Glucose and trehalose PTS permeases of *Spiroplasma citri* probably share a single IIA domain, enabling the spiroplasma to adapt quickly to carbohydrate changes in its environment

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*SPIROPLASMA CITRI* is a plant-pathogenic mollicute phylogenetically related to Gram-positive bacteria. Spiroplasma cells are restricted to the phloem sieve tubes and are transmitted from plant to plant by the leafhopper vector *Circulifer haematoceps*. In the plant sieve tubes, *S. citri* grows on glucose and fructose, whereas in the leafhopper haemolymph the spiroplasma must grow on trehalose, the major sugar in insects. Previous studies in this laboratory have shown that fructose utilization was a key factor of spiroplasmal pathogenicity. To further study the implication of sugar metabolism in the interactions of *S. citri* with its plant host and its leafhopper vector, genes encoding permease enzymes II (EII Glc and EII Tre) of the *S. citri* phosphoenolpyruvate : glucose and phosphoenolpyruvate : trehalose phosphotransferase systems (PTS) were characterized. Mapping studies revealed that the EII Glc complex was split into two distinct polypeptides, IIA Glc and IICB Glc, encoded by two separate genes, *crr* and *ptsG*, respectively. As expected, *S. citri* polypeptides IIA Glc and IICB Glc were more phylogenetically related to their counterparts from Gram-positive than to those from Gram-negative bacteria. The trehalose operon consisted of three genes *treR*, *treP* and *treA*, encoding a transcriptional regulator, the PTS permease (EII Tre) and the amylase, respectively. However, in contrast to the fructose-PTS permease, which is encoded as a single polypeptide (IIABCFru) containing the three domains A, B and C, the trehalose-PTS permease (IIBC Tre) lacks its own IIA domain. No trehalose-specific IIA could be identified in the spiroplasmal genome, suggesting that the IIBC Tre permease probably functions with the IIA Glc domain. In agreement with this statement, yeast two-hybrid system experiments revealed that the IIA Glc domain interacted not only with IIB Glc but also with the IIB Tre domain. The results are discussed with respect to the ability of the spiroplasma to adapt from the phloem sap of the host plant to the haemolymph and salivary gland cells of the insect vector.

**INTRODUCTION**

*SPIROPLASMA CITRI* is a plant-pathogenic bacterium belonging to the class *Mollicutes*, a group of wall-less organisms phylogenetically related to low G+C content, Gram-positive bacteria (Weisburg et al., 1989). Plant-mollicutes are associated with hundreds of diseases affecting a wide variety of plants, including fruit trees, ornamentals, vegetables and grapevine (McCoy et al., 1989; Seemüller et al., 2002). Plant mollicutes range within two taxonomically distinct groups, *Candidatus* Phytoplasma spp. and *Spiroplasma* spp. Spiroplasmas are characterized by their helical morphology and motility. Both phytoplasmas and spiroplasmas multiply in the phloem sieve tubes of their plant hosts and in the haemolymph and other...
tissues of their phloem sap-feeding insect vectors (Fletcher et al., 1998). They also multiply in the herbaceous plant Catharanthus roseus (periwinkle) in which they induce symptoms similar to those observed in their original host. However, despite numerous attempts, phytoplasmas have not yet been cultured in vitro whereas spiroplasmas, and in particular S. citri, have been cultured since 1970 (Saglio et al., 1971, 1973). Since then, S. citri has been extensively characterized at the molecular level and methods for efficient transmission to periwinkle plants by its leafhopper vector Circulifer haematoceps have been developed (Bové et al., 1989; Bové, 1997; Foissac et al., 1996). Eventually, as molecular genetic tools such as transposon and homologous recombination mutagenesis have been made available, S. citri has become a model organism to investigate how plant-pathogenic mollicutes cause diseases in plants and to study the molecular mechanisms of insect transmission (Renaudin, 2002; Bové et al., 2003). In S. citri, several components, including toxins and lactic acid, have been suggested to play a role in disease development (Daniels, 1983). S. citri infection was also shown to cause a shortage of auxins, and it was proposed that utilization of plant sterols could result in a deficit of growth regulators (Chang, 1998). However, no conclusive experiments could confirm these hypotheses. More recently, it was shown that phytoplasma infection of Catharanthus roseus severely impaired carbohydrate partitioning, suggesting that sugar metabolism was involved in the interactions of mollicutes with their host plants (Lepka et al., 1999).

