The role of glucose kinase in carbohydrate utilization and extracellular polysaccharide production in *Xanthomonas campestris* pathovar *campestris*

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The genome of the *Xanthomonas campestris* pathovar *campestris* (*Xcc*) strain 8004 encodes three uncharacterized proteins, XC1166, XC1223 and XC1976, annotated as glucose kinase (Glc) by bioinformatic studies. Here we have investigated the biochemical characteristics and physiological roles of these proteins with particular reference to the synthesis of extracellular polysaccharide (EPS). XC1166, XC1223 and XC1976 were overexpressed as fusion proteins with a His₆ affinity tag and purified by nickel affinity chromatography. The standard Glc activity assay revealed that all three proteins possessed apparent Glc activity, with XC1976-His₆ being the most active; the specific activity values were 1.16×10^6 U mg⁻¹ for XC1166-His₆, 4.36×10^7 U mg⁻¹ for XC1223-His₆ and 2.63×10^8 U mg⁻¹ for XC1976-His₆. TLC analysis showed, however, that only XC1976-His₆ could phosphorylate glucose. Insertional mutants of XC1166, XC1223 and XC1976 were generated using the suicide plasmid pK18mob. Although mutant strains with insertions in XC1166 or XC1223 had Glc activity similar to that of the wild-type strain, the XC1976 mutant had only about 6% of the wild-type activity. Mutation in XC1976 had complex effects on EPS production. In media containing arabinose, glucose, galactose, sucrose or maltose, the XC1976 mutant produced about 40–75% of the wild-type level of EPS, whereas in medium containing fructose, the mutant showed a 30% increase in EPS production compared to the wild-type strain. The XC1976 mutant also showed attenuated virulence on the host plant Chinese radish (*Raphanus sativus*). The results indicate that XC1976 has the most significant role for the parameters tested.

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INTRODUCTION

The phytopathogenic bacterium *Xanthomonas campestris* pathovar *campestris* (hereafter *Xcc*), a strictly aerobic member of the family *Xanthomonadaceae*, is the causal agent of black rot disease of cruciferous crops worldwide (Onsando, 1992; Swings & Civerolo, 1993). This bacterium produces extracellular polysaccharide (EPS) and extracellular enzymes (including amylase, endoglucanase, polygalacturonate lyase and protease), which are collectively essential for pathogenesis (Dow & Daniels, 1994). EPS, also called xanthan gum, is an important industrial biopolymer which is used in a great variety of food and industrial applications as a viscosifying, thickening, stabilizing and suspending agent (Kennedy & Bradshaw, 1984).

Abbreviations: EPS, extracellular polysaccharide; Glc, glucose kinase; *Xcc*, *Xanthomonas campestris* pathovar *campestris*.

Carbohydrate metabolism in *Xcc* has been investigated in some detail, as it is central to both EPS production and the mechanism of bacterial colonization. *Xcc* metabolizes sugars through a central metabolic network similar to that described for *Pseudomonas aeruginosa* (Temple *et al.*, 1998). Glucose and sucrose have been shown to be the best carbon sources for EPS production (García-Ochoa *et al.*, 2000). Two distinct pathways are employed to mobilize extracellular glucose for EPS production and energy generation. In the first pathway, glucose is transported directly into the cell by a permease and undergoes an intracellular phosphorylation catalysed by glucose kinase (EC 2.7.1.2, hereafter Glc) to yield glucose 6-phosphate (Lessie & Phibbs, 1984; Letisse *et al.*, 2001). Glucose 6-phosphate is then converted to gluconate by glucose-6-phosphate 1-dehydrogenase. Extracellular glucose can also be catabolized by an oxidative periplasmic

pathway in which glucose is oxidized to gluconate via a periplasmic NAD(P)⁺-independent glucose dehydrogenase (Whitfield *et al.*, 1982). This enzyme uses pyrroloquinoline quinone (PQQ) as cofactor (Duine & Jongejan, 1989). Gluconate is transported into the cell by an active transport system involving gluconate permease. Gluconate from the two pathways is converted to 6-phosphogluconate and may be further metabolized via the Entner–Doudoroff pathway to yield glyceraldehyde 3-phosphate and pyruvate (Zagallo & Wang, 1967). Some of these products may be recycled via gluconeogenesis for glucose phosphate production (Banerjee *et al.*, 1987), while the remainder is oxidized via the tricarboxylic acid (TCA) cycle.

Glk has been predicted to be important for EPS production in *Xcc* (Letisse *et al.*, 2001, 2002). The enzyme is responsible for phosphorylation of glucose using ATP as a donor to give glucose 6-phosphate and ADP. Although the eukaryotic Glks, such as yeast hexokinase B, *Arabidopsis thaliana* hexokinases and human hexokinase IV (HK4 or GCK, EC 2.7.1.1), are well characterized (Catanzano *et al.*, 1997; Moore *et al.*, 2003; Wilson, 2003), little is known about their bacterial and archaeal counterparts. A survey of the genome sequence data of two *Xcc* strains has revealed three genes predicted to encode Glk enzymes: *XCC2137*, *XCC2886* and *XCC2943* in strain ATCC 33913 (da Silva *et al.*, 2002), and *XC1166*, *XC1223* and *XC1976* in strain 8004 (Qian *et al.*, 2005). However, these genes and their protein products have not been experimentally

