Plant Carbohydrate Scavenging through TonB-Dependent Receptors: A Feature Shared by Phytopathogenic and Aquatic Bacteria

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TonB-dependent receptors (TBDRs) are outer membrane proteins mainly known for the active transport of iron siderophore complexes in Gram-negative bacteria. Analysis of the genome of the phytopathogenic bacterium Xanthomonas campestris pv. campestris (Xcc), predicts 72 TBDRs. Such an overrepresentation is common in Xanthomonas species but is limited to only a small number of bacteria. Here, we show that one Xcc TBDR transports sucrose with a very high affinity, suggesting that it might be a sucrose scavenger. This TBDR acts with an inner membrane transporter, an amyllosucrase and a regulator to utilize sucrose, thus defining a new type of carbohydrate utilization locus, named CUT locus, involving a TBDR for the transport of substrate across the outer membrane. This sucrose CUT locus is required for full pathogenicity on Arabidopsis, showing its importance for the adaptation to host plants. A systematic analysis of Xcc TBDR genes and a genome context survey suggested that several Xcc TBDRs belong to other CUT loci involved in the utilization of various plant carbohydrates. Interestingly, several Xcc TBDRs and CUT loci are conserved in aquatic bacteria such as Caulobacter crescentus, Colwellia psychrerythraea, Saccharophagus degradans, Shewanella spp., Sphingomonas spp. or Pseudoalteromonas spp., which share the ability to degrade a wide variety of complex carbohydrates and display TBDR overrepresentation. We therefore propose that TBDR overrepresentation and the presence of CUT loci designate the ability to scavenge carbohydrates. Thus CUT loci, which seem to participate to the adaptation of phytopathogenic bacteria to their host plants, might also play a very important role in the biogeochemical cycling of plant-derived nutrients in marine environments. Moreover, the TBDRs and CUT loci identified in this study are clearly different from those characterized in the human gut symbiont Bacteroides thetaiotaomicron, which allow glycan foraging, suggesting a convergent evolution of TBDRs in Proteobacteria and Bacteroidetes.

INTRODUCTION

Bacteria are able to colonize a wide variety of habitats, including the most extreme environments and even living organisms. This remarkable feature likely reflects a high degree of adaptability and the presence of specific genetic programs devoted to the exploitation of nutrients present in these diverse habitats. The bacterial ability to use defined carbohydrates to support cell survival or growth implies the availability of these substrates in their habitat. Moreover, it requires the recognition of these molecules and the coordinated induction of particular uptake systems and of metabolic enzymes [1,2]. Characterization of the repertoire of genes involved in signal perception or transduction and in carbohydrate utilization, in conjunction with analysis of the regulation of their expression, is likely to provide key information about the interaction and adaptation of bacteria with their environment [3].

The analysis and comparison of complete genomic sequences of numerous bacteria from diverse phylogenies and habitats have shown a relationship between the ecological niches that a bacterium occupies and the proportion of genes involved in signal perception and transduction. Bacteria that inhabit stable environments (extremophiles, obligate parasites or symbionts), which generally have small genomes, possess fewer sensory and regulatory genes than free-living bacteria found in complex and changing environments such as those living in soil or in association with plants [2]. Therefore, it was proposed that sensors and regulators can be used as “descriptors of bacterial lifestyle” [2].

With the aim to study the molecular mechanisms controlling adaptation of phytopathogenic bacteria to their host-plants, we undertook a global analysis of receptors and regulators of Xanthomonas campestris pv. campestris (Xcc), the causal agent of black rot of crucifers. This pathogen infects a wide range of Brassicaeae plants of economic interest, including cabbage, cauliflower and radish as well as the model plant Arabidopsis thaliana. This epiphytic...
bacterium naturally infects host plants via wounds in the leaves or hydathodes which are specialized pores on the leaf margins of higher plants that connect to the vascular system. Then bacteria multiply and progress in vascular tissues [4,5]. During the past two decades, classical molecular and genetic studies led to the characterization of several determinants controlling pathogenicity of Xcc, such as secretion of extracellular plant cell wall degrading enzymes [6-8], cell-cell signaling [9,10], biofilm formation [11] and hsp genes coding for a type III secretion system [12,13]. The characterization of new virulence factors should now be greatly facilitated by the availability of complete genome sequences of two Xcc strains (strain ATCC33913 [14] and strain 9004 [15]). Moreover, comparative genomics would improve the analysis of virulence and host adaptation in Xanthomonas, since the genomic sequences of four other Xanthomonas strains displaying different host specificities and representing three other species are also available, i.e. Xanthomonas axonopodis pv. citri (Xac), the causal agent of citrus canker [14], Xanthomonas campesstri pv. vesicatoria (Xcv), the causative agent of bacterial spot disease on pepper and tomato plants [16], and Xanthomonas oryzae pv. oryzae (Xoo) (strain KACC10331 [17] and strain MAFF311018 [18]), the causal agent of bacterial blight of rice. Thus, the Xanthomonas genus, which affects two major model plants (Arabidopsis and rice), constitutes a very attractive model to study plant-pathogen interactions.

