Glyceraldehyde-3-phosphate dehydrogenase of *Xanthomonas campestris* pv. *campestris* is required for extracellular polysaccharide production and full virulence

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays an important role in glucose catabolism, converting glyceraldehyde 3-phosphates to 1,3-bisphosphoglycerates. Open reading frame (ORF) *XC_0972* in the genome of *Xanthomonas campestris* pv. *campestris* (*Xcc*) strain 8004 is the only ORF in this strain annotated to encode a GAPDH. In this work, we have demonstrated genetically that this ORF encodes a unique GAPDH in *Xcc* strain 8004, which seems to be constitutively expressed. A GAPDH-deficient mutant could still grow in medium with glucose or other sugars as the sole carbon source, and no phosphofructokinase activity was detectable in strain 8004. These facts suggest that *Xcc* may employ the Entner–Doudoroff pathway, but not glycolysis, to utilize glucose. The mutant could not utilize pyruvate as sole carbon source, whereas the wild-type could, implying that the GAPDH of *Xcc* is involved in gluconeogenesis. Furthermore, inactivation of the *Xcc* GAPDH resulted in impairment of bacterial growth and virulence in the host plant, and reduction of intracellular ATP and extracellular polysaccharide (EPS). This reveals that GAPDH is required for EPS production and full pathogenicity of *Xcc*.

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INTRODUCTION

It is well-known that almost all micro-organisms carry out the catabolic processes described as glycolysis, Entner–Doudoroff (ED) and/or pentose phosphate pathways to break down glucose and other simple sugars for energy and carbon molecules. The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays an important role in the pathways, converting the intermediates glyceraldehyde 3-phosphates (GAPs) to 1,3-bisphosphoglycerates. After a series of reactions, the 1,3-bisphosphoglycerates are converted further to pyruvates that may enter the tricarboxylic acid (TCA) cycle or other cellular processes, simultaneously producing ATP molecules. The GAPDH-encoding

genes of some bacteria have been characterized in some detail. For instance, *Escherichia coli* contains two GAPDH-encoding genes, *gapA* and *gapB*; mutational studies revealed that *gapA* is essential for glycolysis, whilst *gapB* is dispensable for both glycolysis and the pyridoxal biosynthesis pathway (Seta *et al.*, 1997). *Bacillus subtilis* and the cyanobacterium *Synechocystis* also possess two *gap* genes, of which one is essential for glycolysis whilst the other is operative in gluconeogenesis (Fillinger *et al.*, 2000; Koksharova *et al.*, 1998).

The phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*), a strict aerobe member of the family *Xanthomonadaceae*, is the causal agent of black-rot disease of cruciferous crops worldwide (Onsando, 1992). This pathogen naturally infects host plants via wounds or hydathodes, which are specialized pores on the leaf margins of higher plants that connect to the vascular system. After infection, the bacterial cells multiply and progress in vascular tissues, followed by the typical symptoms of vein blackening and V-shaped chlorotic and necrotic lesions extending from leaf margins along veins. The bacterium

Abbreviations: ED, Entner–Doudoroff; EPS, extracellular polysaccharide; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUS, β -glucuronidase; Kan, kanamycin; RACE, rapid amplification of cDNA ends; Rif, rifampicin; TCA, tricarboxylic acid; Tet, tetracycline; *Xcc*, *Xanthomonas campestris* pv. *campestris*.

A supplementary table showing bacterial strains and plasmids used in this work is available with the online version of this paper.

produces a large amount of extracellular polysaccharide (EPS) that plays an important role during bacterial infection (Denny, 1995). The EPS produced by *Xcc* is also called xanthan gum. Because of its physical properties, xanthan gum has been used widely as a viscosifier, thickener, emulsifier or stabilizer in both food and non-food industries (Kennedy & Bradshaw, 1984). Xanthan gum consists of D-glucosyl, D-mannosyl and D-glucuronyl acid residues in a molar ratio of 2:2:1 and variable proportions of O-acetyl and pyruvyl residues (Becker *et al.*, 1998). The ED pathway is implicated in the synthesis of xanthan gum (Hsu & Lo, 2003). Recently, we have determined the whole-genome sequence of *Xcc* strain 8004 (Qian *et al.*, 2005). The genome data show that strain 8004 possesses genes encoding all of the enzymes involved in the glycolysis, ED, pentose phosphate and gluconeogenesis pathways (Qian *et al.*, 2005). Interestingly, the genome contains only one predicted open reading frame (ORF) encoding a protein homologous to GAPDHs. In this paper, we present genetic evidence demonstrating that this ORF encodes a GAPDH that is required for the production of ATP and EPS, as well as the full virulence of *Xcc*.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Supplementary Table S1 (available with the online version of this paper). *E. coli* strains were grown in LB medium (Miller, 1972) at 37 °C. *Xcc* strains were grown at 28 °C in NYG medium (1⁻¹: 5 g peptone, 3 g yeast extract and 20 g glycerol; Daniels *et al.*, 1984), NY medium (NYG medium but without glycerol) or the non-carbohydrate minimal medium NCM (Tang *et al.*, 2005). Antibiotics were added at the following concentrations as required: kanamycin (Kan), 25 µg ml⁻¹; rifampicin (Rif), 50 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; spectinomycin, 50 µg ml⁻¹; and tetracycline (Tet), 5 µg ml⁻¹ for *Xcc* and 15 µg ml⁻¹ for *E. coli*.

