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# Structure and Biosynthesis of a Xanthan-Like Polysaccharide Produced by *Xanthomonas albilineans*

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## ABSTRACT

Leaf scald is a vascular disease of sugarcane plants caused by *Xanthomonas albilineans*. Scalded leaves show white-yellowish streaks alternating with green zones in parallel to the main veins. The white-yellowish streaks show both phloem and xylem completely occluded by the gum and the overall mesophyll appears to be full of this bacterial secretion, as revealed by scanning electron microscopy. The gum in conducting tissues was purified from juices obtained from scalded stalks and was identified as a xanthan-like polysaccharide composed by repeated tetrameric units containing two rests of fructose, one of mannose and one of glucuronic acid. Hydrolysis of xanthan with selective mannosidases and β-1,4-glucanases reveals that the macromolecule consists of a linear, β-1,4-backbone of β-glucose units to which mannose in β-1,3 bonds is linked. Since xanthans contain glucuronic acid, the ability of *Xanthomonas* to produce an active UDP-glucose (UDPglc) dehydrogenase is often seen as a virulence factor. *X. albilineans* produced UDPglc dehydrogenase growing on sucrose. The pI value of the purified enzyme is 8.98 with an estimated molecular mass of about 14 kDa. The enzyme shows a Michaelian kinetics for UDPglc with three different sites that interact with NADPH. The enzyme is inhibited by UDPglc concentrations higher than 1.3 mM. The N-terminal sequence is IQPYNH. *X. albilineans* axenically cultured does not secrete xanthans to liquid media but they are produced in inoculated sugarcane tissues. This host-dependence can be explained on the basis of the action of bacterial proteases upon the dehydrogenase. *In vitro* enzymatic assay of UDPglc dehydrogenase from *X. albilineans* requires the addition of a protease-inhibitors cocktail to cell-free extracts, since bacterial proteases rapidly hydrolyses the enzyme in solution. The dehydrogenase requires > 0.3 mM of both 8-azaguanine and chloramphenicol to inhibit its synthesis. Glycoproteins from sugarcane, the natural host of the bacterium, also assure the production of the active enzyme by inhibiting bacterial proteases.

**Keywords:** Sugarcane, UDPG dehydrogenase, xanthans, *Xanthomonas*, *Xylella*
**Abbreviations:** UDPGDH, uridin diphosphate glucose dehydrogenase

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## INTRODUCTION

Xanthan is an extracellular polysaccharide produced by bacteria of the genus *Xanthomonas*, and it was discovered by the first time in the bacterium *Xanthomonas campestris* B-1459. *X. campestris* belongs to the family *Xanthomonadaceae* and causes diseases such as black rot or black vein in crucifers.

The physical aspect of the xanthan is that of a powder of cremate colour that is dissolved in warm or cold water producing dissolutions of relatively high viscosity to low concentrations. The viscosity is high in a wide range of concentrations and dissolutions are stable in a wide range of pH, salt concentrations and temperatures.

The molecule of xanthan produced by *X. campestris* consists of a β-1,4 linked D-glucopiranoside main chain similar to cellulose. To this backbone, lateral chains of trisaccharide composed by residues of D-mannopiranoside and of acid D-glucuronic acid are annexed. The residues of mannoside that link to the backbone by α-1,2 bonds, have 6-O-acetyl substitutions. An average of approximately the half of the terminal groups of α-D-mannoside have substitutions 4,6-O-(1-carboxyethylidene), for example pyruvic acid which links as 4,6 acetal. Xanthan is assigned the average formula $C_{32.34}H_{49.94}O_{28.34}Na_{1.38}$ (**Fig. 1A**).