Our analysis of the Xcc (ATCC33913) genome revealed an overrepresentation of a particular family of receptors, named TonB-dependent receptors (TBDRs). These proteins are located in the outer membrane of Gram-negative bacteria and are mainly known to transport iron-siderophore complexes and vitamin B12 into the periplasm [19]. In most cases, the expression of the genes encoding these receptors is under the control of the Fur (Ferric uptake regulator) repressor and activated under conditions of iron starvation [20]. In contrast to diffusion through porins, transport via TBDRs requires energy which is provided by the inner membrane energy-coupling TonB-ExbB-ExbD protein complex [21].

The exploration of complete genome sequences of 226 Gram negative bacteria showed that the overrepresentation of TBDRs is restricted to a small proportion of these bacteria, but is a common trait of all sequenced Xanthomonas species. Interestingly, most of the bacteria displaying this particularity have diverse lifestyles and belong to different taxonomical lineages, but they all share the ability to exploit complex carbohydrates. Therefore, we postulated that some Xcc TBDRs might be involved in the transport of plant-derived molecules. This hypothesis was recently reinforced with the characterization of a TBDR, named MalA, in the oligotrophic aquatic bacterium Caulobacter crescentus, which transports maltodextrins [22].

A systematic study of Xcc TBDRs, based on mutagenesis, expression analyses and transport assays identified one Xcc TBDR involved in the transport of sucrose, a major plant sugar. This TBDR gene is required for full virulence on Arabidopsis and is associated with genes required for sucrose metabolism, thus forming a “sucrose utilization locus”. Our study also suggests the existence of other TBDR-containing loci involved in the utilization of plant cell wall compounds which are conserved in a wide range of bacteria displaying TBDR overrepresentation.

RESULTS

Identification of Xcc TBDRs

TBDR proteins consist of two domains, with a C-terminal membrane embedded β-barrel domain that is sealed by the N-terminal plug domain [23-27]. Hidden Markov models (HMMs), PF00593 and PF07715, corresponding to these two domains, are available in the Pfam database [28]. Seventy-six proteins carrying one or both domains were detected in the proteome of the Xcc ATCC33913 strain (Table S1). Among these proteins, 64 possess both domains. The remaining 12 proteins, which possess only one of the two domains, can be divided into three groups: 3 proteins (XCC2206, XCC4131 and XCC4132) which do not seem to have a canonical plug domain, one protein (XCC2497) which has no domain detected by the PF00593 HMM, and finally 8 proteins (XCC3694-3695, XCC1750-1751, XCC3215-3216 and XCC3279-3277) which are truncated and correspond to 4 pseudogenes having a frame-shift in their coding region (Table S1). Each of these 4 pseudogenes was thereafter studied as a unique entity.

Other conserved features of TBDRs were then used to further characterize these identified proteins. In the N-terminal part of the plug domain, TBDRs display a conserved sequence, the TonB-box, that interacts with the TonB protein [26,27,29]. A TonB-box, with the Xcc consensus sequence tLDVXX (lower case indicates less highly conserved amino acid, X indicates any amino acid) was detected in all studied proteins (Table S1). The localization of TBDRs in the outer membrane implies their transport across the inner membrane and thus the presence of a signal peptide at their N-terminal end. The proteins were therefore analyzed for the presence of such a motif. TBDRs which were not predicted to possess a signal peptide in the annotation proposed by da Silva and colleagues [14] were manually re-annotated, thus revealing putative signal peptides (Table S1). Out of 72 proteins, 1 TBDR (XCC2385) does not have a signal peptide. Finally, the 12 C-terminal amino acids of outer membrane proteins (OMPs) form a membrane anchoring β-sheet with the last residue being aromatic (F in the vast majority of OMPs) [30,31]. The 12 C-terminal amino acid of 55 of the proteins studied here are predicted to form a potential β-sheet, ending with an aromatic residue (Table S1). Among the TBDRs which did not display a typical C-terminal sequence, 8 have a β-sheet ending with an aromatic amino acid followed by a short extension of 1 to 4 amino acids (Table S1).

Taking all this information together, 48 proteins possess all typical features of TBDRs in Xcc ATCC33913 and were thus considered as putative functional TBDRs. The other proteins lacking or having degenerated TBDR domains were also included in our study and then named P-TBDRs (for Pseudo-TBDRs) (Table S1).