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

Insertional mutant construction and complementation. An insertional mutant of ORF *XC_0972* was constructed by using the suicide plasmid pK18*mob* (Schäfer *et al.*, 1994; Windgassen *et al.*, 2000) as described previously (Lu *et al.*, 2007a). A 449 bp internal fragment, which spans nt 394–842 of the *XC_0972* ORF sequence, was amplified by PCR using the total DNA of *Xcc* wild-type strain 8004 as template and the following pair of oligonucleotides as primers: 5'-ACAGTTGGATCCTTCGTCTACGGTGTCAAC-3' and 5'-ACAGTTGGATCCACCTTGCTCTTCGGTATAG-3'. Primers were modified to give *Bam*HI-compatible ends (underlined). The amplified DNA fragments were cloned into pK18*mob* in the same orientation as the *lacZ* promoter. The resulting recombinant plasmid was introduced from *E. coli* strain JM109 (Yanisch-Perron *et al.*, 1985) into *Xcc* wild-type strain 8004 by triparental conjugation, using pRK2073 (Leong *et al.*, 1982) as the helper plasmid. The mutant was confirmed by PCR using the oligonucleotides 5'-GCCGATTCATT-AATGCAGCTGGCAC-3', located in pK18*mob*, and 5'-ACAGTTAA-GCTTGCATCAGACGGGAGTGCT-3', located downstream of the

XC_0972 ORF, as primers. The obtained mutant strain was named 0972nk (Supplementary Table S1).

For complementation of mutant 0972nk, a 1166 bp DNA fragment containing the *XC_0972* coding region and extending from 147 bp upstream of the 5' end to 17 bp downstream of the 3' end of the ORF was amplified by using the following pair of oligonucleotides as primers: 5'-ACAGTTGGATCCCAGCAGAGCGTGTGTTCT-3' and 5'-ACAGTTAAGCTTGCATCAGACGGGAGTGCT-3'. Primers were modified to give *Bam*HI- or *Hind*III-compatible ends (underlined). The amplified DNA fragment was cloned into plasmid pLAFR3 (Staskawicz *et al.*, 1987). The obtained recombinant plasmid was transferred into the mutant strain 0972nk by triparental conjugation, resulting in the complemented strain named C0972 (Supplementary Table S1).

Determination of transcriptional start site. The 5'-RACE (rapid amplification of cDNA 5' ends) method was used to determine the transcriptional start site of the *XC_0972* gene. Total RNA was extracted from cultures (10 ml) of *Xcc* wild-type strain 8004 after 12 h incubation (exponential-growth phase) by using an RNeasy Midi kit (Qiagen). The isolated RNA was treated with RNase-free DNase I (Qiagen) at 25 °C for 1 h, followed by a second purification using an RNeasy column. cDNA fragments were obtained by using a 5'-RACE kit (Invitrogen Life Technologies). RNA was reverse-transcribed by using the *XC_0972* sequence-specific primer GSP1 (5'-TTCCTTGGTCAGGAACAG-3'). An anchor sequence was then added to the 3' end of the cDNA by using terminal deoxynucleotide transferase, followed by direct amplification of tailed cDNA using the nested gene-specific primers GSP2 (5'-GCATCCCACTTCAGGTTG-3') and GSP3 (5'-TTGATGGCCACGATCTCG-3'), and an anchor-specific primer (Sangon Biological Engineering Technology and Services Co. Ltd). PCR products were then cloned into the pMD19-T vector (TaKaRa) and sequenced.

Cell-free extract preparation. Cells cultured in NY medium were harvested by centrifugation and washed twice in sterile water. The cells were then resuspended in 2.0 ml 20 mM potassium phosphate (pH 7.5) containing 1 mM dithiothreitol and 0.1 mM EDTA, and disrupted by sonication (three times for 10 s each at 350 W output). Following sonication, the cell debris and intact cells were removed by centrifugation (10 min at 18 000 g) in a 4 °C centrifuge, and the supernatant fractions were collected and used in the enzyme assay.

GAPDH assay. The GAPDH activity from the purified protein or cell-free extracts, based on the conversion of NAD⁺ to NADH, was measured by monitoring the change in A₃₄₀ over time at 37 °C on a Beckman DU730 spectrophotometer (Ganter & Plückthun, 1990). A 10 µl aliquot of the protein sample was transferred to a 5 ml tube containing 1 ml reaction buffer [100 mM Tris/HCl (pH 8.0), 20 µM DL-GAP, 2 µM NAD⁺, 10 µM EDTA, 0.2 µM β-mercaptoethanol]. One unit of enzyme activity was taken as 1 nmol NADH formed min⁻¹ (mg protein)⁻¹; the protein concentration in the samples was determined by using a BCA protein assay kit (Pierce Biotechnology). For characterizing the kinetic parameter of the purified protein, a reaction mixture including 1.0 mg purified GAPDH and a range of DL-GAP concentrations (0.005–10 mM) was used under standard assay conditions. The Michaelis–Menten kinetic parameter was evaluated by the double reciprocal-plot method.