A significant breakthrough came from genetic studies in S. citri, showing that mutants unable to use fructose produced very mild symptoms in spite of multiplication (Foissac produced very mild symptoms in spite of multiplication (Postma et al., 1997; Gaurivaud et al., 1997; Gaurivaud et al., 2000a, b). Functional complementation of the mutants revealed a perfect correlation between the ability of the spiroplasmas to use fructose and their ability to induce severe symptoms in the plant (Gaurivaud et al., 2000b). In the so-called ‘non-pathogenic’ mutants, the defective import system was proved to be the permease of the phosphoenolpyruvate:fructose phosphotransferase system (fructose PTS), indicating that the PTS was the major import system of carbohydrates in S. citri. The bacterial PTS is a multiprotein system used to import a sugar into the cell with concomitant phosphorylation of the sugar. It consists of two general soluble proteins, enzyme I (EI) and HPr, and one membrane-bound sugar-specific permease (enzyme II). Enzyme II (EII) complex is generally made of three functional components (IIA, IIB and IIC), which occur as either protein subunits or domains of a multi-domain polypeptide. The IIC domain is an integral membrane component, whereas IIA and IIB are cytosolic proteins. Based on sequence alignments EIIIs may be grouped in at least four families (Lengeler et al., 1990). The phosphate moiety is transferred from phosphoenolpyruvate (PEP) to the sugar via EI, HPr, EIIA and EIIB (Postma et al., 1993). In addition to fructose, S. citri also metabolizes glucose, which is present in plant and insect cells, and trehalose, which is the major sugar in the insect haemolymph. Therefore, the availability of mutants unable to use glucose or trehalose will be crucial to examine in more detail the role of sugar metabolism in the interactions of S. citri with its plant and insect hosts. With the aim to produce such mutants through inactivation of the relevant permease genes, we first determined the gene organization of the glucose and trehalose PTS enzymes II and showed that they probably function with a single IIA domain to import glucose and trehalose.

**METHODS**

**Strains, growth media and transformation.** *Escherichia coli* TOP10 [F’ lacZAM15 galU galK rpsL Str6 endA1 mupC] was used as the host strain for cloning experiments and plasmid propagation. E. coli competent cells (Invitrogen) were transformed by heat shock at 42°C. S. citri GII-3 wild-type (wt) strain was originally isolated from its leafhopper vector C. haematoceps captured in Morocco (Vignault et al., 1980). Spiroplasmas were grown at 32°C in HSI or SP4 medium (Whitcomb, 1983). Saccharomyces cerevisiae HY (MATa/MTa, ural-52/ural-52, his3-A200/his3-Δ200, trpl-901/trpl-901, leu2-3,112/leu2-3,112, gal4-542/gal4-542, gal80-538/gal80-538, URA3::GAL1-LacZ/URA3::GAL4-17-mer/3'-CYC1LacZ, LYS2/lys2::GAL1-HIS3) used in two-hybrid analyses was obtained through mating of strains HF7c and Y187 (Louve et al., 1997). Yeast cultures were grown either in YPD complete medium or in SD selective medium (Sherman et al., 1986). Yeast transformations were performed by the lithium acetate method (Gietz et al., 1995).

**DNA and RNA analyses.** Total DNA from S. citri cells was extracted with the Wizard Genomic DNA Purification Kit (Promega). Restricted DNA was fractionated by agarose gel electrophoresis, blotted onto positively charged nylon membranes by the alkali transfer procedure and hybridized with appropriate [digoxigenin]-UTP-labelled probes using standard stringency conditions (Sambrook et al., 1989). Hybridization signals were detected with anti-digoxigenin-alkaline phosphatase-conjugate and HNPP (2-hydroxy-3-naphthoic acid-2'-phénylaminol phosphate) as the substrate, following the supplier’s instructions. Fluorescent signals were detected using a Fluor-S Multimag phosphoimager (Bio-Rad). Total RNA was extracted from spiroplasma cells by using the Tri-reagent (Sigma). The RNA samples were treated with RNase-free DNasel (Promega), separated by agarose gel electrophoresis and transferred onto charged nylon membranes according to standard procedures (Sambrook et al., 1989). Hybridization with the appropriate probes was performed as described above for Southern blots except that washing was carried out at 50°C. Probes specific for the S. citri genes trpD, crr, ptsG and spiralin were obtained by PCR amplification of plasmid DNA with primer pairs PER2/PER3, DHA1/DHA2b, GP6/GP7 and SR14/SR16, respectively (Table 1).