Finally, some TBDRs contain a N-terminal extension, located between the signal peptide and the plug domain [32]. Two types of N-terminal extension have been identified: the transducer and the Oar-like extensions [33]. In Xcc, among the 9 TBDRs having an N-terminal extension, one is a member of the TBDR-transducer subclass, seven are in the Oar-like subclass and one displays a N-terminal extension that does not correspond to the two types already identified (Table S1). Among these TBDRs, only two have all canonical features of TBDRs.

TBDRs are overrepresented in Xanthomonas spp. and in bacteria scavenging complex carbohydrates

A survey of TBDRs was performed in 226 eubacterial completely sequenced genomes, by identifying proteins detected by both PF07715 and PF00593 HMMs. This analysis showed that most bacteria (71%) hold less than 14 TBDRs per proteome, whereas the remaining bacteria have a very broad variation in TBDR number, ranging from 14 to 120 (Figure 1 and Table S2). In fact, 27% of the bacteria studied here have no TBDR proteins and...
43% possess between 1 and 13 TBDRs. Only a very small proportion of bacteria (15.5%) possesses more than 30 TBDRs, thus forming a particular class in which TBDRs seem to be over represented. It is worth noting that most bacteria in this class either belong to the α or γ-Proteobacteria classes or to the Bacteroides genus (Figure 1 and Table S2). The ratios between the number of TBDRs found in each proteome and the genome size or the number of annotated coding sequences (CDS) displayed a very similar distribution pattern (Table S2), thus showing that there was no major bias due to annotation. Therefore, the TBDR/ Mbp ratio was used to define several bacterial classes: bacteria displaying a ratio higher than 5 were considered as members of a class showing TBDR overrepresentation, while those having a ratio ranging from 3 to 5 belong to an intermediary class. The 4 Xanthomonas species whose genomes have been sequenced belong to the class displaying TBDR overrepresentation (Table S2). It is worth noting that phytopathogenic bacteria, such as Pseudomonas syringae pathovars or Erwinia carotovora subsp. Atroseptica belong to the intermediary class.

**Seven Xcc TBDR and two Ps-TBDR genes are Fur-regulated**

As TBDRs are mainly known to be involved in iron uptake, it was important to determine how many of these receptors are assigned to this function in Xcc. In most cases, the expression of genes encoding TBDRs involved in iron transport is regulated by the iron status in the medium. These genes are activated under iron depletion conditions and repressed under iron repletion conditions via the Fur repressor (Ferric-uptake regulator), that binds to a specific DNA sequence element called the “Fur-box”, which is found in the target promoters of iron-regulated genes [20,34,35]. These features led us to analyze the regulation by the iron status for all identified Xcc TBDR and Ps-TBDR genes.
The 76 Xcc TBDR and Ps-TBDR genes were mutated by insertion of the suicide plasmid pVO155 [36], leading to transcriptional fusion with the promoterless uidA reporter gene. Two insertions (in XCC1990 and XCC3209) were found unstable. Thus, we constructed deletion-mutations in these genes using the cre-lox system [37,38]. In order to analyze the expression of these 2 deleted TBDR genes, their promoter regions were cloned upstream of a promoterless lacZ gene in a reporter plasmid (see Materials and Methods), and these constructions were introduced into the wild-type strain. Using β-glucuronidase or β-galactosidase expression assays, we monitored the expression of all Xcc TBDRs and Ps-TBDRs in iron-repleted and -depleted media. Seven genes (XCC0158, XCC0768, XCC1391, XCC2772, XCC3050, XCC3518, and XCC4162) and 2 Ps-TBDR genes (XCC3216-3215 and XCC3595) are induced by iron starvation, compared to iron-replete conditions (Figure 2A). With the exception of Ps-TBDR XCC3216-3215, this regulation pattern was confirmed by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) in a wild-type background (data not shown). We then checked whether these genes are under the control of the Fur regulator. XCC1470, the orthologue of the Fur regulator identified in Xoo [39] and in Xanthomonas campestris pv. phaseoli (Xap) [40], was mutated using the manganese method (see Materials and Methods). A point mutant in this latter gene was obtained (fur1). As expected for a fur mutation, this mutant produced more siderophores than the wild-type strain and this production remained unaffected in response to increased iron levels (from 1 to 50 µM), as determined by using the chrome azurol S assay (data not shown). It is worth noting that the fur mutant is unable to induce any disease symptoms (data not shown), as already reported in Xoo [39].