Real-time quantitative RT-PCR. Real-time PCR analysis was carried out by using the DNA Engine OPTICON 2 Continuous Fluorescence Detection system (MJ Research, Inc.) with a commercial kit (RealMasterMix, SYBR green; TIANGEN). Total RNA was isolated by using the SV Total RNA Isolation system (Promega) and cDNA fragments were obtained by using a RevertAid First Strand cDNA Synthesis kit (Fermentas). The resulting first-strand cDNA was diluted

to a final volume of 20 μ l, and SYBR green-labelled PCR fragments were amplified by using primers 5'-TCGACGGCAACACGCTGATC-3' and 5'-TTGGTCAGGAACAGGCCGGT-3', designed from the transcribed region of the *XC_0972* gene. The thermal-cycling programme consisted of an initial activation step at 95 °C for 2 min, followed by 49 cycles of denaturation at 95 °C for 20 s, annealing at 54.6 °C for 20 s and extension at 72 °C for 20 s. The expression level of the 16S rRNA gene was used as an internal standard, and fragments were amplified by using primers 5'-GCCTAACACATGCAAGTCGAACGGC-3' and 5'-AATATTCC-CCACTGCTGCCTCCCG-3'. The comparative-threshold method was used to calculate the relative mRNA level with respect to the corresponding transcript in cells cultured in NY medium. All real-time quantitative RT-PCRs were performed in triplicate.

Overproduction and purification of protein. For overproduction of *XC_0972*, the ORF was amplified by using the following pair of oligonucleotides as primers: 5'-ACAGTTGGATCCATGGCAATCAAGGTTGGC-3' and 5'-ACAGTTAAGCTTCTTGGCCACAACCCG-CAC-3'. Primers were modified to give *Bam*HI- or *Hind*III-compatible ends (underlined). After being confirmed by sequencing, the amplified DNA fragment was cloned into the expression vector pQE-30 (Qiagen) to generate the recombinant plasmid pQE-30-0972. In this plasmid, *XC_0972* is fused (N-terminally and in frame) to the His₆ tag-coding region of plasmid pQE-30. The recombinant plasmid pQE-30-0972 was transformed into *E. coli* strain JM109, resulting in strain JM109/pQE-30-0972. For overproduction and purification of the fused protein, strain JM109/pQE-30-0972 was grown to an OD₆₀₀ of 0.6 and then induced by addition of 1.0 mM IPTG. After the culture was grown for a further 4 h, the fused protein was purified by Ni-NTA resin (Qiagen). The purified protein was checked by SDS-PAGE (12 % gel) and diluted in 25 % (v/v) glycerol for enzymic assay.

β -Glucuronidase (GUS) activity assay. *Xcc* strains were cultured in NY medium overnight and diluted to an OD₆₀₀ of 0.5, and 1.0 ml of each was inoculated into 200 ml NY medium held in 500 ml flasks. GUS activities were determined at 8 h intervals until 48 h by

measurement of A_{415} , using *p*-nitrophenyl β -D-glucuronide as the substrate, as described by Henderson *et al.* (1985).

EPS assay. To estimate EPS production, strains were cultured in 100 ml NY medium supplemented with 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).

ATP determination. Intracellular ATP levels were determined by a modification of the luciferin-luciferase bioluminescence assay (Kimmich *et al.*, 1975), using prepared standard reagents on a Sirius Single Tube Luminometer (Berthold Detection Systems). After culture in NY medium supplemented with 2 % glucose for 12, 24 or 36 h, cells were harvested quickly and immediately resuspended in Tris (100 mM)/EDTA (2 mM) buffer (pH 7.75), followed by cooling in an ice bath. Measurements were done by addition of 80 μ l luciferase reagent (3 mg ml⁻¹) to a 20 μ l sample. All samples were measured at least in triplicate. Standard ATP curves were generated with standards containing known amounts of ATP in Tris/EDTA buffer. Intracellular ATP levels were also measured by the modified method developed by Schneider & Gourse (2004).

Virulence assay. The virulence of *Xcc* to the host plant Chinese radish (*Raphanus sativus*) was tested by the leaf-clipping method (Dow *et al.*, 2003). Lesion length was measured 10 days after inoculation and data were analysed by *t*-test. The growth of bacteria in radish leaf tissues was determined as described previously (Lu *et al.*, 2007a).

RESULTS

ORF *XC_0972* of *Xcc* strain 8004 encodes a GAPDH protein

In the reported genome sequence of *Xcc* strain 8004, only the protein encoded by ORF *XC_0972* (NCBI Protein

Fig. 1. Genetic and physical map of the *gapA* gene in the genome of *Xcc* strain 8004. Open arrows indicate length, location and orientation of the ORFs. ∇ above ORF *XC_0972* indicates the insertion site of a transposon Tn5*gusA5* derivative in mutant 069F10. The middle elements show the PCR fragments amplified in this work; the 449 bp fragment was cloned into the *Bam*HI site within suicide plasmid pK18*mob* for mutation of the *gapA* gene, the 1166 bp fragment was cloned into the *Bam*HI/*Hind*III site within plasmid pLAFR3 for complementation of insertional mutant 0972nk, and the 999 bp fragment was cloned into the *Bam*HI/*Hind*III site within expression vector pQE-30 for the overexpression of GAPDH in *E. coli*. The lower element shows the 100 nt sequence upstream of the start codon ATG (included) of the ORF of the *gapA* gene. The transcriptional start site of the *gapA* promoter is indicated by an arrow. The putative -10 and -35 regions and the Shine–Dalgarno sequence (SDS) are underlined.

accession no. YP_242066.1) was assigned as a GAPDH (Qian *et al.*, 2005), which displays 73 and 82 % identity and similarity, respectively, to the GAPDH encoded by the *gapA* gene of *E. coli* (NCBI Protein accession no. P0A9B2) (data not shown). Sequence comparison showed that no other ORFs in the genome of *Xcc* strain 8004 display DNA sequences homologous to GAPDH-encoding genes. ORF *XC_0972* is 1002 bp in length and located in the genome sequence at position 1164956–1165957 (Fig. 1). The transcriptional start site of *XC_0972* was determined to be 42 nt upstream of the ORF start codon ATG by 5'-RACE PCR (Fig. 1). Putative -10 and -35 sequences and a potential Shine-Dalgarno sequence are present in the promoter region (Fig. 1).