The synthesis of xanthan is achieved at the envelope of *X. campestris*. Proteins encoded by the *gum* genes are responsible of the complete synthesis of the xanthan. In the first reaction the glycosyltransferase GumD transfers a glucose-phosphate residue from UDP-glucose (UDPglc) to an undecaprenylphosphate lipid carrier located at the inner face of the cell membrane. In the four subsequent reaction steps GumM, GumH, GumK and GumI add sequentially a second glucose residue, two mannose residues, and a glucuronate residue from UDPglc, GDP-mannose (GDPman) and UDP-glucuronate (UDPglcA) to form the carbohydrate

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Invited Mini-Review

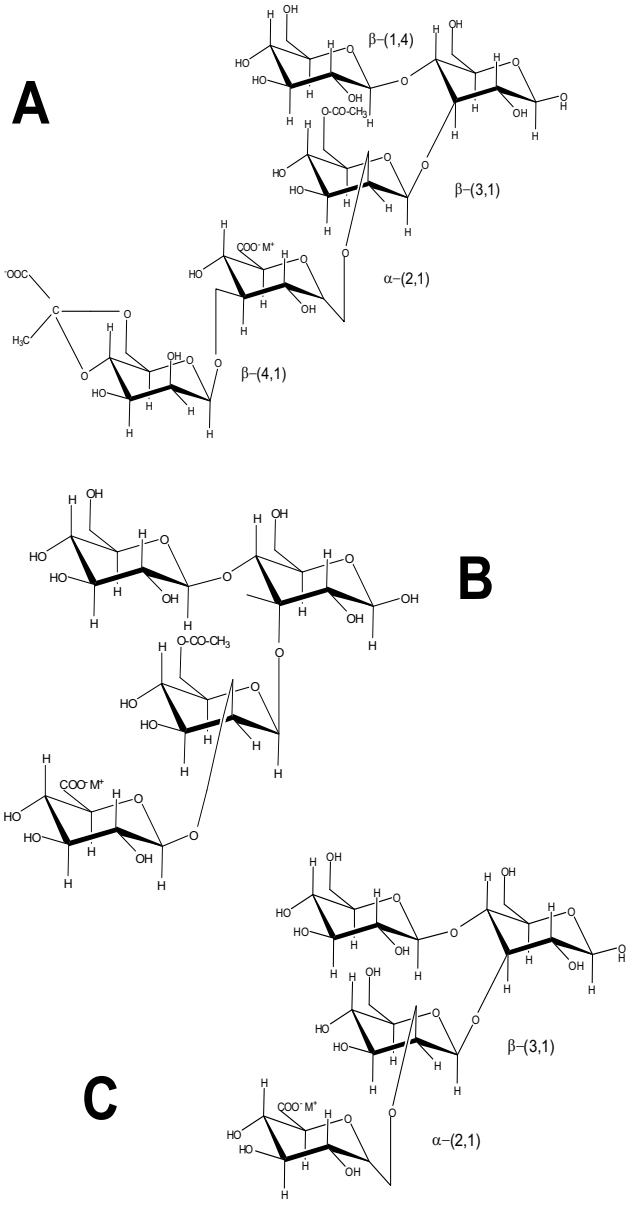


**Fig. 1** Constitutive repeats of xanthans produced by *Xanthomonas campestris* (**A**), *Xylella fastidiosa* (**B**) and *Xanthomonas albilineans* (**C**).