We then compared by qRT-PCR analysis the relative expression of the TBDR and Ps-TBDR genes induced by iron starvation, in the wild-type strain versus a fur mutant, in an iron-containing medium. As presented in Figure 2B, the expression of all these genes was repressed by the Fur protein. However, the repression level of the 2 Ps-TBDR genes was low (less than twofold). We confirmed that the deregulation phenotype of the fur mutant was the result of mutation in the fur gene by complementation experiments: the repression by iron of the 7 TBDR and the 2 Ps-TBDR genes was restored by providing the wild-type Fur protein on the pL-XCC1470 plasmid (see Materials and Methods) in the fur mutant (Figure 2B).

The 9 Fur-regulated TBDR/Ps-TBDR genes, but not the other Xcc TBDR genes, possess a putative Fur-box

The DNA regions located upstream of the 9 Fur-repressed TBDR and Ps-TBDR genes were analyzed using the MotifSampler program [41,42] to identify putative Fur-boxes. For genes arranged in putative operons (XCC3050 and XCC0768b), the DNA region located upstream of the first gene of the putative operon was also studied. XCC3595, which belongs to the TBDR transducer subclass, is preceded by two genes displaying significant similarities with the fecI (XCC3593) and fecR (XCC3594) genes.
Two TBDR genes belong to the hrp regulon of Xcc

During the study of TBDR gene promoters, we identified a pip/hrp box in the promoter of 2 TBDR genes: XCC1041 and XCC1719. In Xanthomonas, hrp genes coding for a TTSS, as well as other functions related to pathogenicity are positively regulated by the HrpG regulon [51]. HrpG regulates the expression of the hrpX, which controls the expression of genes possessing a pip-box (TTCGNC<sub>2</sub>TTGCC) or a hrpR-box (TTCGNC<sub>2</sub>TTGC) [52,53]. By qRT-PCR experiments, we showed that XCC1041 and

**Table 1. Candidate Fur-boxes identified in the promoter regions of Xanthomonas campestris pv. campestris Fur regulated TonB-dependent receptor (TBDR) genes, TBDR containing operons and the XCC3593 FecI sigma factor gene.**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Name</th>
<th>Putative function</th>
<th>Putative Fur box sequences</th>
<th>No. of matches/total</th>
<th>Distance from the start codon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XCC0158</td>
<td>fpvA</td>
<td>ferrigypoverdine</td>
<td>AAGGATAACGGATTACATT</td>
<td>14/19</td>
<td>136</td>
</tr>
<tr>
<td>XCC0768</td>
<td>phuR</td>
<td>conserved</td>
<td>AATGAGAATGCTACTGAT</td>
<td>13/19</td>
<td>7</td>
</tr>
<tr>
<td>XCC7391</td>
<td>fluA</td>
<td>outer membrane</td>
<td>TTGTAACACATTCCCTC</td>
<td>15/19</td>
<td>159</td>
</tr>
<tr>
<td>XCC2772</td>
<td>fluA</td>
<td>TonB-dependent</td>
<td>AAGAAGAATGCTATTATT</td>
<td>14/19</td>
<td>9</td>
</tr>
<tr>
<td>XCC3049</td>
<td>mhpE</td>
<td>4-hydroxy-2-oxoalde</td>
<td>CTGGAATACATTCCCTC</td>
<td>14/19</td>
<td>41</td>
</tr>
<tr>
<td>XCC3216</td>
<td>fluA</td>
<td>outer membrane</td>
<td>AATGGAATGCTACTGAT</td>
<td>15/19</td>
<td>102</td>
</tr>
<tr>
<td>XCC3518</td>
<td>fpvA</td>
<td>ferrigypoverdine</td>
<td>AATGGAATGCTACTGAT</td>
<td>15/19</td>
<td>53</td>
</tr>
<tr>
<td>XCC3593</td>
<td>fecI</td>
<td>RNA polymerase</td>
<td>AATGAGAATGCTACTGAT</td>
<td>17/19</td>
<td>48</td>
</tr>
<tr>
<td>XCC4162</td>
<td>nexA</td>
<td>ferrichrome-iron</td>
<td>GATAAGAATGCTACTGAT</td>
<td>15/19</td>
<td>12</td>
</tr>
</tbody>
</table>

*Gene identification (ID), name and putative function from Xanthomonas campestris pv. campestris strain ATCC33913 as previously reported [14].

*Matches to the Fur consensus sequence AATGAGAATACATTACATT, determined in this study, appear in boldface.

*Distance after start codon reannotation (see Table S1).