**PCR and RT-PCR.** First-strand cDNA was synthesized from 1 μg DNase-treated RNA mixed with 2-4 μl 200 mM dNTP, 2-4 μl 0-1 mM DTT, 3 μl 100 mM 3’ primer (DHA2b or DHGBG2b) and 30 units RNase-OUT (Invitrogen) to a total volume of 20 μl. RNA was heated-denatured at 65°C for 5 min before adding 200 units Superscript II reverse transcriptase (Invitrogen). The reverse transcription was performed over 70 min at 40°C and the enzyme was heat-denatured for 5 min at 70°C. PCR was performed with 2 μl of the product of the reverse transcription or 40 ng genomic DNA. Primers used for amplification are listed in Table 1. PCR conditions were 94°C for
**RESULTS**

**Gene organization of the trehalose and glucose PTS enzymes II**

From data of the ongoing *S. citri* genome sequencing project, short sequences encoding putative peptides with high similarities to the trehalose- and glucose-specific PTS enzyme II (permeases) were identified. Then, the DNA inserts from the relevant plasmids were sequenced to determine the complete sequences of the PTS permease genes. The restriction maps as well as the gene organization of the regions encompassing the trehalose operon and the glucose PTS permease genes are presented in Fig. 1. Sequences of the 6.2 kbp region of the trehalose operon were found to contain six ORFs in the same orientation, each one starting with an ATG initiation codon preceded by a RBS sequence and finishing with a TAA or TAG stop codon (Fig. 1a). The encoded polypeptides were 282, 199, 87, 329, 522 and 549 aa long, respectively. The first two polypeptides (ORFs 1 and 2) were found to share significant homology with rRNA methylases and GTPases, respectively. The highest scores were 41 % identity and 60 % similarity between ORF1 and a *Mycoplasma pulmonis* rRNA methylase, and 49 % identity and 68 % similarity between ORF1 and a GTPase (ORF2). The resulting constructions pAA (pACT2 + IIBGlc), pB (pACT2 + IIBTre) and the amylase, respectively. Inverted repeat terminators were found downstream of the GTP-binding enzymes II (permeases) were identified. Then, the DNA inserts from the relevant plasmids were sequenced to determine the complete sequences of the PTS permease genes. The restriction maps as well as the gene organization of the regions encompassing the trehalose operon and the glucose PTS permease genes are presented in Fig. 1. Sequences of the 6.2 kbp region of the trehalose operon were found to contain six ORFs in the same orientation, each one starting with an ATG initiation codon preceded by a RBS sequence and finishing with a TAA or TAG stop codon (Fig. 1a). The encoded polypeptides were 282, 199, 87, 329, 522 and 549 aa long, respectively. The first two polypeptides (ORFs 1 and 2) were found to share significant homology with rRNA methylases and GTPases, respectively. The highest scores were 41 % identity and 60 % similarity between ORF1 and a *Mycoplasma pulmonis* rRNA methylase, and 49 % identity and 68 % similarity between ORF2 and a GTP-binding protein of *Streptococcus pneumoniae*. In contrast, ORF3 showed no significant homology with known proteins. Also, based on similarities of their predicted translation products with proteins in the databases, the last three ORFs (ORFs 4, 5 and 6) were found to share significant homology with rRNA methylases and GTPases, respectively. The highest scores were 41 % identity and 60 % similarity between ORF1 and a *Mycoplasma pulmonis* rRNA methylase, and 49 % identity and 68 % similarity between ORF2 and a GTP-binding protein of *Streptococcus pneumoniae*. In contrast, ORF3 showed no significant homology with known proteins. Also, based on similarities of their predicted translation products with proteins in the databases, the last three ORFs (ORFs 4, 5 and 6) were identified as the treR, treP and treA genes, encoding a transcriptional regulator, the trehalose PTS permease (EIITre) and the amyrase, respectively. Inverted repeat sequences characteristic of *p*-independent transcription terminators were found downstream of the GTP-binding

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<th>Primer</th>
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<th>Accession no.</th>
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*Bold letters indicate restriction enzyme sites. Lower-case letters indicate mismatched nucleotides.

**Table 1. Primers used in this study**

45 s, 53 °C for 45 s and 72 °C for 45 s for RT-PCR and for 90 s for PCR. The number of cycles was 40 for PCR and 20, 30 and 40 for RT-PCR experiments.