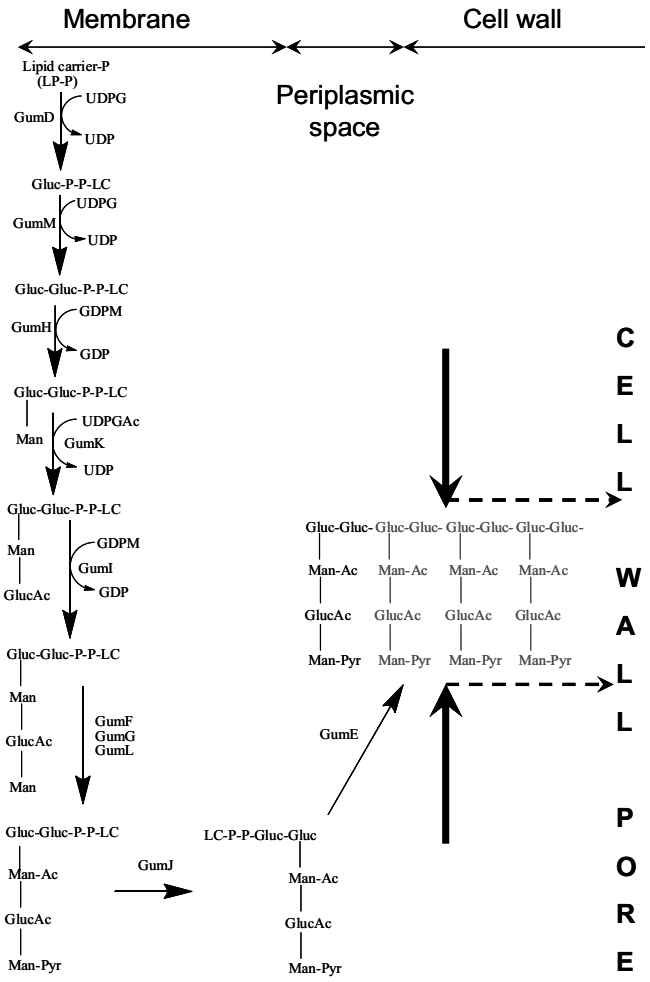


**Fig. 2 Genes involved in xanthan biosynthesis and location of each one of the enzymatic reactions conduction to xanthan secretion from *Xanthomonas campestris* cells.**

structure of a xanthan repeat unit. The outer, last-added mannose can be pyruvylated by GumL, both mannose moieties can be specifically acetylated by GumF and GumG, respectively. Finished repeat units are translocated by GumJ to the outer face of the inner membrane. In the periplasm, xanthan could be polymerized by GumE, which transfers immature xanthan polymers to newly translocated repeat units. Finally, xanthan is exported. This involves GumC, a protein that is anchored in the inner membrane with a substantial periplasmic domain. When GumC gets into contact with the outer membrane protein GumB, which is assumed to have a large periplasmic domain (**Fig. 2**), too, complexes of both proteins can form open pores which permit exportation of the mature xanthan (Vorhölter *et al*. 2008).

The action of UDPGDH has largely been studied and defined as a "*bi-uni-uni-ping-pong*" mechanism (Campbell *et al.* 1997) for humans and animals, acting in the following way: firstly UDPG, and then $NAD^+$, are joined to the enzyme. UDPG is oxidized to an aldehyde and $NAD^+$ reduced to NADH and removed from the enzyme-substrate complex. Then, a second molecule $NAD^+$ binds to the enzyme and it is reduced to NADH whereas aldehyde is oxidized to UDP-glcA, which is removed from the complex after the liberation of the second molecule of NADH. UDPGDH from plants work in the same way and sequence, as revealed by Turner and Botha (2002) for sugarcane. In *X. campestris*, the gene *udgH* encodes for UDPGDH and it play a determinant role in the bacterial pathogenicity. Mutations of *udpgH* gene *X. campestris pv. campestris* and *X. campestris pv vessicatoria*, produce a complete loss of virulence (Chang *et al.* 2001).

*Xylella fastidiosa* is a phytopathogen bacterium restricted to the plant xylem that causes a wide range of diseases with very dire economic consequences. *X. fastidiosa* produces variegated citric chlorosis. By comparing the gene sequence of *X. fastidiosa* with that from *X. campestris* pv. campestris, the genes *gumI* (that codes for glycosyltransferase V, responsible for the final mannose linkage), *gumL* (coding for cetolase that incorporates pyruvate to the polymer) and *gumG* (coding for an acetyltransferase which incorporates acetate) have not been found. This fact implies that the xanthan from *X. fastidiosa* is less viscous than that produced by *Xanthomonas*. The basic structure of the polysaccharide produced by *X. fastidiosa* is a highly repeated tetrasaccharide (**Fig. 1B**) produced by the sequential addition of UDP-glc, GDP-mannose and UDP-glucuronic acid (Rodrigues *et al.* 2001).