To determine whether the protein encoded by ORF *XC_0972* has GAPDH activity, ORF *XC_0972* was cloned into the expression vector pQE-30 to generate the recombinant plasmid pQE-30-0972 and overexpressed with His₆ tags in *E. coli* strain JM109, as outlined in Methods. Separation of crude cell lysates by SDS-PAGE showed an intense protein band migrating at the expected molecular mass of 38 kDa (Fig. 2a). The intense protein band was also present in the crude cell lysate without IPTG induction (Fig. 2a). To verify whether the protein band was expressed from the vector pQE-30, the crude cell lysate of strain JM109 harbouring pQE-30 was examined; no such protein band was observed (data not shown). It is possible that the intense protein band present in the crude cell lysate without IPTG induction was due to leaky expression from pQE-30-0972. The fusion protein with a His₆ tag was purified by using an Ni-NTA column, to obtain preparations apparently free from contaminating proteins (Fig. 2a). The GAPDH activity of the purified fusion protein was tested as described by Ganter & Plückthun (1990), with NAD⁺ as a coenzyme. The results showed that the fusion protein exhibited GAPDH activity over a broad pH range and was optimally active at pH 9.5 (Fig. 2b). The optimum temperature for the enzyme activity was 35 °C and activity dropped sharply at 45 °C (Fig. 2c). At 35 °C and pH 9.5, the maximum GAPDH activity value of the fusion protein was determined to be 5.26×10^5 U (mg protein)⁻¹ and the apparent kinetic constant (K_m) value for GAP was 680 μM. The enzymic activity of the purified protein was tested further by using NADP⁺, instead of NAD⁺, as coenzyme; no activity was detected. As a control, Ni-NTA column-purified crude cell extract of *E. coli* strain JM109 was also tested for GAPDH activity; no significant activity was detected. These results demonstrate that ORF *XC_0972* of *Xcc* strain 8004 encodes an NAD-dependent GAPDH enzyme. Therefore, we designated ORF *XC_0972* as *gapA* gene in *Xcc*.

GAPDH is important for utilization of different carbohydrates

To investigate the physiological role of the GAPDH in *Xcc*, a mutant carrying insertional disruption within the GAPDH-

Fig. 2. Purification of His₆-tagged recombinant protein XC₀₉₇₂ from *E. coli* JM109/pQE-30-0972 and its characterization. (a) SDS-PAGE analysis of His₆-tagged recombinant protein XC₀₉₇₂. Lanes: 1, crude extract of *E. coli* JM109; 2, crude extract of *E. coli* JM109/pQE-30-0972 before induction with IPTG; 3, crude extract of *E. coli* JM109/pQE-30-0972 after induction with IPTG; 4, affinity-purified His₆-XC₀₉₇₂ protein; M, molecular mass marker. (b, c) Effect of pH (b) and temperature (c) on the GAPDH activity of the His₆-XC₀₉₇₂ protein. Values are the means $\pm$ SD of triplicate measurements from a representative experiment; similar results were obtained in two other independent experiments.

encoding ORF *XC_0972* was constructed by homologous suicide-plasmid integration (see Methods for details) and named 0972nk (see Supplementary Table S1, available with the online version of this paper). Simultaneously, a complemented strain, named C0972 (Supplementary Table S1), was also constructed by introducing the recombinant plasmid pL0972 (Supplementary Table S1), in which a 1166 bp DNA fragment from 147 bp upstream to 17 bp downstream of the ORF *XC_0972* sequence (Fig. 1) was cloned into vector pLAFR3 (Supplementary Table S1), into

Fig. 3. Growth curves of *Xcc* strains in minimal medium NCM containing glucose, sucrose, mannose, fructose, galactose or pyruvate as the sole carbon source. □, Wild-type strain 8004; ○, *gapA* mutant 0972nk; △, complemented strain C0972. As a control, growth of mutant strain 0972nk in the nutrient-rich complex medium NY is shown. Strains were inoculated into 100 ml liquid medium and incubated at 28 °C with shaking at 200 r.p.m. Aliquots were taken in triplicate at intervals, and bacterial growth was determined by measuring OD₆₀₀ against the medium blank. Data presented are from a representative experiment; similar results were obtained in two other independent experiments.

the mutant strain 0972nk. The mutant and the wild-type grew identically in modified nutrient-rich complex NY medium (Fig. 3). No GAPDH activity was detected in the cultures of the mutant grown in NY medium alone or supplemented with a range of various sugars, whereas the wild-type and the complemented strain produced significant GAPDH activity (Table 1). This further validates that *Xcc* strain 8004 possesses only one GAPDH protein, which is encoded by ORF *XC_0972*.