*doi:10.1371/journal.pone.0000224.g003

Figure 3. Palindromic consensus Fur-box sequence identified upstream of 9 Xanthomonas campestris pv. campestris Fur regulated TonB-dependent receptor genes. (A) Sequence logos generated by WebLogo (http://weblogo.berkeley.edu/,[123]) of the Xcc Fur-box motifs as predicted by the MotifSampler program [41,42]. (B and C) Multiple alignment generated with Multalin program [116] with previously proposed Fur-box consensus sequences of Bacillus subtilis [46], Shewanella oneidensis [45], Xcc (Xcc subtilis and Escherichia coli) [44] (B) and after a 3 bp shift of the E. coli sequence (C). doi:10.1371/journal.pone.0000224.g003

Coding for a sigma factor of the ECF subfamily and its anti-sigma factor, respectively. In most cases, the FecI/FecR system is associated with a TBDR of the transducer subclass and is involved in iron signaling and transport by regulating the expression of the TBDR gene and other "iron-associated" genes (for review see [33,43]). We thus also analyzed the DNA regions upstream of these two genes. This analysis allowed the identification of a single significant 19-bp palindromic motif (9-1-9 inverted repeat) in the coding region of this gene (Table 1). For XCC3593, the palindromic motif was detected in the upstream region of XCC3593 (fecI). For XCC0768, two motifs were identified: one in the upstream region of XCC0767 and one in the coding region of this gene (Table 1). This motif (AATGAGAATCATTACATT; lower case indicates less highly conserved) only showed a weak similarity with the E. coli 19 bp Fur-box consensus sequence (GATAAGAATACATTACATT) [44]: it matched this sequence in 10 bp positions out of 19 (52% identity). However, the conservation was significantly higher with Fur-box consensus sequences identified in Shewanella oneidensis (AATGAGAATACATTACATT; 84% identity) [45] and Bacillus subtilis (AATGAGAATTACATTACATT; 84% identity) [46]. Interestingly, a multiple alignment of these 4 Fur-box consensus sequences showed that although the E. coli consensus sequence seems more divergent, it perfectly matches the 3 other sequences after a 3 bp shift (Figure 3B and C). These results suggest that the palindromic motif identified in this study might correspond to a possible Xcc Fur-box. We then explored DNA sequences located upstream of the other Xcc TBDR or Ps-TBDR genes as well as the entire Xcc genome sequence to detect putative Fur-boxes, using the MotifScanner program [42] or the PatScan pattern matcher software [47]. No putative Fur-box was detected in the promoter region of any other TBDR gene. These analyses identified more than 20 genes having candidate Fur-boxes in the region from −300 to +20 relative to the start of translation (data not shown). Several of these genes are orthologs or homologs of genes regulated by Fur in other bacteria, such as feoA (XCC1834) [48] and hfeL (XCC0481) [49], or involved in iron storage such as puaB (XCC0565) [50], thus reinforcing the validity of our Fur-box consensus sequence. Altogether, this analysis suggests that only 7 TBDR and 2 Ps-TBDR are directly associated with iron uptake in Xcc. The role of the other TBDRs remained to be deciphered.
TBDR genes are activated by HrpG and HrpX (Figure 4). Interestingly, a mutant in the XCC1719 TBDR gene is weakly affected in pathogenicity (data not shown, see below). This gene seems to be specific to Xcc as it is absent in the genomes of Xca, Xac, Xoo and Xf, although its surrounding genes are conserved in syntenic regions in all these strains (Table S3). On the other hand, XCC1041 is present in all Xanthomonas species (but absent in Xf) and encodes a TBDR with an atypical N-terminal extension.

**Xcc TBDRs and plant interactions: XCC3358 plays a major role in pathogenicity**

In order to assess whether Xcc TBDRs control the interaction with plants, we tested the 76 TBDR mutants constructed in this study for pathogenicity on the Arabidopsis thaliana Sf-2 ecotype. Among these mutants, 17 were altered in symptom development (data not shown). However, the alteration was weak and was not reproducible in all the tests we performed, suggesting either that their involvement is not crucial or that functional redundancy mask their individual contribution. However, the XCC3358 insertion mutant was clearly and reproducibly altered in pathogenicity, showing a clear delay in symptom development in comparison to the wild-type strain (Figure 5A). The XCC3358 gene is likely to form an operon with the downstream gene, XCC3359 (Figure 6). The ortholog of the XCC3359 gene, named suh, has been characterized in Xanthomonas axonopodis pv. glycines (Xag), the causal agent of bacterial pustule disease on soybean. This gene codes for a sucrose hydrolase, SUH, which plays a major role in sucrose metabolism in Xag. A mutant in this gene is moderately affected in pathogenicity on soybean [54]. This prompted us to construct a non polar deletion-mutation in XCC3358 (XCC3358D1, see Materials and Methods and Figure 6). A deletion-mutant was also generated into XCC3359 (XCC3359D1, see Materials and Methods and Figure 6). These two mutants showed an altered phenotype, i.e. delayed symptom development, similar to that observed with the pVO155 insertion mutant in XCC3358 (Figure 5B). The non polar mutation in XCC3358 was complemented by introducing the plasmid pL-XCC3358, which carries a functional XCC3358 gene, into XCC3358D1 mutant (Figure 5B). This result confirms that the XCC3358 TBDR plays a role in virulence. Similarly, the deleted mutant in XCC3359 was complemented by a plasmid carrying this gene (Figure 5B).