**Yeast two-hybrid analysis.** The use of plasmids pACT2 (Clontech) and pODB80 (accession no. 008932) to study protein–protein interactions in a *Saccharomyces cerevisiae* two-hybrid system has been described previously (Louvet et al., 1997). DNA sequences corresponding to domains IIA Glc, IIB Glc and IIB Tre of the *S. citri* permeses were amplified with primer pairs DHA1n/DHAb2, DHBGn1/DHBGb2 and DHTn1/DHHTb2, respectively (Table 1).

PCR amplification was performed with the proofreading Platinum Pfx DNA polymerase (Invitrogen) as recommended by the supplier. Restriction sites Nol or BamHI were included in the primers to fuse the amplified DNA fragments either to the GAL4 DNA-binding domain of plasmid pODB80 or to the GAL4-activating domain of pACT2. The resulting constructions pAA (pACT2 + IIAGlc), pB (pACT2 + IIBGlc), pAT (pACT2 + IIBTre), pOA (pODB80 + IIAGlc), pOG (pODB80 + IIBGlc), pAT (pACT2 + IIBTre) and pOT (pODB80 + IIBTre) carrying the translational fusions were verified by sequencing. The insert-free pACT2 and pODB80 plasmids were also tested as negative controls. Transformed cells carrying the various plasmid combinations were selected by plating on SD medium lacking tryptophan and leucine (SD-TL). Then, protein interactions were tested by streaking transformants on SD medium lacking tryptophan, leucine and histidine (SD-TLH). To confirm protein interactions β-galactosidase assays were carried out as described previously (Barthe et al., 1998).

**In silico analyses.** DNA and protein sequence analyses were performed using the programs proposed by Infobiogen (http://www.infobiogen.fr/index.html). The BLAST program was used to search homologies in general databases (http://www.ncbi.nlm.nih.gov/BLAST/) or in the *Spiroplasma kunkelii* partially sequenced genome (http://www.genome.ou.edu/spiro_BLAST.html). Multiple alignments were done with MULTALIN (Corpet, 1988; http://www.toulouse.inra.fr/lgc/multalin/multalin.html). Phylogenetic trees were constructed by using the MEGA2 program (Kumar et al., 2001; http://evolution.genetics.washington.edu/phylip.html).
protein gene and downstream of the treA gene, suggesting that ORF3 and the treR, treP and treA genes are transcribed to a single polycistronic mRNA. Indeed, Northern blot analyses revealed that the IIA\textsubscript{Glc} and IICB\textsubscript{Glc} domains of the permease were encoded by two distinct genes, each one occurring as a single copy. These genes were annotated crr and ptsG as they certainly represent orthologues of the crr and ptsG genes of E. coli (sequence alignments are included as supplementary data with the online version of this paper at http://mic.sgmjournals.org). As shown in Fig. 2, none of the restriction fragments were found to hybridize with both the crr- and ptsG-specific probes. For example, probe crr hybridized with the 7-3 kbp Nsil and 11-8 kbp Hpal fragments (Fig. 2a, lanes 4 and 5) whereas the ptsG probe hybridized with Nsil and Hpal fragments of 4-2 and 3-6 kbp, respectively (Fig. 2b, lanes 4 and 5). Sequence analyses of the 4-2 kbp Nsil fragment hybridizing with the ptsG probe (Fig. 2b, lane 4) showed this fragment to contain four ORFs, including the ptsG gene (ORF2), which encodes the glucose PTS permease polypeptide IICB\textsubscript{Glc} (Fig. 1b).

Upstream of ptsG, ORF1 encodes an 82 aa polypeptide for which no significant homology was found. In contrast, the 350 aa ORF3 and the truncated ORF4 located downstream of ptsG encode hypothetical, conserved proteins with no known function in the case of ORF3 and with striking similarities to serine-threonine protein phosphatases in the case of ORF4. These two ORFs are orthologues of the yloN and yloO genes of Bacillus subtilis (Foulger & Errington, 1998).