investigated. Here we have examined the biochemical characteristics and physiological roles (particularly in EPS production) of the multiple Glk enzymes in *Xcc* strain 8004.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium (Miller, 1972) at 37 °C. *Xcc* strains were grown at 28 °C in NYG medium (per litre: 5 g peptone, 3 g yeast extract and 20 g glycerol; Daniels *et al.*, 1984b) or the modified minimal medium MMX [per litre: 2.0 g (NH₄)₂SO₄, 4.0 g K₂HPO₄, 6.0 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 1.0 g citric acid, 5.0 g sugar; Daniels *et al.*, 1984a]. Antibiotics were added at the following concentrations as required: kanamycin (Kan), 25 µg ml⁻¹; rifampicin (Rif), 50 µg ml⁻¹; ampicillin (Amp), 100 µg ml⁻¹; spectinomycin (Spc), 50 µg ml⁻¹ and tetracycline (Tet), 5 µg ml⁻¹ for *Xcc* and 15 µg ml⁻¹ for *E. coli*.

DNA manipulations. The methods described by Sambrook *et al.* (1989) were used for preparation of the plasmid and chromosomal DNAs, restriction digestion, DNA ligation, agarose gel electrophoresis and DNA transformation of *E. coli*. The conjugation between *Xcc* and *E. coli* strains was performed as described by Turner *et al.* (1984). The restriction endonucleases, T4 DNA ligase and *Pfu* polymerase were provided by Promega (Shanghai).

Construction of insertional mutants. Mutants of the ORFs *XC1166*, *XC1223* and *XC1976* were constructed using the method previously described by Lu *et al.* (2007). A 300–500 bp internal

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
JM109	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>supE44</i> , <i>relA1</i> Δ(<i>lac-proAB</i>)/F' [<i>traD36</i> , <i>lacI</i> ^r , <i>lacZ</i> ΔM15]	Yanisch-Perron <i>et al.</i> (1985)
DH5α	φ80 Δ <i>lacZ</i> M15 <i>recA1</i> <i>endA1</i> <i>deoR</i>	Gibco-BRL, Life Technologies
HB101	<i>recA</i> 13rps	Boyer & Roulland-Dussoix (1969)
<i>Xcc</i>		
8004	Wild-type, Rif ^r	Daniels <i>et al.</i> (1984b)
1976nk	As 8004, but <i>XC1976</i> ::pK18 <i>mob</i> , Rif ^r , Kan ^r	This work
1166nk	As 8004, but <i>XC1166</i> ::pK18 <i>mob</i> , Rif ^r , Kan ^r	This work
1223nk	As 8004, but <i>XC1223</i> ::pK18 <i>mob</i> , Rif ^r , Kan ^r	This work
C1976nk	1976nk harbouring pLATC1976, Rif ^r , Kan ^r , Tc ^r	This work
Plasmids		
pLAFR3	Broad-host-range cloning vector, Tra ⁻ , Mob ⁺ , Tc ^r IncP replicon	Staskawicz <i>et al.</i> (1987)
pLAFR6	A promoterless derivative of pLAFR3, Tc ^r	Huynh <i>et al.</i> (1989)
pRK2073	Helper plasmid, Tra ⁺ , Mob ⁺ , ColE1, Spc ^r	Leong <i>et al.</i> (1982)
pK18 <i>mob</i>	pUC18 derivative, <i>lacZα</i> Km ^r , <i>mob</i> site. Suicide plasmid in <i>Xcc</i>	Schäfer <i>et al.</i> (1994)
pK1976	pK18 <i>mob</i> containing a 484 bp internal fragment of <i>XC1976</i> , Kan ^r	This work
pK1166	pK18 <i>mob</i> containing a 357 bp internal fragment of <i>XC1166</i> , Kan ^r	This work
pK1223	pK18 <i>mob</i> containing a 430 bp internal fragment of <i>XC1223</i> , Kan ^r	This work
pLATC1976	pLAFR3 containing a 1222 bp fragment including the <i>XC1976</i> gene, Tc ^r	This work
pQE-30	Expression vector, allow the production of fusion proteins containing N-terminal 6 × His-tagged sequences. Amp ^r	Qiagen
pQE-30-1166	pQE-30 containing a 1032 bp fragment of <i>XC1976</i> coding region	This work
pQE-30-1223	pQE-30 containing a 1014 bp fragment of <i>XC1976</i> coding region	This work
pQE-30-1976	pQE-30 containing a 1005 bp fragment of <i>XC1976</i> coding region	This work

fragment of the target ORF was amplified by PCR using the corresponding primers (Table 2). The amplified DNA fragments were cloned into suicide plasmid pK18mob (Schäfer *et al.*, 1994; Windgassen *et al.*, 2000) in the same orientation as the *lacZ* promoter to guarantee the construction of non-polar mutants. The resulting recombinant plasmid was introduced from *E. coli* strain JM109 (Yanisch-Perron *et al.*, 1985) into the *Xcc* wild-type strain 8004 by triparental conjugation using pRK2073 (Leong *et al.*, 1982) as the helper plasmid. Mutants were confirmed by PCR using oligonucleotide P18conF, and the corresponding oligonucleotide located downstream of the target ORF, as primers (Table 2).