## PROPERTIES AND APPLICATIONS

Some industrial applications need that the content of pyruvate exceeds 3.3% in weight, this value being the best indicator of the quality of the product (Flores-Candia 1998). The molecular weight of xanthan ranges from 2 to $16 \times 10^6$ Da and depends on the fermentation conditions (García-Ochoa *et al.* 2000). The most exceptional property of xan-

than is its reactivity with galactomannans as gum guar and the gum of the carob tree. The addition of any of these galactomannans to a solution of xanthan at ambient temperature causes a synergic increase in viscosity. The viscosity of these mixtures depends on xanthan and on the galactomannan structure (Dea *et al.* 1986; Casas and García-Ochoa 1999). Conformational changes of xanthan in solution depend mainly on temperature.

When xanthan is dissolved at low temperatures (< 40ºC), it acquires a tidy conformation that allows a better interaction between xanthan and galactomannan molecules (Dea *et al.* 1977; Tako *et al*. 1984; Casas and García-Ochoa 1999).

Xanthan is joined to food to control the rheology of the final product. The polymer produces a great effect on some properties as texture, liberation of aroma and appearance, which contribute to the acceptability of the product and hence its consumption. Regarding its pseudoplastic nature in solution, xanthan produces a less gummy sensation in the mouth that gums with Newtonian behaviour (Flores-Candia 1998; García-Ochoa *et al*. 2000). Its behavior as an antioxidant is higher than that of other polysaccharides due to its great aptitude to join metals and its viscous nature.

In the pharmaceutical and cosmetic industries, xanthan is used as an emulsifier and to give body to prepared products. Personal care products as shampoos, creams, lotions, make-up, and products of capillary and tooth care can be formulated containing xanthan. In the pharmaceutical industry, xanthan is used to support antibiotics and other medicines in suspension and to achieve formulations of uniform dose and to stabilize creams containing medicines.

In agriculture, xanthan is used for improving the efficiency of fungicides, herbicides and insecticides by allowing solid components of formulations to be uniformly suspended in watery systems or by stabilizing emulsions and multiphasic liquid systems.

Xanthan's rheological properties are: facilitating pulverization, reducing wind dispersion and increasing the persistence and adhesion of pesticides. In the petroleum industry, it is used in perforation muds as a lubricant and as a flood polymer for controlling the mobility of water in the tertiary processes of improved oil recovery (Littman 1988; Song *et al*. 2006). In the secondary recovery of oil it is added to reduce the permeability and mobility of water by increasing its viscosity.

## INDUSTRIAL PRODUCTION

An inoculum is added to a bioreactor prior to production before being sterilized. Production contains a carbon source (sucrose, glucose or corn syrup) at 0-40 g $L^{-1}$, inorganic sources of nitrogen and other nutrients in minor quantities. Fermentation is carried out in aerobic conditions, while temperature, pH, dissolved oxygen, foam and agitation are all controlled. At the end of fermentation, cells are separated by centrifugation or filtration (Flores-Candia 1998; García-Ochoa and Gómez 2001). The xanthan formed can typically be separated with iso-propanol, ethanol or acetone, or by adjusting pH.