The gene organization of the trehalose operon is similar to that of the fructose operon described previously (Gaurivaud et al., 2000b). However, alignment of the amino acid sequences of the two permeases indicated that, in contrast to the fructose permease, which contains all three domains (IIABC\textsuperscript{Fru}), the treP-encoded trehalose permease (IIBC\textsuperscript{Tre}) lacked domain IIA (see Fig. 5). In addition, no trehalose-specific IIATre domain could be identified elsewhere in the spiroplasmal genome. In the case of the glucose-specific PTS, PCR amplification of genomic DNA with primer pair GP1/GP2 (see positions of the primers in Fig. 1b and c) yielded negative results, suggesting that, in contrast to the situation in Mycoplasma genitalium and Mycoplasma pneumoniae, the three domains IIA, IIB and IIC are not fused into a single polypeptide IICBA\textsuperscript{Glc} encoded by a single gene. Indeed, Southern blot analyses revealed that the IIA\textsuperscript{Glc} and IICB\textsuperscript{Glc} domains of the permease were encoded by two distinct genes, each one occurring as a single copy. These genes were annotated crr and ptsG as they certainly represent orthologues of the crr and ptsG genes of E. coli (sequence alignments are included as supplementary data with the online version of this paper at http://mic.sgmjournals.org). As shown in Fig. 2, none of the restriction fragments were found to hybridize with both the crr- and ptsG-specific probes. For example, probe crr hybridized with the 7-3 kbp Nsil and 11-8 kbp Hpal fragments (Fig. 2a, lanes 4 and 5) whereas the ptsG probe hybridized with Nsil and Hpal fragments of 4-2 and 3-6 kbp, respectively (Fig. 2b, lanes 4 and 5). Sequence analyses of the 4-2 kbp Nsil fragment hybridizing with the ptsG probe (Fig. 2b, lane 4) showed this fragment to contain four ORFs, including the ptsG gene (ORF2), which encodes the glucose PTS permease polypeptide IICB\textsuperscript{Glc} (Fig. 1b).

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![Fig. 1. Partial restriction map and gene organization of the regions surrounding the trehalose operon (a), ptsG (b) and crr (c) genes of S. citri. For each region, ORFs are numbered according to their location, from 5’ to 3’. The thick bars indicate the probes used in the hybridization experiments shown in Figs 2 and 3. Positions of primers are indicated by short arrows. T, transcription terminator-like structure; C, Clal; E, EcoRI; EV, EcoRV; H, HindIII; Hc, HindII; Hp, Hpal; K, KpnI; N, NsiI.](image-url)

![Fig. 2. Southern blot hybridization between restricted DNA of S. citri and crr (a) or ptsG (b) probes. Lanes: 1, EcoRV; 2, HindIII; 3, EcoRI; 4, NsiI; 5, Hpal; 6, HindII.](image-url)
Northern blot hybridizations with the ptsG probe revealed a unique mRNA of 2.2 kb, the size of which was in agreement with a monocistronic ptsG transcript (Fig. 3c). Accordingly, a typical transcription terminator structure was found immediately downstream of ptsG and sequences resembling the −10 and −35 consensus sequences of eubacterial promoters were identified in the intergenic region between ORF1 and ptsG. Sequencing a 5-2 kbp region encompassing the IIAGlc-encoding crr gene (ORF4) revealed four additional, putative ORFs, three of which (ORFs 1, 3 and 5 in Fig. 1c) did not show any significant homology with known proteins. Interestingly, the 578 aa ORF2 showed striking similarities with the ptsI-encoded, PTS enzymes I. In particular, it shared 88% identical and 90% similar amino acids with enzyme I of S. kunkelii, and 55% identity and 73% similarity with enzyme I of Mycoplasma capricolum. In this organism, ptsI and crr constitute a dicistronic operon that includes an independent promoter for the crr gene. The intergenic region between ptsI and crr is only 85 bp and contains no transcription terminator (Zhu et al., 1994). In S. citri, the occurrence of a ρ-independent type transcription terminator structure immediately downstream of ptsI strongly suggests that, in contrast to M. capricolum, the S. citri ptsI and crr genes are transcribed to two distinct mRNAs. A similar organization of the ptsI and crr genes was also found to occur in S. kunkelii. However, unlike the situation in E. coli where ptsH, encoding the HPr protein, ptsI and crr are organized in a single operon, in the S. citri genome, ptsI and crr on the one hand and ptsH on the other hand are located at two distinct loci on the chromosome.