**Figure 4.** Regulation of Xanthomonas campestris pv. campestris TonB-dependent receptor genes XCC1041 and XCC1719 by hrp regulators. The relative expression was analyzed by real-time quantitative RT-PCR (qRT-PCR), performed on RNA extracted from the wild-type strain and hrpG or hrpX insertion mutants (strains XP082 and XP083, respectively). Experiments were repeated at least three times. Calculation of relative expression includes normalization against the endogenous control gene 16S RNA. doi:10.1371/journal.pone.0000224.g004

**Figure 5.** Quantitative analysis of the interaction between Xanthomonas campestris pv. campestris (Xcc) and Arabidopsis thaliana Sf-2 plants. (A) Pathogenicity tests with the Xcc wild-type strain and the XCC3358 insertion mutant (XCC3358::pVO). (B) Pathogenicity and complementation tests with the Xcc wild-type strain, the XCC3356 and XCC3357 insertion mutants (XCC3356::pVO and XCC3357::pVO, respectively), the XCC3358 and XCC3359 deleted mutants (XCC3358D1 and XCC3359D1, respectively) and their corresponding complemented strains (XCC3358D1 (pL-XCC3358) and XCC3359D1 (pL-XCC3359), respectively). Disease symptoms were scored 5 to 8 days after inoculation. Each inoculated leaf was individually scored as: no symptom = 0; weak chlorosis surrounding the wound sites = 1; strong V-shaped chlorosis = 2; developing necrosis = 3; leaf death = 4. The represented average disease scores and the standard deviations were calculated from the values of four plants with four inoculated leaves per plant. doi:10.1371/journal.pone.0000224.g005
XCC3358 TBDR belongs to a locus required for full pathogenicity

In Xcc genome, XCC3358 and XCC3359 are preceded by two other genes that might be related to sugar metabolism: XCC3356 codes for a putative transcriptional repressor of the LacI/GalR-family and XCC3357 encodes a putative sugar transporter of the major facilitator family, allowing the transport of substrate molecules through the inner membrane. We constructed insertion mutants in these two genes, presuming that they are monocistronic (Figure 6). These insertions lead to transcriptional fusions with the promoterless uidA reporter gene. The insertion mutant in XCC3357 showed an altered phenotype on the A. thaliana Sf-2 ecotype, resembling that using XCC3358 or XCC3359 mutants, whereas the insertion mutant in XCC3356 was not affected in pathogenicity (Figure 5B). These results suggest that XCC3357, XCC3358 and XCC3359 are required in the same process related to sucrose metabolism and/or transport. The phenotype of a mutant in XCC3356 was not surprising since this gene encodes a putative repressor, which might negatively control the expression of the other genes of the locus. A mutation in this gene should induce the constitutive expression of the other genes without affecting their function (see below).

The XCC3356-3359 locus is required for sucrose utilization

Insertion mutants in XCC3357, XCC3358 and XCC3359 and the deletion mutant XCC3359Δ1 grew like the wild-type strain on minimal medium containing glucose or fructose (data not shown), but were all affected in growth on sucrose (20 mM) (Figure 7A). The growth of the insertion mutant in XCC3356 and the XCC3358Δ1 mutant was not impaired in minimal medium containing sucrose. The fact that the insertion mutant in XCC3358 was impaired in growth on sucrose whereas the deletion mutant in the same gene was not, suggested that the phenotype of the insertion mutant was due to a polar effect of the insertion on XCC3359 gene expression. This hypothesis was confirmed by complementation experiments. The growth of insertion mutants in XCC3358 and XCC3359 on sucrose media was restored when

Figure 6. Genetic organization of the Xanthomonas campestris pv. campestris sux locus. Location of mutations are indicated above the map: arrowheads indicate pVO155 insertions and deleted sequences are represented by horizontal dotted bars. Regions cloned in plasmids are indicated below the map: horizontal thick black bars indicate sequences used for plasmid constructions.