Complementation of the insertional mutants. For mutant complementation, a 1222 bp DNA fragment containing the *XC1976* coding region and extending from 212 bp upstream of the 5' end to 2 bp downstream of the 3' end of the ORF was amplified using the primers C1976F/R (Table 2), and the amplified DNA fragment was cloned into the plasmid pLAFR3 (Staskawicz *et al.*, 1987) to generate the recombinant plasmid pLATC1976 (Table 1). The recombinant plasmid was transferred into the *XC1976* mutant by triparental conjugation, resulting in strain C1976nk (Table 1).

RT-PCR analysis. To analyse transcriptional expression levels, total RNA was isolated using Trizol reagent (Promega) and treated with RNase-free DNase (Promega). RT-PCR analysis was carried out with total RNA and specific primers for *XC1166* (O1166F/R), *XC1223* (O1223F/R) and *XC1976* (O1976F/R) (Table 2). One microgram of total RNA was used for each reaction in a 20 µl volume, and 4 µl of the reaction mixture was subjected to PCR amplification. The PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels, and verified by DNA sequencing.

Overproduction and purification of protein. For overproduction of *XC1166*, *XC1223* and *XC1976*, their corresponding ORFs were amplified using the primers O1166F/R, O1223F/R and O1976F/R (Table 2), respectively. After being confirmed by sequencing, the amplified DNA fragments were cloned into the expression vector pQE-30 (Qiagen) (Table 1) to generate recombinant plasmids pQE-30-1166, pQE-30-1223 and pQE-30-1976 (Table 1), respectively. In

these plasmids, *XC1166*, *XC1223* or *XC1976* is fused N-terminally in-frame to the His₆-tag coding region of the plasmid pQE-30. The recombinant plasmids obtained were transformed into *E. coli* strain JM109, resulting in strains JM109/pQE-30-1166, JM109/pQE-30-1223 and JM109/pQE-30-1976 (Table 1), respectively. For fused proteins *XC1166*-His₆, *XC1223*-His₆ and *XC1976*-His₆ overproduction and purification, strains were grown to OD₆₀₀ 0.6, and then induced by addition of 1.0 mM IPTG. The cultures were grown for a further 4 h. The fused proteins were purified by Ni-NTA resin (Qiagen). The proteins were checked by 12% SDS-PAGE and used for enzymic assay.

Cell-free extract preparation. *Xcc* strains were grown in NYG medium supplemented with 2% glucose or fructose for 20 h. Cells were harvested and washed twice in sterile water by centrifugation. The cells were then resuspended in 2 ml 20 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol and 0.1 mM EDTA and disrupted by sonication. The sonicates were centrifuged and the supernatant fractions were used in the enzyme assay.

Glk assay. Glk activity from purified proteins or cell-free extracts was measured by an enzyme-linked assay based on the NADP⁺/NADPH ratio (Gonzali *et al.*, 2001). Ten microlitres of the protein sample was transferred to a 5 ml tube containing 2 ml reaction buffer [100 mM Tris/HCl (pH 7.4), 100 mM KCl, 7.5 mM MgSO₄, 5 mM ATP, 0.5 mM NADP⁺, 5 mM β-mercaptoethanol, 100 mM glucose and 0.2 U glucose-6-phosphate dehydrogenase ml⁻¹]. After incubation for 10 min at 37 °C, A₃₄₀ was measured. A unit of enzyme activity was taken as 1 nmol NADPH formed min⁻¹ (mg protein)⁻¹; the protein concentration in the samples was determined by the BCA protein assay kit (Pierce Biotechnology). For characterizing kinetic parameters of the purified proteins, the reaction mixture including 1.0 µg purified Glk, and a range of D-glucose or ATP concentrations (0.05–20 mM), was used under standard assay conditions. Michaelis-Menten kinetic parameters were evaluated by the double reciprocal-plot method.

The phosphorylation of different sugars by the purified proteins was monitored by TLC, as described by Meyer *et al.* (1997). Purified proteins were incubated with 50 mM sugar in 50 mM Tris/HCl (pH 7.4) containing 10 mM MgCl₂ and 50 mM ATP. The assay

Table 2. Primers used in this study

Underlined sequences indicate restriction sites for *Bam*HI and *Hind*III.

Primer	Nucleotide sequence (5'–3')	Amplified segment or primer location
1976kF	ACAGTTAAGCTTTGCTCCTTCCAAGCCGGTGCTGGT	484 bp DNA fragment spans nt 6–489 of the <i>XC1976</i> ORF sequence
1976kR	ACAGTTGGATCCAAAACACGCGGCCGTTGCGGATGAT	
C1976F	ACAGTTAAGCTTACTCATCGCTGCTCTCCC	1222 bp DNA fragment spans 212 bp upstream and 2 bp downstream of the <i>XC1976</i> ORF sequence
C1976R	ACAGTTGGATCCTATGAACGCTTGTTCTGTG	
1166F	ACAGTTAAGCTTCATGCTCGATCGGCAGAT	357 bp DNA fragment spans nt 278–634 of the <i>XC1166</i> ORF sequence
1166R	ACAGTTGGATCCTCAGCAACAACCTGCCCT	
1223F	ACAGTTGGATCCTTGACCTGGTCAACGAT	430 bp DNA fragment spans nt 343–772 of the <i>XC1223</i> ORF sequence
1223R	ACAGTTAAGCTTAGAACGTCTGCAGCGCCT	
P18conF	GCCGATTCATTAATGCAGCTGGCAC	Located in pK18mob
O1976F	ACAGTTAAGCTTTCGCTGCTCTCCCGGCAA	1005 bp of the <i>XC1976</i> ORF
O1976R	ACAGTTGGATCCATGACTGCTCCTTCCAAG	
O1166F	ACAGTTAAGCTTCGACACCTCGGTGTGGTG	1032 bp of the <i>XC1166</i> ORF
O1166R	ACAGTTGGATCCGTGGCTGCAACCTCGCAC	
O1223F	ACAGTTGGATCCGTGAGCGCAAGCAGCCCA	1014 bp of the <i>XC1223</i> ORF
O1223R	ACAGTTAAGCTTGCGGCCGTGCTGCAGGAA	