Transcription of the trehalose operon, ptsG and crr genes

In the experiment shown in Fig. 3, total RNA was extracted from S. citri cultures grown in HSI medium containing glucose (lanes 1), fructose (lanes 2) or trehalose (lanes 3) and hybridized with three different probes, treP (Fig. 3a), spiralin (Fig. 3b and c) and ptsG (Fig. 3c). Fig. 3a shows that the treP-specific probe hybridized with a 4.5 kb signal corresponding to the transcription product of the whole operon. Interestingly enough, the hybridization signal was significantly stronger in the presence of trehalose, indicating that transcription of the trehalose operon is stimulated by trehalose (lane 3), but not by glucose (lane 1) and fructose (lane 2). The amount of spiralin mRNA (used as the control) was not significantly affected (Fig. 3b). Similarly, hybridization with the ptsG probe revealed that glucose, but not fructose and trehalose, stimulated transcription of the glucose permease gene (Fig. 3c, lanes 1–3). Surprisingly, the crr transcription product could not be detected by Northern blot hybridization (data not shown). However, its presence in spiroplasma cells was revealed by RT-PCR experiments (Fig. 4). As shown in the figure, the 428 bp amplified DNA fragment corresponding to the crr mRNA could only be detected through at least 30 cycles of amplification (lanes 6 and 9) whereas the 278 bp fragment corresponding to the ptsG transcript was detected through 20 cycles only (lanes 4, 7 and 10).

Sequence analyses of the S. citri glucose-PTS enzyme II

The amino acid sequences of the S. citri enzyme II polypeptides were aligned with those of various Gram-positive and Gram-negative bacteria (multiple alignments are included as supplementary data with the online version of this paper at http://mic.sgmjournals.org). To maximize the alignment agreement of the IICBGlc sequences, insertion loops were introduced into the S. citri and S. kunkelii spiroplasmal sequences between regions IIC1 and IIC2 as well as between regions IIC2 and IIC3. Additional insertions

**Fig. 3.** Northern blot hybridization between total RNA and probes specific for trehalose permease gene treP, spiralin and ptsG. RNAs were extracted from S. citri cultures grown in HSI medium supplemented with glucose (lanes 1), fructose (lanes 2) or trehalose (lanes 3). (a and b) The membrane was successively hybridized with the trehalose permease (a) and the spiralin (b) probes. (c) Hybridization was carried out with a mixture of both ptsG and spiralin probes. The spiralin probe was used as a control.

**Fig. 4.** Reverse transcription analysis of ptsG and crr transcripts. cDNAs obtained from the crr and ptsG mRNAs were amplified through 20 (lanes 3 and 4), 30 (lanes 6 and 7) or 40 (lanes 9 and 10) PCR cycles with primer pairs DHAn1/DHAb2 for crr (lanes 3, 6 and 9), and GP6/GP7 for ptsG (lanes 4, 7 and 10). Lanes: 1 and 2; negative controls without DNA; 5 and 8, 1 kb ladder molecular markers.
were introduced between regions IIC2 and IIC3 of the IICBaGlc sequences of the human mycoplasmas *M. genitalium* and *M. pneumoniae*. The alignments indicate that the *S. citri* polypeptides do possess the PTS permease-specific domains IIA, IIB and IIC (Fig. 5). In particular, they show that the histidine residues at positions 66 and 81 of the domains IIA, IIB and IIC (Fig. 5). In particular, they show that the histidine residues at positions 66 and 81 of the domains IIA, IIB and IIC (Fig. 5. Comparison of domain arrangement within the glucose-, trehalose- and fructose-PTS enzymes II of *S. citri* and the glucose-PTS enzyme II of *E. coli*. Symbols: stippled boxes, IIA; hatched boxes, IIB; open boxes, IIC; X, presence of a linker, filled circle, essential residues. The numbers indicate the first (no. 1) and the last residues of the peptides as well as the position of the essential residues within each polypeptide.

It was found that the histidine residues at positions 66 and 81 of the domains IIA, IIB and IIC (Fig. 5). In particular, they show that the histidine residues at positions 66 and 81 of the domains IIA, IIB and IIC (Fig. 5. Comparison of domain arrangement within the glucose-, trehalose- and fructose-PTS enzymes II of *S. citri* and the glucose-PTS enzyme II of *E. coli*. Symbols: stippled boxes, IIA; hatched boxes, IIB; open boxes, IIC; X, presence of a linker, filled circle, essential residues. The numbers indicate the first (no. 1) and the last residues of the peptides as well as the position of the essential residues within each polypeptide.