To examine the effect of the GAPDH on carbohydrate utilization of *Xcc*, bacteria were grown in the minimal medium NCM supplemented with glucose, sucrose, mannose, fructose or galactose as the sole carbon source. The GAPDH-deficient mutant 0972nk displayed smaller colonies than the wild-type on all plates tested (data not

shown). In liquid NCM supplemented with the sugars listed above as the sole carbon source, growth of the mutant was significantly slower than that of the wild-type (Fig. 3), suggesting that the capability of the mutant to utilize these sugars is diminished. The mutant stopped growing in NCM supplemented with pyruvate as the sole carbon source (Fig. 3). The growth of the complemented strain C0972 was identical to that of the wild-type in NCM supplemented with different carbohydrates as the sole carbon source (Fig. 3), indicating that the capability of the mutant to utilize various carbohydrates could be restored completely by the wild-type *gapA* gene *in trans*. These results show that the *gapA* gene is important for efficient utilization of sugars and other glycolytic carbon sources, but absolutely required for utilization of gluconeogenic ones.

Table 1. GAPDH activity in *Xcc* strains

GAPDH activity in *Xcc* strains was assayed with cell-free extracts, prepared as described in Methods. Strains were cultured in NY medium alone or supplemented with various sugars for 24 h. Data presented are the means $\pm$ SD of triplicate measurements from a representative experiment; similar results were obtained in two other independent experiments. ND, Not detectable.

Strain	GAPDH activity [nmol NADH min ⁻¹ (mg protein) ⁻¹]					
	No sugar	Glucose	Sucrose	Mannose	Fructose	Galactose
8004 (wild-type)	333 $\pm$ 38	321 $\pm$ 35	326 $\pm$ 53	346 $\pm$ 46	337 $\pm$ 12	331 $\pm$ 26
0972nk (<i>XC_0972</i> mutant)	ND	ND	ND	ND	ND	ND
C0972 (complemented mutant)	427 $\pm$ 43	488 $\pm$ 46	437 $\pm$ 71	485 $\pm$ 62	419 $\pm$ 52	442 $\pm$ 48
8004/pL0972 (8004 harbouring pL0972)	442 $\pm$ 52	456 $\pm$ 48	431 $\pm$ 63	471 $\pm$ 67	422 $\pm$ 42	434 $\pm$ 59

GAPDH is required for EPS production by *Xcc*

To define whether GAPDH is required for EPS production by *Xcc*, we measured EPS production by the GAPDH-deficient mutant 0972nk. Strains were grown on NY agar plates supplied with glucose, sucrose, mannose, fructose or galactose. The mutant displayed smaller colonies than the wild-type strain on all plates tested (data not shown). Interestingly, the complemented strain formed colonies similar to those of the wild-type on plates containing glucose or sucrose, but formed colonies larger than those of the wild-type on plates containing mannose, fructose or galactose. To verify whether the smaller-colony phenotype of the *gapA* mutant was due to lower EPS production or impaired growth, we measured bacterial growth and EPS production of the *gapA* mutant in NY medium supplemented with 2 % glucose over a time-course. As shown in Fig. 4(a), the mutant produced significantly less EPS than the wild-type at each time point tested. However, the bacterial growth rates of the mutant and the wild-type were almost identical. These indicate that the *gapA* mutant is deficient in EPS production.

To estimate the level of EPS produced by the mutant, strains were cultured in NY liquid medium supplemented with 2 % various sugars for 3 days, and EPS was extracted from the cultures (see Methods for details). As summarized in Fig. 4(b), the mutant produced about 70–80 % less EPS than the wild-type in all of the tests. In addition, the EPS yield of the complemented strain showed no significant difference from that of the wild-type when cultured in medium containing glucose or sucrose; however, the complemented strain produced about 10 % more EPS than the wild-type when cultured in medium containing mannose, fructose or galactose. These results demonstrate that the GAPDH is involved in EPS production by *Xcc*.

GAPDH affects the intracellular ATP level of *Xcc*

To clarify whether GAPDH has any effect on intracellular energy production in *Xcc*, the GAPDH-deficient mutant strain 0972nk was cultured in NY medium supplemented with 2 % glucose, and its intracellular ATP level was measured by luciferin–luciferase bioluminescence assay (Kimmich *et al.*, 1975). The result showed that the intracellular ATP level of the mutant 0972nk was 85.6, 74.8 and 64.2 % of that of the wild-type when cultured for 12, 24 and 36 h, respectively (Table 2). The ATP levels of the complemented strain C0972 and the wild-type strain 8004 were almost identical (Table 2), indicating that the ATP production of the mutant could be restored completely by the wild-type *gapA* gene *in trans*. The intracellular ATP levels of the *Xcc* strains were also determined by the method described by Schneider & Gourse (2004). The result also displayed that the *gapA* mutant produced significantly less intracellular ATP than the wild-type (Table 2). These results reveal that GAPDH is essential to maintain a high level of intracellular ATP in *Xcc*.