volume was 100 µl containing 10 µg protein. After 2 h incubation, an 8 µl sample was spotted onto the silica-coated TLC plate (Qingdao Haiyang Chemical) and the plate was then developed with butanol-ethanol-water (5:3:2, by vol.) for 3 h. After drying the TLC plate, the sugar-containing spots were visualized by dipping the plate in methanol containing 2 % concentrated H₂SO₄, followed by drying and charring for 8 min at 150 °C.

EPS assay. To estimate EPS production, strains were cultured in 100 ml NYG liquid medium containing 2 % (w/v) various sugars at 28 °C with shaking at 200 r.p.m. for 3 days. EPS was precipitated from the culture supernatant with ethanol, dried and weighed as described by Tang *et al.* (1991).

Virulence assay. The virulence of *Xcc* to Chinese radish (*Raphanus sativus*) was tested by the leaf-clipping method (Dow *et al.*, 2003). Leaves were cut with scissors dipped in the bacterial suspensions of an OD₆₀₀ of 0.1. Lesion length was measured 10 days after inoculation, and data were analysed by *t* test. The growth of bacteria in radish leaf tissue was determined as previously described (Lu *et al.*, 2007).

RESULTS

Enzyme characteristics of the predicted Glks XC1166, XC1223 and XC1976

The three hypothetical proteins annotated as Glk from *Xcc* strain 8004 are the 344 aa protein XC1166 (YP_242256), the 338 aa protein XC1223 (YP_242312) and the 335 aa protein XC1976 (YP_243059). Sequence comparison (Vector NTI suite 9.0) revealed only limited amino acid sequence similarity among these proteins, which displayed only 24.0 % identical residues (Fig. 1). This suggests that the three proteins have distinct functions. The three predicted Glk proteins in *Xcc* possess the same Pfam domain (PF02685) and they are the only proteins with such a domain in the *Xcc* proteome. Wider similarity searches revealed that these proteins also share limited identical residues with characterized Glks from other microbial sources: XC1976 has 38.4 and 19.3 % identity, respectively, to the *E. coli* enzyme (accession no. AAC75447) and the *Streptomyces coelicolor* A3(2) enzyme (accession no. CAA46727). XC1166 and XC1223 share 31 and 27.9 % identity, respectively, with the *E. coli* enzyme, and 16.3 and 14.5 % identity with the *S. coelicolor* enzyme.

To explore the biochemical characteristics of the products of these genes, XC1166, XC1223 and XC1976 were overexpressed with His₆-tags in *E. coli* strain JM109, as outlined in Methods. Separation of crude cell lysates on SDS-PAGE showed intense protein bands migrating at the expected molecular mass of 35.5 kDa (Fig. 2). Fusion proteins with a His₆-tag were purified using a Ni-NTA column, to obtain preparations apparently free from contaminating proteins (Fig. 2).

The standard assay for measuring Glk activity, in which the phosphorylation of glucose is coupled to the formation of NADPH by glucose-6-phosphate dehydrogenase (Gonzali *et al.*, 2001), was used to test enzyme properties and kinetic constants of the fusion proteins (see Methods). All three

Fig. 1. Comparison of the predicted amino acid sequences of XC1976, XC1166 and XC1223 with that of the *E. coli* Glk (AAC75447). Residues that are identical in at least three sequences are shown with a grey background. The black line above the sequence (ATP) indicates the putative ATP-binding site (Meyer *et al.*, 1997).

fusion proteins exhibited Glk activity in the presence of ATP by this method; however, no significant enzymic activity was observed without ATP (data not shown). The specific activity values were determined to be 1.16×10^6 U (mg protein)⁻¹ for XC1166-His₆, 4.36×10^7 U mg⁻¹ for XC1223-His₆ and 2.63×10^8 U mg⁻¹ for XC1976-His₆. The apparent *K_m* values for glucose were 4.02 mM for XC1166-His₆, 4.20 mM for XC1223-His₆ and 2.52 mM for XC1976-His₆, while the apparent *K_m* values for ATP were 2.50 mM (XC1166-His₆), 1.85 mM (XC1223-His₆) and 1.25 mM (XC1976-His₆).

The enzymic activities of the purified proteins were further assessed by examination of glucose phosphorylation by

Fig. 2. SDS-PAGE analysis of the His₆-tagged recombinant proteins XC1166, XC1223 and XC1976 of *Xcc*. Lanes: 1, crude extract of *E. coli* JM109; 2, crude extract of *E. coli* JM109/pQE-30-1166 after induction with IPTG; 3, crude extract of *E. coli* JM109/pQE-30-1223 after induction with IPTG; 4, crude extract of *E. coli* JM109/pQE-30-1976 after induction with IPTG; 5, affinity-purified His₆-XC1166 protein; 6, affinity-purified His₆-XC1223 protein; 7, affinity-purified His₆-XC1976 protein; M, molecular mass markers.