**Domr. IIA**

The hypothesis that the IIA**Glc** domain could function either with IIC**Glc** or with IIBC**Tre** was suggested by the following observations: (i) *S. citri* metabolizes fructose, glucose and trehalose; (ii) no IIA**Tre** was identified in the genomes of *S. citri* and *S. kunkelii*; (iii) the glucose permease gene organization IIA**Glc**+IIC**Glc** in Gram-positive bacteria seemed to correlate with the ability to grow in trehalose-rich environments such as insect haemolymph; and (iv) IIA**Glc** and IIB**Tre** of *S. citri* show strong sequence similarities in agreement with the fact that they belong to the same EIi family. Therefore the ability of the *S. citri* IIA**Glc** domain to bind the IIB**Tre** as well as the IIB**Glc** domains was tested through yeast two-hybrid experiments. Construction of pACT2 and pODB80 recombinant plasmids carrying either one of the IIA**Glc**, IIB**Glc** or IIB**Tre** domains is described in Methods. *Saccharomyces cerevisiae* HY was transformed with various plasmid pairs (1–11) as indicated in the legend to Fig. 6 and transformants were selected by plating on SD-TL medium. For each plasmid combination, four individual transformants were selected. Colonies were dispersed in water and the undiluted cell suspensions, as well as 10⁻¹ and 10⁻² dilutions, were plated on selective medium SD-TLH. For each plasmid combination, all four transformants yielded identical results, which are presented in Fig. 7. Whereas all transformants grew on the SD-TL medium (Fig. 7b), only those carrying plasmid pairs 8, 9, 10 or 11 grew on the SD-TLH selective medium (Fig. 7a), indicating interactions between the relevant protein domains. The results clearly indicated that, in addition to the expected interaction between IIA**Glc** and IIB**Glc** (plasmid combinations 8 and 10), the IIA**Glc** domain interacted also with domain IIB**Tre** (plasmid combinations 9 and 11). These results were further confirmed by β-galactosidase assays, showing that the GAL4 promoter-lacZ fusion was also activated in the yeast transformants carrying plasmid pairs 8, 9, 10 and 11 (data not shown). These data strongly suggested that the *S. citri* trehalose permease IIBC**Tre**, which lacks its own IIA domain, might function with the glucose permease IIA**Glc** domain to import trehalose.

**DISCUSSION**

The carbohydrate PTS catalyses transport and phosphorylation of carbohydrates in various obligate and facultative
anaerobic bacteria. The sugar-specific component of the system (EII complex) generally consists of three functional domains, two cytoplasmic proteins IIA and IIB and one integral membrane protein IIC, which occur as either protein subunits or domains of a multi-domain polypeptide (Saier & Reizer, 1992). In *Bacillus subtilis*, the glucose-PTS permease consists of a single polypeptide chain in which the three domains are fused in the order IICBA Glc (Zagorec & Postma, 1992), whereas in *E. coli*, the glucose-specific permease EIIGlc is split into two distinct polypeptides IIAGlc and IICB Glc encoded by two separate genes, *crr* and *ptsG*, respectively (Lengeler et al., 1994). The *ptsG*-encoded IICB\textsuperscript{Glc} protein confers sugar specificity and mediates transport of glucose. In *S. citri*, orthologues of *crr* and *ptsG* were identified at two distinct loci on the chromosome. Protein comparisons showed that the *crr* and *ptsG* predicted products shared striking homologies with the previously described glucose PTS permeases. In agreement with the phylogeny of mollicutes, which have arisen from ancestors of low G+C, Gram-positive bacteria, the *S. citri* proteins IIA\textsuperscript{Glc} and IICB\textsuperscript{Glc} are more similar to their counterparts from Gram-positive than to those from
Gram-negative organisms. Unexpectedly however, the gene organization IIA\textsuperscript{Glc} + IICB\textsuperscript{Glc} of the \textit{S. citri} glucose PTS permease resembles that of \textit{E. coli} rather than that (IICBA\textsuperscript{Glc}) found in \textit{Bacillus subtilis} and the mollicutes \textit{M. pulmonis}, \textit{M. pneumoniae}, \textit{M. genitalium} and \textit{M. penetrans}. Our studies showed that, in Gram-positive bacteria, the IIA\textsuperscript{Glc} + IICB\textsuperscript{Glc} organization was found to occur only in multiple-host bacteria such as \textit{Borrelia burgdorferi}, \textit{M. capricolum} (Zhu et al., 1994) and the spiroplasmas \textit{S. citri} and \textit{S. kunkelii}, all of which have the ability to multiply in an arthropod host. Knowing that trehalose is the major sugar in arthropod haemolymph (Becker et al., 1996), it is consistent that genes encoding the trehalose-PTS enzyme II (EIIT\textsuperscript{Tre}) were identified in the genomes of the insect-transmitted spiroplasmas \textit{S. citri} and \textit{S. kunkelii}, but not in the human and animal mycoplasmas \textit{M. genitalium} (Fraser et al., 1995), \textit{M. pneumoniae} (Himmelreich et al., 1996) and \textit{M. pulmonis} (Chambaud et al., 2001).