Fig. 4. EPS production of *Xcc* strains. (a) Time-course of EPS production in NY medium supplemented with 2 % (w/v) glucose. EPS yield and bacterial growth were determined at 12 and 4 h intervals, respectively, until 72 h. Bars represent the EPS yield; open and shaded indicate the wild-type strain and the *gapA* mutant, respectively. Curves represent bacterial growth, measured by counting bacterial c.f.u.; ● and △ indicate the wild-type strain and the *gapA* mutant strain, respectively. Data are the means $\pm$ SD of three replicates. (b) EPS production in nutrient-rich complex NY medium supplemented with 2 % (w/v) various sugars. Values given are the means $\pm$ SD of triplicate measurements from a representative experiment; similar results were obtained in two other independent experiments.

GAPDH is required for full virulence and growth of *Xcc* in the host plant

To determine whether GAPDH is involved in the pathogenicity of *Xcc*, the virulence of the GAPDH-deficient mutant 0972nk was tested on the host plant Chinese radish by the leaf-clipping method (Dow *et al.*, 2003). Ten days after inoculation, the mutant caused black-rot symptoms with a mean lesion length of 5.5 mm, whereas the wild-type produced a mean lesion length of about 12 mm (Fig. 5a, b). As analysed by *t*-test, the mean lesion length caused

Table 2. Intracellular ATP levels in *Xcc* strains

Strains were cultured in NY medium supplemented with 2% glucose. Data presented are the means $\pm$ SD of triplicate experiments. Assays A and B used the methods described by Kimmich *et al.* (1975) and Schneider & Gourse (2004), respectively.

Strain	ATP content [$\mu\text{mol (g dry cell weight)}^{-1}$]					
	Assay A			Assay B		
	12 h	24 h	36 h	12 h	24 h	36 h
8004 (wild-type)	3.60 ± 0.17^a	2.82 ± 0.08^a	1.95 ± 0.11^a	3.32 ± 0.12^a	2.45 ± 0.08^a	1.42 ± 0.07^a
0972nk (<i>gapA</i> mutant)	3.08 ± 0.14^b	2.11 ± 0.12^b	1.25 ± 0.07^b	3.02 ± 0.05^b	2.08 ± 0.11^b	1.17 ± 0.05^b
C0972 (complemented mutant)	3.72 ± 0.15^a	2.99 ± 0.18^b	1.83 ± 0.11^a	3.21 ± 0.15^a	2.35 ± 0.11^a	1.35 ± 0.13^a

Different letters *a* and *b* in each column indicate significant differences at $P \leq 0.05$ (*t*-test).

by the mutant was significantly shorter than that caused by the wild-type ($P=0.01$). Interestingly, the complemented strain C0972 induced disease symptoms with a mean lesion length of about 8.2 mm (Fig. 5b), which is significantly longer than that caused by the mutant ($P=0.01$ by *t*-test), but significantly shorter than that generated by the wild-type ($P=0.01$ by *t*-test), showing that the virulence of the mutant could not be restored completely to that of the wild-type by the *gapA* gene cloned in pLAFR3 (i.e. the recombinant plasmid pL0972). There was no significant difference between the mean lesion lengths caused by strain 8004/pL0972 and the wild-type ($P=0.05$ by *t*-test); the mean lesion length of the former was 11.5 mm (Fig. 5b).

To ascertain the role of GAPDH in growth of *Xcc* inside the host, the populations of the GAPDH-deficient mutant 0972nk, the wild-type strain 8004 and the complemented strain C0972 in the infected radish leaves were compared. The results indicated that the number of bacteria (c.f.u.) of the mutant recovered from the infected leaves was approximately 10- to 100-fold lower than that of the wild-type strain at each of the test points (Fig. 5c). The bacterial number of the complemented strain was significantly higher than that of the mutant, at each of the test points ($P=0.01$ by *t*-test) (Fig. 5c). The stability of the recombinant plasmid pL0972 in the complemented strain *in planta* was assessed. Leaves inoculated with the complemented strain C0972 were homogenized, diluted in sterile water and plated on NYG agar plates supplemented with Rif and Kan only. The colonies formed on the plates were then replicated onto NYG agar plates supplemented with Rif, Kan and Tet. Respectively 90, 84 and 84% of the Rif/Kan-resistant colonies from the infected leaves at 2, 4 and 8 days post-inoculation could grow on the Rif/Kan/Tet-containing plates, suggesting that the recombinant plasmid pL0972 in the complemented strain is relatively unstable and that 10–16% bacterial cells of the complemented strain C0972 lost plasmid pL0972 and reverted to mutant status *in planta*. This may be the cause for the phenomenon that the virulence of the mutant could not be restored completely to the wild-type level by pL0972. Overall, these data reveal that

GAPDH activity is required for full virulence and growth of *Xcc* in the host plant.

GAPDH appears to be constitutively expressed in *Xcc*

A transposon Tn5gusA5 insertional mutant of the *GapA*-encoding ORF XC_0972, named 069F10, was obtained from the mutant collection of strain 8004 in our laboratory. In this mutant strain, the transposon Tn5gusA5 is inserted at nt 987 of ORF XC_0972 and the promoterless *gusA5* gene is fused in the same translational direction as XC_0972 (Fig. 1). Therefore, the GUS activity level in the mutant strain 069F10 might mirror the expression of *gapA*. To monitor the expression level of *gapA*, the GUS activity of the mutant strain 069F10 was measured after culture in NY medium. As shown in Fig. 6(a), the GUS activity of the mutant increased markedly in the stationary phase and decreased when bacterial growth entered the decline phase. To determine the effect of sugars on the expression of *gapA*, the GUS activity of mutant 069F10 was also assessed when cultured in NY medium supplemented with glucose, sucrose, mannose, fructose or galactose as sole carbon source. In all cases, the GUS expression profile resembled the profile shown in Fig. 6(a) (data not shown), suggesting that these sugars do not affect the expression of *gapA* in *Xcc*. To verify this observation, a real-time quantitative RT-PCR was employed to assay the *gapA* transcripts in the wild-type grown in NY medium alone and supplemented with the various sugars. The results showed that the level of *gapA* transcripts between the different media differed only within 1.3-fold (Fig. 6b), suggesting that the *gapA* expression of *Xcc* is not affected by any of the sugars tested.