TLC (see Methods). Purified proteins were incubated with 50 mM glucose and 50 mM ATP for 2 h at 37 °C before TLC separation of substrates and products. Although phosphorylation of glucose by XC1976-His₆ could be observed by this method (Fig. 3), no phosphorylation of glucose by XC1166-His₆ and XC1223-His₆ was detected. To examine the sugar substrate specificity of XC1166, XC1223 and XC1976, these assays were repeated under the same conditions using a range of sugars, including arabinose, xylose, sorbitol (glucitol), mannose, mannitol, sorbose, fructose, galactose, rhamnose, sucrose and maltose. No phosphorylation of any of these sugars by XC1166-His₆, XC1223-His₆ or XC1976-His₆ could be detected (data not shown). Taken together, these results indicate that XC1976 is a Glk and not a hexokinase.

Analysis of the genome sequence of *Xcc* strain 8004 revealed that XC1976 is probably transcribed as the

Fig. 3. Phosphorylation of glucose by the His₆-tagged recombinant proteins XC1166, XC1223 and XC1976 revealed by TLC. An 8 µl sample of each reaction mixture was spotted onto TLC plates. Lanes: 1, glucose standard; 2, glucose 6-phosphate standard; 3, His₆-XC1166; 4, His₆-XC1223; 5, His₆-XC1976.

XC1977-XC1976-XC1975 operon. XC1977 is annotated as a putative glucose-6-phosphate 1-dehydrogenase, whereas XC1975 is annotated as an orthologue of 6-phosphogluconolactonase. The linked genes XC1974 and XC1973 are predicted to encode 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, respectively. Therefore, the genes XC1977-XC1973 might encode five enzymes probably responsible for glucose catabolism by the Entner-Doudoroff pathway (Conway, 1992), suggesting a role for XC1976 in this pathway of glucose metabolism.

XC1976 makes the major contribution to Glk activity in *Xcc*

To demonstrate the physiological role of the three genes, non-polar mutants carrying insertional disruption within the ORF XC1166, XC1223 or XC1976 were constructed and named 1166nk, 1223nk and 1976nk, respectively (Table 1; see Methods). RT-PCR analysis showed that full-length transcripts for each gene are present in the wild-type strain but not in the corresponding mutant (Fig. 4).

The Glk activities in the wild-type and mutant strains were then measured. Cell extracts were prepared from cultures grown in NYG liquid medium containing 2% glucose or fructose. A level of activity was found in all the tested strains in the absence of ATP (Table 3); this might reflect the glucose dehydrogenase activity of *Xcc*. As summarized in Table 3, the Glk activities in the XC1166 and XC1223 mutants were not different from that in the wild-type strain 8004. However, the XC1976 mutant showed a substantial reduction in enzymic activity, whereby the level of ATP-dependent activity was only 6% of the wild-type level. XC1976 was cloned as a 1222 bp DNA fragment in plasmid pLAFR3 to give pLATC1976 (see Methods). Introduction of pLATC1976 into the XC1976 mutant resulted in a strain that expressed the XC1976 transcript (Fig. 4) and that had a level of Glk activity that was considerably elevated over that of the wild-type (Table 3). XC1976 is the central gene in the XC1977-XC1975 operon (see above) and is unlikely to have its own promoter. The *lac* promoter of the vector pLAFR3, which is active in *Xcc* (Soby & Daniels, 1996), probably drives expression of XC1976 in pLATC1976. Consistent with this interpretation, when the 1222 bp fragment was cloned into the promoterless vector pLAFR6 (Huynh *et al.*, 1989), the resulting construct could not restore the Glk activity of the XC1976 mutant (data not shown). Overall, these results indicate that XC1976 makes the most significant contribution to the total Glk activity in *Xcc*, when the bacterium grows in NYG medium supplemented with glucose or fructose.

XC1976 influences the utilization of glucose, sucrose and maltose

To examine the effect of mutation in XC1166, XC1223 and XC1976 on the ability of *Xcc* to utilize various carbon sources, the growth of different bacterial strains was tested.

Fig. 4. RT-PCR analysis of the expression of *XC1166*, *XC1223* and *XC1976*. Total RNA from cells grown in NYG medium for 20 h was used as template for reverse transcription. WT, wild-type strain; M1, M2 and M3, mutant strains 1166nk, 1223nk and 1976nk, respectively; M4, the complemented mutant strain C1976nk.

Bacteria were grown on minimal medium MMX agar plates containing arabinose, xylose, glucose, sorbitol, mannose, mannitol, sorbose, fructose, galactose, rhamnose, sucrose or maltose as the sole carbon source. The growth of the *XC1166* and *XC1223* mutants was similar to that of the wild-type strain 8004 under all conditions tested (data not shown). However, the *XC1976* mutant displayed smaller colonies than the wild-type strain on plates containing glucose, sucrose or maltose. Introduction of pLATC1976 (which carries the *XC1976* gene) into the *XC1976* mutant restored the wild-type phenotype (data not shown). When cultured in liquid medium, the *XC1976* mutant grew with reduced growth rates compared to the wild-type. The doubling times in glucose were 7.1 h for the mutant and 4.4 h for the wild-type, in sucrose 5.9 h for the mutant and 4.6 h for the wild-type, and in maltose 6.8 h for the mutant and 4.7 h for the wild-type. However, when cultured in the nutrient-rich complex medium NYG containing glucose, sucrose or maltose, the *XC1976* mutant grew in a similar fashion to the wild-type strain (data not shown). These

results indicate that the Glk-defective mutant is still able to utilize glucose, sucrose or maltose, although to a more limited extent than the wild-type.