Interestingly, the \textit{S. citri} trehalose permease (IIBC\textsuperscript{Tre}) encoded by the \textit{treP} gene of the trehalose operon lacks its own IIB\textsuperscript{Tre} domain. In several \textit{E. coli} EII complexes and in particular that for trehalose, the IIA domain has been lost and it has been functionally replaced by the glucose IIA\textsuperscript{Glc} protein. In this case, the IIA domain is not sugar-specific and phosphorylates a subset of several PTS IIB domains (Lengeler et al., 1994). In \textit{S. citri}, which uses both glucose and trehalose, the yeast two-hybrid experiments clearly indicated that the IIA\textsuperscript{Glc} domain could interact not only with the IIB\textsuperscript{Glc} domain but also with the trehalose-specific IIB\textsuperscript{Tre} domain. Crystallographic studies of the IIA\textsuperscript{Glc} of \textit{M. capricolum} have suggested that interactions between domains IIA\textsuperscript{Glc} and IIB\textsuperscript{Glc} are hydrophobic with no sequence specificity (Huang et al., 1998). Therefore, the ability of the \textit{S. citri} IIA\textsuperscript{Glc} to bind the two distinct domains IIB\textsuperscript{Glc} and IIB\textsuperscript{Tre} would be explained by the non-discriminating nature of the interactions. However, genetic studies will be required to definitively demonstrate that in vivo the \textit{S. citri} trehalose permease functions with the IIA\textsuperscript{Glc} domain to import trehalose.

The occurrence of two distinct genes \textit{crr} and \textit{ptsG} encoding IIA\textsuperscript{Glc} and IICB\textsuperscript{Glc} polypeptides is consistent with the ability of IIA\textsuperscript{Glc} to function with both IICB\textsuperscript{Glc} and IIBC\textsuperscript{Tre}. However, its biological significance is not known. The constituents of the bacterial PTS have undergone extensive shuffling during their evolution (Reizer & Saier, 1997). From our studies, the gene organization of the glucose permeases in Gram-positive bacteria did not correlate with a specific phylogenetic branch, but instead seemed to be more related to the ecological niches of the bacteria. In vitro analyses of the \textit{E. coli} glucose PTS showed that a fusion protein made of IIC\textsuperscript{Glc}, IIB\textsuperscript{Glc} and IIA\textsuperscript{Glc} domains linked together exhibited higher phosphotransferase activity than a mixture of the isolated subunits (Mao et al., 1995). These data suggested that the linking of functional domains could be an advantage regarding the activity of the permease. It is noteworthy that, among mollicutes, the mycoplasmas exhibit a glucose permease in which the three domains are linked, whereas the \textit{S. citri} enzyme is split into two distinct polypeptides. Selection of such an organization, which could be disadvantageous regarding enzyme activity, might be related to the spiroplasma life cycle. In vitro, \textit{S. citri} metabolizes fructose, glucose and trehalose. The finding that the transcription of the PTS permease genes was stimulated by the presence of the relevant carbohydrate in the medium (Gaurivaud et al., 2001; this study) reflects the adaptive capacity of \textit{S. citri} to grow in ecologically different niches. In the plant sieve tubes, \textit{S. citri} grows on fructose and glucose, whereas in the leafhopper, the spiroplasma must grow on trehalose, the major sugar in insect haemolymph. Therefore, when \textit{S. citri} is acquired by the insect from the plant, adaptation from glucose and fructose to trehalose could be achieved with an IIBC + IIA organization, provided that the IIA...
component functions with both IIICrGlc and IIICrTre. Similarly, when the spiroplasma moves from the insect haemolymph, where it relies on trehalose, to the cells of the salivary glands where glucose is the main sugar, a common IIA domain would help the spiroplasma to rapidly adapt from one host or host-compartment to another.

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