Figure 7. Growth of Xanthomonas campestris pv. campestris wild-type strain and mutants in minimal medium containing 20 mM sucrose. (A) Bacterial growth of insertion mutants XCC3356::pVO to XCC3359::pVO or deletion mutants XCC3358Δ1 and XCC3359Δ1, compared to the wild-type strain. (B) Growth of strains carrying plasmids pL-XCC3358 or pL-XCC3359 allowing the constitutive expression of XCC3358 and XCC3359 genes, respectively. Bars represent standard deviations from 3 independent experiments.
XCC3359 was supplied in trans on the expression plasmid pL-XCC3359, which allows constitutive expression of the XCC3359 gene. No complementation was observed when the XCC3359 gene was supplied in trans in the XCC3359 insertion mutant (Figure 7B). These results confirmed that XCC3358 and XCC3359 form an operon. They also pointed out that this putative operon and the entire locus is important for sucrose utilization in Xcc. Thus, we renamed this locus sux, for sucrose utilization in Xanthomonas. For the purpose of this study, the TBDR-amylosucrase operon was renamed suxAB, the putative sugar transporter gene, suxC, and the putative regulatory gene, suxR (see Figure 6). However, the role of the TBDR SuxA in sucrose utilization remained elusive: this transporter gene was not required for growth on sucrose, but a non polar mutant in this gene was altered in pathogenicity, like suxB and suxC insertion mutants. This observation suggested that this suxA gene might have a particular function.

**SuxA and SuxC allow sucrose uptake**

To clarify the role of the SuxA TBDR, we investigated its involvement in sucrose transport, by performing sucrose uptake experiments using [14C]sucrose. First, the uptake rates into the Xcc wild-type strain were compared after an overnight preculture in the presence (induced) or absence (uninduced) of sucrose. As shown in Figure 8A, induced cells took-up [14C]sucrose quicker than uninduced cells, but the values obtained after 2 hours of incubation were lower for induced cells. For further experiments, we worked with uninduced cells, in order to study the effect of different mutations on the transport induction.

Competition experiments were performed to test transport specificity. A 10-fold excess of unlabelled sucrose reduced the transport rate to 52% of the level obtained without unlabelled sucrose, and with a 100-fold or 200-fold excess, the transport rates were reduced to 10.5 and 7% respectively (Table 2). Moreover, the addition of unlabelled fructose or glucose (sucrose degradation sub-products) slightly affected sucrose transport; a 200-fold excess of these carbon sources reduced the sucrose transport to about 80% of the control level (Table 2). The involvement of the SuxA TBDR in sucrose transport was then checked by comparing the uptake rate of [14C]sucrose into the wild-type strain and into a suxA non polar mutant (ΔsuxA). Sucrose transport was much lower in the ΔsuxA strain, compared to the wild-type strain (Figure 8B and Table 2): after 8 minutes, it reached only 3.5% of the transport rate into the wild-type strain. When suxA was supplied in trans on a constitutive expression plasmid, sucrose transport was more efficient and rapid, with a transport rate value at 8 minutes corresponding to 484% of that of the wild-type strain. This result confirms that SuxA is required for sucrose entry into Xcc.

We then studied sucrose transport in suxR and suxC insertion mutants (suxR::pVO and suxC::pVO, respectively), and in a suxB deletion mutant (ΔsuxB). When the inner membrane transporter encoded by suxC was absent, the sucrose transport rate reached only 2.1% of the value obtained for the wild-type strain. Thus, this protein is necessary for sucrose entry into Xcc. On the contrary, sucrose transport was greatly enhanced in the suxR repressor mutant and in the ΔsuxB amylosucrase mutant (488% and 241% respectively of the value obtained for the wild-type strain after 8 minutes) (Figure 8C and Table 2). These results confirm the repressor function of SuxR (see below) and suggest that SuxB also has a repressor activity on suxA expression. When suxB was supplied in trans in the ΔsuxB mutant, the sucrose transport was reduced to about the same level as the wild-type strain (76.4% of the transport rate level of the wild-type strain).

Concentration-dependent sucrose transport experiments showed a biphasic kinetics (Figure 9), with a fast rate between

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**Figure 8.** [14C]sucrose transport into Xanthomonas campestris pv. campestris (Xcc). (A) Transport of [14C]sucrose over 120 minutes into the Xcc wild-type strain precultured in minimal medium without sugar (uninduced) or supplemented with 20 mM sucrose (induced). (B and C) Transport of [14C]sucrose over 8 minutes into the Xcc wild-type strain, the insertion mutants in suxR and in suxC (suxR::pVO and suxC::pVO, respectively), the deleted mutants in suxA and suxB (ΔsuxA and ΔsuxB, respectively) and their corresponding complemented strains (ΔsuxA (pL-suxA) and ΔsuxB (pL-suxB), respectively). Cells were grown in minimal medium without sucrose. Transport was measured using 0.5 μM [14C]sucrose.