***XC1976* influences EPS production**

To evaluate the EPS production of these mutants, strains were grown on NYG agar plates supplied with a range of sugars. The *XC1166* and *XC1223* mutants showed the same colony size as the wild-type strain 8004 on all agar plates tested, indicating equivalent production of EPS. However, the *XC1976* mutant displayed colonies that were smaller than the wild-type on plates containing arabinose, glucose, sorbitol, mannitol, galactose, sucrose or maltose, larger than the wild-type on plates containing fructose or sorbose, and the same as the wild-type on plates containing xylose, mannose or rhamnose. These findings suggest that the *XC1976* gene might have complex effects on EPS production, promoting it in arabinose-, glucose-, sorbitol-, mannitol-, galactose-, sucrose- or maltose-containing medium, but inhibiting it in fructose- or sorbose-containing medium.

To quantitatively estimate the EPS production of the mutants, strains were grown in NYG liquid medium supplemented with 2 % of various carbohydrates for 3 days, and EPS was extracted from the cultures (see Methods). As summarized in Table 4, the *XC1976* mutant produced about 25–60 % less EPS than the wild-type strain 8004 when cultured in arabinose-, glucose-, galactose-, sucrose- or maltose-containing medium. In addition, the EPS yield of the complemented mutant strain showed no significant difference from that of the wild-type. Conversely, the *XC1976* mutant produced more EPS than the wild-type when cultured in fructose-containing medium. The EPS yield of the mutant was increased about 30 % compared to that of the wild-type strain, and reached 8.5 g l⁻¹, which is approaching the level of the wild-type strain cultured in glucose- or sucrose-containing medium. Surprisingly, complementation did not restore EPS production on fructose to wild-type level.

Table 3. Glk activity in *Xcc* strains

Strains were cultured in NYG liquid medium supplemented with glucose or fructose. Data presented are from a representative experiment and similar results were obtained in two other independent experiments. Data are mean $\pm$ SD of triplicate measurements. Different letters in each data column indicate significant differences at $P=0.05$ (t test).

Strain	Glk activity [nmol NADPH min ⁻¹ (mg protein) ⁻¹]					
	Glucose			Fructose		
	Without ATP (A)	With ATP (B)	(B)–(A)	Without ATP (A)	With ATP (B)	(B)–(A)
8004 (wild-type)	21.8 $\pm$ 4.3	101.9 $\pm$ 18.3	80.1a	20.1 $\pm$ 5.2	94.9 $\pm$ 12	74.8a
1976nk (<i>XC1976</i> mutant)	20.3 $\pm$ 4.4	24.9 $\pm$ 5.2	4.6b	16.8 $\pm$ 4.3	19.2 $\pm$ 3.7	2.4b
C1976nk (complemented mutant)	15.4 $\pm$ 7.8	262.6 $\pm$ 57	247.2a	18.6 $\pm$ 5.6	128.4 $\pm$ 28	109.8a
1166nk (<i>XC1166</i> mutant)	21.0 $\pm$ 5.2	96.9 $\pm$ 15.3	75.9a	18.2 $\pm$ 4.8	91.4 $\pm$ 14.2	73.2a
1223nk (<i>XC1223</i> mutant)	21.5 $\pm$ 5.5	91.4 $\pm$ 12.2	77.6a	19.2 $\pm$ 6.2	91.3 $\pm$ 15	72.1a

Table 4. EPS production in *Xcc* strains

Strains were cultured in NYG liquid medium supplemented with various carbon sources. Data presented are from a representative experiment and similar results were obtained in two other independent experiments. Data are mean $\pm$ SD of triplicate measurements. Different letters in each data column indicate significant differences at $P=0.05$ (t test).