The GAPDH activity of wild-type strain 8004 was also determined during growth in NY medium. The result showed that the GAPDH enzymic-activity profile of the wild-type is similar to the GUS expression profile of mutant 069F10 (data not shown). The GAPDH enzymic activity reached a maximum level in the stationary phase and decreased afterwards. The GAPDH activity of the wild-

Fig. 5. GAPDH is required for full virulence of *Xcc* to Chinese radish. (a) Symptom production on leaves 10 days after inoculation by clipping with the *gapA* mutant 0972nk, the wild-type strain 8004 and the complemented mutant C0972. (b) Mean lesion lengths caused by different *Xcc* strains. Mutant 0972nk has attenuated virulence. However, complementation did not restore virulence to that of the wild-type. Values given are the means $\pm$ SD from 15 measurements. (c) Bacterial populations of mutant 0972nk (○), wild-type strain 8004 (◇) and complemented mutant C0972 (▲) in host plant leaves. Inoculated leaves for each strain were taken daily and homogenized in sterile water. The homogenates were diluted and plated on NYG plates. Bacterial c.f.u. were counted after incubation for 3 days. Data are the means $\pm$ SD from three replicates.

sugar induction was observed. The above results suggest that the GAPDH might be constitutively expressed in *Xcc*.

DISCUSSION

In this work, we have clarified that *Xcc* strain 8004 possesses only one GAPDH that is encoded by the *gapA* gene (ORF XC_0972). The GAPDH was overexpressed with His₆ tags in *E. coli*. The apparent K_m value of the His₆-tagged GAPDH for GAP was determined to be 680 μ M, which is significantly higher than those of most GAPDHs identified from other microbial sources. The apparent K_m values of GAPDHs from different micro-organisms for GAP differ largely, e.g. 0.01, 100 and 150 μ M for *Bacillus stearothermophilus* (Suzuki & Imahori, 1973), *Streptomyces arenae* (Maurer *et al.*, 1983) and *Trypanosoma brucei* (Lambeir *et al.*, 1991), respectively. A very high K_m value (730 μ M) for GAP has been also reported for the GAPDH of *Thermus aquaticus* (Harris *et al.*, 1980). The very high K_m value of the *Xcc* GAPDH for GAP would deserve a specific enzymological study. Like many bacterial counterparts, the *Xcc* GAPDH acquires its enzymic activity by using NAD⁺ specifically as a cofactor. However, differing from the observation that the expression of the *gap* gene is induced by glucose in *Streptomyces aureofaciens*, *E. coli* and *B. subtilis* (Kormanec *et al.*, 1997; Charpentier *et al.*, 1998; Tobisch *et al.*, 1999; Doan & Aymerich, 2003), the *Xcc gapA* gene seems to be constitutively expressed. We have also demonstrated that inactivation of the *Xcc* GAPDH results in impairment of bacterial growth and virulence in the host plant, deficiency of the capability to utilize various carbohydrates, and reduction of ATP and EPS. Although metabolic pathways are generally not considered to be virulence factors, elucidation of the mechanism to acquire and metabolize carbohydrates is critically important for fully understanding the pathogenicity, as well as the EPS production, of *Xcc*.

As described above, *E. coli*, *B. subtilis* and the cyanobacterium *Synechocystis* possess two GAPDH-encoding *gap* genes and one of them is essential for glycolysis (Seta *et al.*, 1997;

type grown in medium supplemented with various sugars (glucose, sucrose, mannose, fructose or galactose) as the sole carbon source was also tested. The results displayed that all of the GAPDH-activity profiles were similar to that of the wild-type in NY medium (data not shown). No

Fig. 6. Expression of the *gapA* gene in *Xcc*. (a) GUS activities in mutant strain 069F10 (*gapA* :: Tn5*gusA5*) cultured in NY medium. GUS activities and bacterial growth were determined at 8 and 4 h intervals, respectively, until 48 h. Bars represent GUS activities; the curve represents bacterial growth. Values given are the means $\pm$ SD of triplicate measurements from a representative experiment; similar results were obtained in two other independent experiments. (b) Real-time quantitative RT-PCR analysis. RNA was isolated from cultures of *Xcc* wild-type strain 8004 grown in NY medium alone and supplemented with various sugars as the sole carbon source for 24 h. The relative mRNA level was calculated with respect to the level of the corresponding transcript in cells cultured in NY medium (equalling 1). Values given are the means $\pm$ SD of triplicate measurements from a representative experiment; similar results were obtained in two other independent experiments.