## THE ROLE OF UDP-GLC DEHYDROGENASE

UDP-glc dehydrogenase catalyses the $NAD^{+}$(H)-dependent oxidation of UDP-glc to UDP-glucuronic acid. It belongs to a small group of dehydrogenases that are able to carry out the two-fold oxidation of an alcohol to an acid without the release of an aldehyde as intermediate (Campbell *et al*. 1997). This enzyme has a wide range of functions. In plants, UDP-glc dehydrogenase is the main enzyme in the pathway of synthesis of hemicelluloses and pectins, which are the components of newly formed cell walls (Kärkönen *et al.* 2005). Nucleotide sugars are energy-rich compounds and are costly for the cell to form. Therefore, it is vital that these compounds be channelled to where they are needed and not wasted. UDPglc dehydrogenase has been suggested to be a regulatory or rate-limiting enzyme that controls part of the polysaccharide biosynthesis in plants and animals (De Luca *et al.* 1976; Robertson *et al.* 1995; Hickery *et al.* 2003). Thus, the rate of its production is not primarily governed by the amount of substrate but by the activity of the rate-limiting enzyme, UDP-glc dehydrogenase, which in turn is regulated by one or several factors. These factors include the amount of enzyme, and covalent and/or allosteric modification of the enzyme (Wegrowki *et al*. 1998).

## XANTHAN FROM *X. ALBILINEANS*

Identification of the gum produced by *X. albilineans* invading sensitive sugarcane cv. 'Louisiana' was achieved using leaves of diseased sugarcane plants showing scald symptoms (Fontaniella *et al*. 2002; Solas *et al.* 2003). Analysis of the fraction collected after filtration through Sephadex columns of the *iso*-propanol-precipitated fraction from aqueous extracts of diseased leaves revealed peaks identified as mannose (11.79 min), glucose (13.8 min), glucose-1-P (19.49 min), and glucuronic acid (19.90 min). Sometimes, a small peak corresponding to cellobiose (10.33 min) appeared. A peak at 22.45 min was not identified. The occurrence of both mannose and glucuronic acid (mannose/glucuronic acid ratio = 0.78) and glucose could be considered as indicative of the existence of a xanthan-like polysaccharide in extracts obtained from diseased sugarcane laves, although the amount of glucose was too high in the hydrolysate to consider it as a true xanthan. This large amount of glucose could have derived from starch obtained from bundle-sheath cells and was partially extracted with *iso*-propanol.

To test this hypothesis, the gum obtained from sugarcane stalks, which mainly accumulated sucrose instead of starch, was analyzed. The hydrolysate obtained from *iso*-propanol precipitated juice always contained a large amount of cellobiose, glucose, mannose, glucose-1-P and glucuronic acid. In this case, the mannose to glucuronic acid ratio was 0.82. Moreover, cellobiose (β-D-glucosyl-[1→4]-D-glucose, which appeared in the electropherogram, was considered as a potential source of glucose. As described in the literature, incomplete hydrolysis of xanthan produced a large amount of cellobiose (Christensen and Smidsrod 1996). The ratio of both free glucose and that occurring as cellobiose, to mannose or glucuronic acid, was calculated as 2.5 and 2.05, respectively, obtained from stalk extract fractions.

During infection, *X. campestris* pv. campestris produces a gum that has been described as a xanthan (Li *et al.* 2001), composed of a repeated pentamer formed by three rests of glucose, to which a dimer formed by mannose and glucuronic acid is attached. This pathogen bacterium is able to produce xanthan even in culture (Papagianni *et al*. 2001). Other pathogenic bacteria, such as *Xylella fastidiosa* (da Silva *et al.* 2001) and *X. albilineans* (Fontaniella *et al.* 2002a), also produce xanthan-like polymers, but they are composed of a repeated tetramer of glucose–glucose–mannose–glucuronic acid (**Fig. 1C**). However, *X. albilineans* does not produce xanthans in culture (Blanco *et al*. 2005). We found that these bacteria actively produce proteases that rapidly hydrolyze UDP-glc dehydrogenase, the enzyme responsible for the production of glucuronic acid, the most characteristic monomer of the xanthan macromolecule (Blanch *et al.* 2007a). However, some of the glycoproteins produced by sugarcane after infection act as powerful inhibitors of protease activity. Thus, this could be the reason for restricting xanthan production to the infective status. According to this rationale, stalk segments of sugarcane experimentally infected with *X. albilineans* not only produced sugarcane polysaccharides (HMMC and MMMC), but also produced polysaccharides of molecular mass higher than that which defined sugarcane HMMC, according to Martínez *et al.* (1990). A fundamental difference between both classes of polysaccharides concerns the protein content; whereas HMMC coelutes with protein from the chromato-

