identical conditions had different compositions. This result differed from that for the wild-type polymer which showed remarkably consistent composition from batch to batch. The results shown in Table 3 for the composition of polymers from crenated strains are those from typical batches; the limits of variability have not yet been established.

Significantly, the ratios of glucose to mannose were not 1:1 in exopolysaccharides from the crenated mutants, suggesting that fewer side-chains were present. Values for glucuronic acid content support this. Preliminary results also suggest that polymers from the crenated mutants could be partially degraded using a purified commercial cellulase preparation. The wild-type polymer was resistant to attack by cellulase under identical conditions.

The occurrence of significant amounts of galactose and rhamnose was difficult to explain since the proportions of the lipopolysaccharide-specific sugars did not reflect simple contamination by lipopolysaccharides. All attempts to separate a possible mixture of polymers using conventional gel chromatography techniques were unsuccessful.

**DISCUSSION**

The composition of lipopolysaccharides isolated from crenated and non-mucoid mutants of *X. campestris* resembles that of other such polymers. Water-soluble polysaccharides have been reported to contain rhamnose, galactose, glucose and ketodeoxyoctonic acid, but no heptose (Volk, 1968; Schlabach, 1970). Proportions of sugars have been reported only in phenol-soluble lipopolysaccharides (Hickman & Ashwell, 1966), which contain rhamnose (58%), 3-acetamido-3,6-dideoxy-d-galactose (25%) and smaller amounts of glucose and galacturonic acid, but no ketodeoxyoctonic acid. We could detect no ketodeoxyoctonic acid in these strains and no galacturonic acid; however, the lability of these sugars or their linkages (Volk, 1968) may have resulted in their absence.

Chromatography of partially hydrolysed lipopolysaccharide from the crenated strains indicated the absence of substantial O-antigen side-chains and the presence of chemically identical 'soluble' polysaccharides. Attachment of O-antigen to core is possibly defective in these strains, by analogy with *Salmonella* Rfa mutants with defective core or, alternatively, Rfh mutations affecting O-antigen transferases (Stocker & Mäkelä, 1978).

Some indirect evidence tends to suggest that the core polysaccharide is altered in the crenated mutants. Examination of the major outer membrane proteins (C. Whitfield, unpublished results) showed marked diminution of several protein species found in wild-type and non-mucoid strains. Similar observations have been made with lipopolysaccharide mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames et al., 1974; Chatterjee et al., 1976; Koplow & Goldfine, 1974). The report of a *Salmonella* Re mutant exhibiting altered colonial morphology (Wilkinson et al., 1972) may be significant with respect to the crenated mutants of *X. campestris*. Such results are difficult to interpret in *X. campestris* since previous reports have indicated that its core polysaccharide is significantly different from those of *E. coli* and *S. typhimurium*. The most notable difference is the absence of heptose. Instead the core is thought to consist of a single ketodeoxyoctonic acid residue substituted with mannose 1-phosphate (Volk et al., 1972); however, phosphate was not reported by Hickman & Ashwell (1966).

The presence in strain T646 of a significantly different lipopolysaccharide containing predominantly glucose is difficult to explain. It is likely that under conditions where considerable amounts of extracellular polysaccharide are produced in addition to lipopolysaccharide, co-ordination of the synthesis of these two polymers is subject to complex regulation.

The exopolysaccharide produced by strain T646 had a composition characteristic of authentic xanthan gum; the non-mucoid strain produced small amounts of similar material. Analysis of the enzymes involved in exopolysaccharide biosynthesis did not reveal any defect.
which could account for the non-mucoid phenotype (C. Whitfield, unpublished results). Previous reports concerning the biosynthesis of lipopolysaccharide in Salmonella species (Nikaido, 1968) and exopolysaccharide biosynthesis in Enterobacter aerogenes (Norval & Sutherland, 1969) have suggested that mutations affecting the later stages of polysaccharide biosynthesis occur more frequently than do mutations affecting precursor synthesis. The production of small amounts of exopolysaccharide by non-mucoid mutants of X. campestris tends to suggest that the mutation affected either transferase or polymerase activity, or the regulatory genes co-ordinating synthesis of exopolysaccharide and lipopolysaccharide, possibly accounting for alterations in the lipopolysaccharide.

The occurrence of non-mucoid or poorly mucoid strains of X. campestris has been reported elsewhere (Cadmus et al., 1976). 'Non-mucoid' polysaccharide contained less pyruvate, as did that of strain T646NM2 in our study. Cadmus et al. (1976) suggested the existence of genetic instability in mucoid X. campestris. We found no evidence to support this suggestion in batch cultures, although the selection of less mucoid bacteria may occur in carbon-limited continuous cultures.

Several strains of Xanthomonas, including X. stewartii (Gorin & Spencer, 1961) and Xanthomonas strain S19 (Fareed & Percival, 1976), contain galactose in their exopolysaccharides. Studies with X. fuscans, a subspecies of X. campestris (Buchanan & Gibbons, 1974), indicated the presence of glucose, mannose and small amounts of both 6-deoxy-L-mannose and L-glucuronic acid in the exopolysaccharide (Konishek et al., 1977). An exopolysaccharide closer in composition to lipopolysaccharide was identified in a Xanthomonas species isolated from soil (Yadomae et al., 1978). This polymer contained glucose, mannose, O-acetyl-manno-octulosonic acid and an unidentified deoxyhexose; no glucuronic acid or pyruvate was detected. The polysaccharide was unstable in 2% acetic acid at 100 °C, but did not contain lipid or phosphate. Unfortunately, no data concerning the lipopolysaccharide from this unique strain were presented.

C. W. acknowledges receipt of an S.R.C. CAPS award; I. W. S. was in receipt of S.R.C. grant GR/A/3004.9.

REFERENCES