Strain	EPS yield (g l ⁻¹)											
	Arabinose	Xylose	Glucose	Sorbitol	Mannose	Mannitol	Sorbose	Fructose	Galactose	Rhamnose	Sucrose	Maltose
8004 (wild-type)	3.41 $\pm$ 0.25a	2.52 $\pm$ 0.17a	9.35 $\pm$ 0.25a	1.15 $\pm$ 0.13a	6.23 $\pm$ 0.28a	1.02 $\pm$ 0.08a	1.23 $\pm$ 0.23a	6.26 $\pm$ 0.24a	7.11 $\pm$ 0.32a	4.32 $\pm$ 0.19a	9.12 $\pm$ 0.28a	9.10 $\pm$ 0.28a
1976nk (<i>XC1976</i> mutant)	2.16 $\pm$ 0.47b	2.34 $\pm$ 0.22a	4.03 $\pm$ 0.35b	1.17 $\pm$ 0.22a	6.57 $\pm$ 0.34a	1.14 $\pm$ 0.16a	1.78 $\pm$ 0.53a	8.75 $\pm$ 0.36b	5.33 $\pm$ 0.36a	4.25 $\pm$ 0.37a	4.55 $\pm$ 0.31b	3.28 $\pm$ 0.52b
C1976nk (complemented mutant)	3.43 $\pm$ 0.17a	2.48 $\pm$ 0.15a	9.65 $\pm$ 0.58a	1.08 $\pm$ 0.16a	6.68 $\pm$ 0.45a	1.18 $\pm$ 0.11a	1.25 $\pm$ 0.26a	8.56 $\pm$ 0.27b	6.95 $\pm$ 0.24a	4.35 $\pm$ 0.31a	8.75 $\pm$ 0.21a	8.62 $\pm$ 0.28a
1166nk (<i>XC1166</i> mutant)	3.22 $\pm$ 0.28a	2.55 $\pm$ 0.24a	9.44 $\pm$ 0.33a	1.13 $\pm$ 0.24a	6.75 $\pm$ 0.53a	1.05 $\pm$ 0.21a	1.31 $\pm$ 0.34a	6.35 $\pm$ 0.42a	7.15 $\pm$ 0.35a	4.27 $\pm$ 0.41a	9.09 $\pm$ 0.19a	9.15 $\pm$ 0.25a
1223nk (<i>XC1223</i> mutant)	3.17 $\pm$ 0.37a	2.43 $\pm$ 0.08a	9.32 $\pm$ 0.23a	1.05 $\pm$ 0.14a	6.51 $\pm$ 0.44a	1.13 $\pm$ 0.19a	1.27 $\pm$ 0.42a	6.52 $\pm$ 0.15a	7.13 $\pm$ 0.24a	4.18 $\pm$ 0.36a	9.23 $\pm$ 0.26a	9.06 $\pm$ 0.33a

***XC1976* is required for full virulence and *in planta* growth**

The virulence assay for the *Xcc* strains was performed on Chinese radish by the leaf-clipping method and measurement of the length of lesions (Dow *et al.*, 2003). Ten days after inoculation, the *XC1976* mutant showed a mean lesion length of 5.48 mm on the leaves, while the wild-type strain, as well as the mutants of *XC1166* and *XC1223*, showed a mean lesion length of about 12 mm. As analysed by t test, the mean lesion length caused by the *XC1976* mutant was significantly shorter than that caused by the wild-type strain ($P=0.01$). In contrast, when the *XC1976* mutant was transformed with plasmid pLATC1976, the virulence of the mutant could be restored to wild-type; the lesion lengths caused by the complemented strain and the wild-type strain were not significantly different ($P=0.05$) (Fig. 5b). These results show that Glk activity is required for the full virulence of *Xcc*.

To determine whether mutation in *XC1976* results in a decrease in the proliferation of *Xcc* in the host, the numbers of the mutant bacterial cells in infected radish leaves were calculated. The number of the mutant bacterial cells was approximately three- to eightfold lower than that of the wild-type strain 1 day post-inoculation and after (Fig. 5c). The growth of the mutant *in planta* could be completely restored by pLATC1976. These results show that Glk activity affects the growth of *Xcc* as well as the production of symptoms *in planta*.

DISCUSSION

In this work, we have demonstrated that *XC1976* in *Xcc* is the most active and makes the largest contribution to the total Glk activity, and that the protein influences EPS production in a complex fashion and is required for full virulence towards plants.

The insertional mutants of *XC1166* and *XC1223* still had Glk activity similar to that of the wild-type (Table 3), and no phosphorylation of glucose by either *XC1166*-His₆ or *XC1223*-His₆ could be observed on silica-coated TLC plates. We thus suppose that *XC1166* and *XC1223* do not serve as a Glk in *Xcc*, despite the close similarity of their amino acid sequences to those of other microbial Glks (Fig. 1) and the Glk activity of the purified proteins as measured in the linked assay (Table 3). These proteins could not phosphorylate any of a range of other sugars tested. Analysis of the deduced protein sequence using the SMART algorithm (<http://smart.embl-heidelberg.de>) showed differences in domain structure among *XC1166*, *XC1223* and *XC1976*. All three proteins contain a Glk domain (PF02685); however, *XC1166* and *XC1223* have in addition a Rok domain [residues 24–218 of *XC1166* (8.70×10^{-3}) and residues 20–215 of *XC1223* (1.10×10^{-2})]. The family of proteins with a Rok domain consists of repressors, ORFs with unidentified functions, and sugar kinases (Titgemeyer *et al.*, 1994). This difference in domain composition suggests that *XC1166* and *XC1223* fulfil other cellular functions, which remain obscure and require more work before they can be understood.

In *Xcc*, glucose uptake relies on two discrete systems: the intracellular phosphorylation pathway and the periplasmic oxidative pathway (Whitfield *et al.*, 1982; Lessie & Phibbs, 1984). Extracellular glucose entering cellular metabolism via the intracellular phosphorylation pathway will lead to the production of glucose 6-phosphate. Some of the glucose 6-phosphate will enter the pathways for polysaccharide production via phosphoglucomutase, and the remainder will enter the central metabolic network via glucose-6-phosphate dehydrogenase. Metabolic flux modelling predicts that if all external glucose enters the cytosol via the periplasmic pathway, no EPS will be synthesized. Conversely, if most glucose enters metabolism via intracellular phosphorylation, less EPS will be produced