graphic column (in fact some of these HMMC are really glycoproteins, according to Legaz *et al.* (1995), polysaccharide eluates of very high molecular mass, only appearing after stalk infection, do not contain protein. In fact, separation of both fractions by CE reveals that sugarcane HMMC at least produces a peak absorbing at 280 nm whereas this peak does not appear after analysis of HMMC from infected stalks.

Since fructose and galactitol are the only components of both HMMC and MMMC (Vicente *et al.* 1991; Legaz *et al.* 2005), characteristic polysaccharides produced by sugarcane cells after infection, whereas glucuronic acid is the most singular monomer of xanthans produced by bacteria (Fontaniella *et al*. 2002a, 2002b), the occurrence of one or others in hydrolysates of HMMC polysaccharides obtained from infected tissues could be enough to characterize the origin (from plant or bacteria) of the hydrolyzed macromolecule. Only polysaccharides obtained from sugarcane stalks previously infected with *X. albilineans* release glucuronic acid as well as mannose and glucose after acidic hydrolyses, whereas those obtained from non-inoculated stalks seem to be glucans, since only glucose is released from the polymer. Thus, it can be concluded that *X. albilineans* invading the storage tissue of sugarcane stalks is able to produce xanthans that can be conveniently separated from sugarcane polysaccharides. This could be explained on the basis of the action previously found of bacterial protease inhibition caused by sugarcane polysaccharides in such a way that a non-proteolyzed, active UDP glucose dehydrogenase would assure xanthan production (Blanch *et al.* 2007b).

*In vitro* enzymatic assay of UDP-glc dehydrogenase from *X. albilineans* requires the addition of a protease inhibitors cocktail to cell-free extracts, since bacterial proteases rapidly hydrolyse the enzyme in solution. The addition of low amounts of 8-azaguanine and chloramphenicol to the culture medium do not impede the production of the dehydrogenase that requires concentrations higher than 0.3 mM of both antimetabolites to inhibit its synthesis. Glycoproteins from sugarcane, the natural host of the bacterium, also assure the production of the active enzyme by inhibiting bacterial proteases.

UDPG dehydrogenase activity was completely nullified by increasing the concentration of inhibitors from 100 to 300 μM. These results could be explained by considering that 100 μM 8-azaguanine or chloramphenicol mainly inhibits protease synthesis whereas the same inhibitors at 300 μM could inhibit the synthesis of both proteases and UDPG dehydrogenase. A similar explanation could be used to justify the effect of both HMMG and MMMG since, in addition, the recovery of protein per mg of dry bacteria from those growing on sugarcane glycoproteins was always higher than that recovered from bacteria growing on control media. *Xylella fastidiosa*, another pathogenic bacterium of plants producing xanthan-like gums (da Silva *et al.* 2001), also synthesized extracellular proteases into the culture broth. These proteases produced by strains of *X. fastidiosa* from citrus and grape, belong to the serine- and metalloprotease group, respectively (Fedatto *et al.* 2006).

However, in the absence of the plant host, bacterial proteases could be used to hydrolyse bacterial proteins, such as UDPG dehydrogenase. When cell-free extracts from *X. albilineans*, showing protease activity, were pre-incubated for 5 min with HMMG or MMMG obtained from sugarcane plants, variable inhibition of protease activity against casein as a substrate was revealed. Whereas HMMG slightly inhibited bacterial proteases (16.4% inhibition), MMMG produced 42.6% of protease inhibition. The characteristics of xanthan production could then be explained on the basis of the inhibitory action of bacterial proteases caused by sugarcane glycoproteins in such a way that a non-proteolysed, active UDP-glucose dehydrogenase would assure xanthan production (**Fig. 3**). Since sugarcane glycoproteins inhibit bacterial proteases, almost for *X. albilineans*, and simultaneously permit the production of UDPG dehydrogenase, it can be interpreted as an interdependence between host and pathogen, probably derived from a coevolutionary process. This could explain why *X. albilineans* did not produce xanthans in culture whereas the gum was secreted from bacteria invading sugarcane tissues (Blanco *et al.* 2005).