Fillinger *et al.*, 2000; Koksharova *et al.*, 1998). In many bacteria, glucose is catabolized through the glycolysis pathway, which functions primarily to provide energy in the form of ATP (Gottschalk, 1986). In *E. coli*, the GAPDH encoded by the *gapA* gene is essential for glycolysis and the *gapA*-mutant strain cannot grow in medium with glucose as the sole carbon source (Seta *et al.*, 1997). However, the

Xcc gapA mutant could still grow in minimal medium with glucose as the sole carbon source, although the growth rate was slower than that of the wild-type (Fig. 3). Early enzymic studies showed that *Xcc* lacks phosphofructokinase activity, which converts fructose 6-phosphate to fructose-1,6-bisphosphate in glycolysis and is essential for a functional glycolytic pathway (de Crécy-Lagard *et al.*, 1991; Whitfield *et al.*, 1982). We have also tested the phosphofructokinase activity of *Xcc* strain 8004 and no detectable enzymic activity was observed (data not shown), although a putative phosphofructokinase-encoding gene was annotated in the genome of *Xcc* strain 8004 (Qian *et al.*, 2005). In addition, glucose-flow studies demonstrated that, in *Xcc*, glucose is catabolized mostly via the ED pathway and slightly through the pentose phosphate pathway (Whitfield *et al.*, 1982; Pielken *et al.*, 1988). In the ED pathway, a glucose molecule is broken down via four steps to a pyruvate molecule, which may enter the TCA cycle or other cellular processes, and a GAP molecule that is catabolized further to a pyruvate molecule after five reactions, including the GAPDH dehydrogenation. Therefore, without GAPDH activity, the *Xcc gapA* mutant can obtain a certain amount of pyruvate via the ED pathway to keep a basic TCA cycle and provide a certain amount of ATP energy in medium with glucose as the sole carbon source. In consequence, the *Xcc gapA* mutant could still grow in medium with glucose as the sole carbon source, although the bacterium possesses only one *gap* gene.

The *Xcc gapA* mutant completely lost the capability to grow in minimal medium supplemented with pyruvate as the sole carbon source (Fig. 3). This is probably due to a break in the gluconeogenesis pathway of the mutant. It is well-known that bacteria may employ gluconeogenesis to synthesize glucose from non-sugar C₂ or C₃ compounds or the intermediates of the TCA cycle when there is not sufficient hexose in their niches. In the first phase of gluconeogenesis, pyruvates are converted after several reactions to 1,3-bisphosphoglycerates, which are converted further to GAPs by GAPDH. It has been demonstrated that, in *B. subtilis* and the cyanobacterium *Synechocystis*, one of the two GAPDH-encoding *gap* genes is involved in gluconeogenesis (Fillinger *et al.*, 2000; Koksharova *et al.*, 1998). Our previous work has shown that *Xcc* strain 8004 possesses a functional gluconeogenesis pathway that is indispensable for the bacterium to utilize pyruvate as the sole carbon source (Tang *et al.*, 2005). Therefore, we deduce that, in addition to functioning in the ED pathway, the GAPDH encoded by the unique *gapA* gene in *Xcc* is also operative in the gluconeogenesis pathway.

The EPS produced by *Xcc* consists of D-glucosyl, D-mannosyl, D-glucuronyl acid, O-acetyl and pyruvyl residues (Becker *et al.*, 1998). The biosynthesis of EPS in *Xcc* is a complex process that requires a series of enzymes and a significant proportion of total cellular nicotinamide cofactors and ATP (Letisse *et al.*, 2001; Jarman & Pace, 1984). The *Xcc* GAPDH-deficient mutant produced

significantly less EPS and intracellular ATP than the wild-type. The EPS reduction of the mutant is probably due to ATP diminution and short supply of carbon precursors such as *O*-acetyl and pyruvyl residues. Interestingly, the complemented strain produced about 10 % more EPS than the wild-type when cultured in medium containing mannose, fructose or galactose. The reason for this is unknown.

The attenuation in virulence and growth *in planta* of the GAPDH-deficient mutant may result, at least partially if not completely, from the reduction in EPS synthesis and weakened ability to utilize carbohydrates. EPS has been shown to act as an important virulence factor in *Xcc*. 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). *Xcc* is a xylem-colonizing systemic pathogen that invades host plants via hydathodes or wounds in the leaves, and propagates and spreads in the apoplasts (Hayward, 1993). After entering the host, the pathogen needs to acquire nutrients such as carbon from the host to establish a successful infection. Therefore, the reduction in ability of the GAPDH-defective mutant to utilize available carbohydrates in the host reduces its growth, with consequences for its aggressiveness. Consistent with this, it has recently been demonstrated that glucose and sucrose utilization is required for full virulence in *Xcc* (Blanvillain *et al.*, 2007; Lu *et al.*, 2007b). Previously, we have shown that an *Xcc ppsA* (a gene encoding phosphoenolpyruvate synthase, the key enzyme in gluconeogenesis) mutant, which has lost the capability to utilize pyruvate and C₄ dicarboxylates as carbon sources, has limited growth in host tissues and significantly reduced virulence compared with the wild-type (Tang *et al.*, 2005). Finally, we would like to point out that, in addition to the metabolic function, GAPDH has been shown to be implicated in other cellular processes, including transcription activation and initiation of apoptosis in eukaryotes (Tarze *et al.*, 2007; Zheng *et al.*, 2003). Whether the GAPDH in *Xcc* has any other functions in addition to catalysing carbon metabolism, especially those implicated in pathogenicity, remains to be further investigated.

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