Fig. 5. XC1974 is required for full virulence of *Xcc* to Chinese radish. (a) Symptom production on leaves 10 days after inoculation by clipping with the XC1976 mutant, the wild-type and the complemented mutant. (b) Mean lesion lengths caused by different *Xcc* strains. 1166nk and 1223nk have full virulence, and 1976nk has attenuated virulence; as analysed by the *t* test, the mean lesion length caused by 1976nk was significantly shorter ($P=0.01$) than that caused by the wild-type. Values given are the mean $\pm$ SD from 15 measurements. (c) Bacterial populations of the XC1976 mutant, the wild-type and the complemented mutant strain in host plant leaves. Inoculated leaves for each strain were taken daily and homogenized in sterile water. The homogenates were diluted and then plated on agar plates. Bacterial c.f.u. were counted after incubation for 3 days. Data are the mean $\pm$ SD from three replicates.

because the availability of biochemical energy is limited (Letisse *et al.*, 2002). Here we have shown that inactivation of XC1976 greatly reduced the Glk activity in *Xcc*. One consequence of this may be to impede glucose mobilization via the permease/phosphorylation pathway and lead to a decrease in the cellular level of precursors for EPS synthesis, with a resulting reduction in EPS yield.

The XC1976 mutant also produced less EPS than the wild-type when cultured in medium supplemented with arabinose, galactose, sucrose or maltose as carbon source. The sucrose-utilization system in *Xcc* has recently been described in some detail and is clearly different from that of other bacteria (Blanvillain *et al.*, 2007; Kim *et al.*, 2004; Reid & Abratt, 2005). In *Xcc*, extracellular sucrose is

transported through the outer membrane via a TonB-dependent receptor (SuxA), and through the inner membrane via a sugar transporter (SuxC). The intracellular sucrose is then hydrolysed by sucrose hydrolase (SUH) to yield glucose and fructose. The impeding of glucose phosphorylation through inactivation of XC1976 may thus lead to less sucrose utilization via EPS synthesis. Little is known about the pathways for utilization of arabinose, galactose or maltose in *Xcc*, although conversion of maltose (a disaccharide of glucose) to glucose seems likely.

Interestingly, the XC1976 mutant produced more EPS than the wild-type in fructose-containing medium. When plasmid pLATC1976, which resulted from the cloning of XC1976 in plasmid pLAFR3, was introduced into the XC1976 mutant, the resulting complemented strain

displayed about 50 % higher Glk activity than the wild-type strain when cultured in NYG medium containing 2 % fructose, but still produced more EPS than the wild-type (Table 3). To confirm the effect of *XC1976* mutation, three other isolates of the *XC1976* mutant were selected at random to test in fructose-containing medium; all produced more EPS than the wild-type (data not shown).

A similar phenomenon has been observed in *S. coelicolor*, in which both inactivation and overexpression of Glk result in a loss of carbon catabolite repression (CCR) of glycerol kinase and agarase (Kwakman & Postma, 1994). Glk has been implicated in the mechanism of CCR in several bacteria from the genera *Streptomyces*, *Staphylococcus* and *Bacillus* (Angell *et al.*, 1992, 1994; Kwakman & Postma, 1994; Wagner *et al.*, 1995; Spath *et al.*, 1997). CCR ensures that micro-organisms adjust their metabolic activities to utilize specific carbon sources in their environment (Brückner & Titgemeyer, 2002). A phosphoenolpyruvate-dependent phosphotransferase system (PTS) identified to be responsible for fructose transportation and phosphorylation in other bacteria (Prior & Kornberg, 1988; Geerse *et al.*, 1989; Wu *et al.*, 1990) has been demonstrated in *Xcc*, and a pathway for fructose catabolism has also been proposed (de Crécy-Lagard *et al.*, 1991a, b, 1995). However, although some details of fructose metabolism are understood, little is known about either the regulation of fructose utilization or CCR in *Xcc*. Further investigations are needed to fully understand the contribution of *XC1976* to such regulatory processes.

Mutation of *XC1976* also attenuates the virulence of *Xcc* to Chinese radish and leads to a reduction of growth in this host plant. It is possible that these effects result from an impairment of EPS synthesis by bacteria *in planta*. EPS plays an important role during bacterial infection. It can enhance the susceptibility of host plants by suppressing defence responses such as callose formation (Yun *et al.*, 2006), and contribute to biofilm formation (Dow *et al.*, 2003) and bacterial resistance against host defences. It may also serve to mask the bacterium to prevent recognition by the host and to enable colonization of host tissues (Alvarez, 2000). An alternative, but not mutually exclusive, view is simply that the reduction in ability to utilize available carbohydrates in the plant reduces bacterial growth with consequences for the aggressiveness of the pathogen. Consistent with this, it has recently been demonstrated that sucrose utilization is required for full pathogenicity in *Xcc* (Blanvillain *et al.*, 2007). Previously, we have shown that an *Xcc ppsA* mutant, which is unable to utilize C4-dicarboxylates as carbon source, has limited growth in plant hosts and reduced virulence compared to the wild-type (Tang *et al.*, 2005). Collectively, our findings suggest that the utilization of plant carbohydrates as well as C4-dicarboxylates is required for the full virulence of *Xcc*. These carbon sources may be available in different amounts at different stages of infection, as bacteria initially utilize components of the xylem fluid before mobilizing carbon sources by enzymic degradation of host polymers as black rot disease progresses.

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