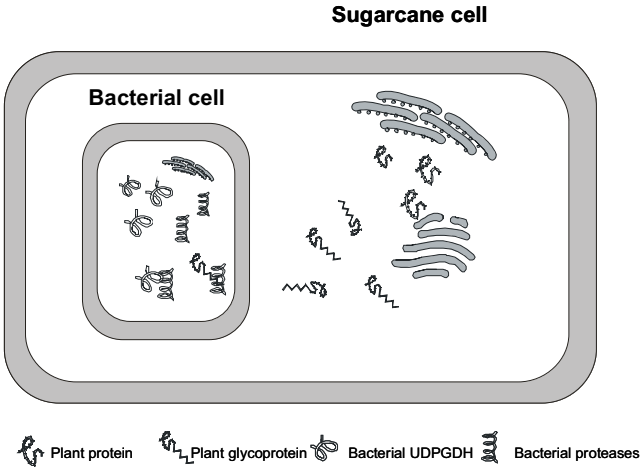


**Fig. 3 Inhibition of bacterial proteases by sugarcane glycoproteins facilitates UDPG dehydrogenase stability and xanthan production by *Xanthomonas albilineans*.**

## The enzyme UDPG dehydrogenase from *X. albilineans*

*X. albilineans* produces a UDP-glucose dehydrogenase growing on sucrose. The enzyme oxidizes UDP-glucose to UDP-glucuronic acid by using molecular oxygen and NADPH (**Fig. 4**). The kinetics of enzymatic oxidation of NADPH is linearly dependent on the amount of oxygen supplied. The enzyme has been purified at homogeneity. The value of pI of the purified enzyme is 8.98 and its molecular mass has been estimated as about 14 kDa. The molecular mass is very low by comparison with those reported for other UDPG dehydrogenases, such as the dimeric enzyme of *Escherichia coli* (Mr 72 kDa), as described by Sieberth *et al.* (1995) or the monomeric protein from *Streptococcus pyogenes*, Mr 45.5 kDa (Campbell *et al.* 1997). The enzyme shows a Michaelian kinetics for UDP-glucose concentrations. The value of Km for UDP-glucose is 0.87 mM and 0.26 mM for NADPH, although the enzyme has three different sites to interact with NADPH. The Km value for UDP-glucose, 0.87 mM, is lower than that described for the enzyme from *E. coli* (Schiller *et al.* 1973; Sieberth *et al.* 1995) but higher than that described for UDPG dehydrogenase from A group streptococci (Campbell *et al.* 1997) or soybean nodules (Stewart and Copeland 1999). Since UDPG dehydrogenase shows only one catalytic centre for UDPG and assuming that the binding of NADPH molecules to the regulatory centres changes the kinetic constants of the enzyme, UDPG dehydrogenase from *X. albilineans* can be defined as a mixed, allosteric enzyme for its coenzyme, according to Klotz and Hunston (1975). This could explain the sigmoidal response of the enzyme to increasing concentrations of the effector as well as the inhibition of the enzyme to NADPH concentrations higher than 0.22 mM. The enzyme is inhibited by UDP-glucose concentrations higher than 1.3 mM. The N-terminal sequence has been determined as IQPYNH (**Table 1**).

In contrast to other UDPGDHs, including that from *X. campestris*, and other enzymes that catalyze the oxidation of an alcohol to an acid without release of an aldehyde intermediate through a bi-uni-uni-bi ping pong mechanism (Campbell *et al.* 1997), the catalytic action of UDPGDH from *X. albilineans* can be described as a tri-uni-uni-tri ping pong mechanism. This model is imposed by the requirement of molecular oxygen in the reaction. According to this,

**Table 1** Properties of UDPG dehydrogenase from *Xanthomonas albilineans* (from Blanch *et al*. 2008).

| | |
|---|---|
| Substrates | UDPglucose and $O_2$ |
| Cofactor | NADPH |
| pI | 8.98 |
| Molecular mass | 14 kDa |
| pH optimum | 6.0 |
| Optimal temperature | 37°C |
| $K_m$ for UDPG | 0.87 mM |
| Inhibited by UDPG concentrations higher than | 1.30 mM |
| $K_m$ for NADPH | 0.26 mM |
| Inhibited by NADPH concentrations higher than | 0.30 mM |
| N-terminal end | IQPYNH |

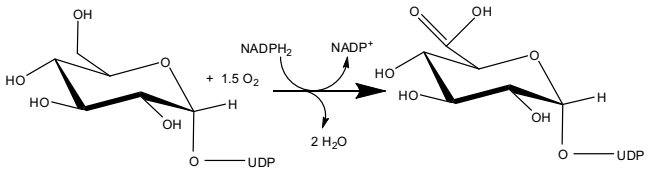


**Fig. 4 Reaction catalyzed by UDPG dehydrogenase of *Xanthomonas albilineans*.**

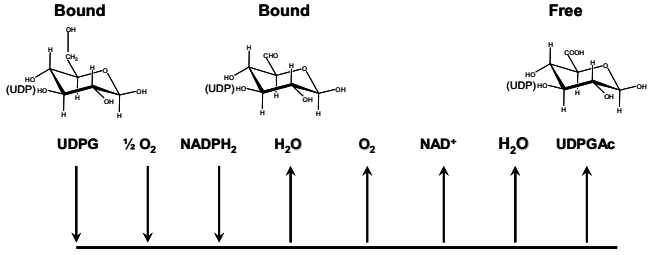


**Fig. 5 Proposed tri-uni-uni.tri ping pong mechanism for the action of UDPG dehydrogenase from *Xanthomonas albilineans*.**

the alcohol (the C6 hydroxyl of UDPG) is bound first, the coenzyme $NADPH_2$ remains bound whereas water is released, as deduced from the enzyme inhibition by $NADPH_2$ higher than 0.22 mM, and the acid (UDPglucuronic acid) is released last (**Fig. 5**).

## Virulence

In plant pathogenic bacteria like *X. campestris* and *X. albilineans*, UDP-glucose dehydrogenase is not only required for the production of xanthan gum but it is also considered as a determinant factor for virulence. In addition, other virulence factors have been described. Virulence of the black rot pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) is regulated by cell–cell signalling involving the diffusible signal factor DSF (a derivative from dodecenoic acid that acts as quorum sensing signal). Synthesis and perception of DSF require products of genes within the *rpf* (regulation of pathogenicity factors) cluster. RpfF directs DSF synthesis whereas RpfC and RpfG are involved in DSF perception. Wild-type (WT) *Xcc* formed microcolonies that developed into a structured biofilm. In contrast, an *rpfF* mutant (DSF-minus) and an *rpfC* mutant (DSF overproducer) formed only unstructured arrangements of bacteria. A *gumB* mutant, defective in xanthan biosynthesis, was also unable to develop the typical WT biofilm. Mixed cultures of *gumB* and *rpfF* mutants formed a typical biofilm *in vitro*. In contrast, in mixed cultures, the *rpfC* mutant prevented the formation of the structured biofilm by the WT and did not restore WT biofilm phenotypes to *gumB* or *rpfF* mutants. These effects on structured biofilm formation were correlated with growth and disease development by *Xcc* strains in *Nicotiana benthamiana* leaves. These findings suggest that DSF signalling is finely balanced during both biofilm formation and virulence (Torres *et al.* 2007).

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