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0.01 and 0.1 μM sucrose (Kd = 0.033 μM), and a slow rate between 0.25 and 5 μM sucrose (Kd = 0.59 μM). This biphasic pattern is very similar to those observed in energy coupled transports of vitamin B12 into *E. coli* through the BtuB TBDR [55,56] and more recently of maltose transport into *C. crescentus* through the MalA TBDR [22]. In both systems, it was concluded that the first phase reflects binding of the transported molecule to the OM TBDR and that the second slower phase reflects binding to a cytoplasmic membrane transporter. Thus, we presume that the low Kd value (0.033 μM) mainly reflects binding to SuxA and that the higher Kd value (0.59 μM) binding to SuxC.

Altogether, these data indicate that both SuxA and SuxC are required for sucrose transport. It is worth noting that if the ΔsuxA mutant and the ΔsuxC insertion mutant have a different phenotype regarding their growth ability in presence of sucrose (see figure 7A), both mutants show a very similar alteration in sucrose transport rate after 8 minutes. However, after 240 minutes, sucrose transport rate in the ΔsuxA-deleted mutant reached almost 23% of the value obtained for the wild-type strain, whereas in the ΔsuxC insertion mutant, this rate was less than 7% of that of the wild-type strain. This result suggested the existence of two sucrose uptake pathways, an active and a passive pathway, both requiring transport through SuxC. The active transport pathway depends on SuxA for translocation through the outer membrane, whereas the passive pathway does not. It is assumed that the sucrose uptake rate observed into the ΔsuxA-deleted mutant is determined by slow and passive diffusion of sucrose across the outer membrane, which is sufficient to support growth in the presence of 20 mM sucrose in the medium.

<table>
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<th>Time (min) c</th>
<th>% Transport</th>
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</tr>
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<td>100.0</td>
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The expression of *sux* genes is induced by sucrose and repressed by SuxR

Using qRT-PCR analyses, we observed that the expression of *suxR, suxC* and *suxAB* is specifically induced by the presence of sucrose in the medium (Table 3).

As SuxR has a high degree of similarity with members of the LacI/GalR family of transcriptional repressors [62,63], we tested whether this protein regulates the expression of *sux* genes by comparing their expression by qRT-PCR in the *suxR* insertion mutant and in the wild-type strain, cultivated in minimal medium with or without sucrose. *suxAB, suxC* and *suxR* were over-expressed in a *suxR* mutant as compared to the wild-type strain in the absence of sucrose (Table 4), suggesting a negative and effector-dependent control of *sux* genes by SuxR.

We also studied the expression of the *suxAB* operon in the presence of different sucrose concentrations. For this purpose, the promoter region of this operon was cloned upstream of a minimal promoter in a reporter plasmid (Materials and Methods). This plasmid, named pPr-*suxAB*, was introduced into the *Xcc* wild-type strain, the *suxR* and *suxC* insertion mutants (*suxR::pVO and suxC::pVO, respectively*), and the *suxA* and *suxB* deletion mutants (*AsuxA* and *AsuxB*, respectively).

These strains were used to perform β-galactosidase assays after 6 hours growth in minimal medium containing a range of sucrose concentrations (Figure 10A). Induction of the expression of the reporter gene was detected for sucrose concentrations ranging from 20 mM to 20 mM. Maximal induction (3.5 to 4-fold induction) was observed for concentrations higher than 200 mM sucrose.

### Table 3. Relative expression ratios of *suxR, suxC, suxA* and *suxB* in the wild-type strain, in minimal medium containing 20 mM sucrose vs minimal medium without sucrose.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Ratio (+/- SD) a</th>
</tr>
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<tr>
<td><em>suxR</em></td>
<td>5.87 (+/- 1.98)</td>
</tr>
<tr>
<td><em>suxC</em></td>
<td>11.14 (+/- 0.4)</td>
</tr>
<tr>
<td><em>suxA</em></td>
<td>20.92 (+/- 8.5)</td>
</tr>
<tr>
<td><em>suxB</em></td>
<td>44.97 (+/- 2.64)</td>
</tr>
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</table>

a

### Table 4. Relative expression ratios of *suxR, suxC, suxA* and *suxB* in the *suxR* insertion mutant (*suxR::pVO*) vs wild-type strain.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Ratio (+/- SD) a</th>
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<td><em>suxR</em></td>
<td>10.4 (+/- 2.3)</td>
</tr>
<tr>
<td><em>suxC</em></td>
<td>23.6 (+/- 4.8)</td>
</tr>
<tr>
<td><em>suxA</em></td>
<td>154 (+/- 83.2)</td>
</tr>
<tr>
<td><em>suxB</em></td>
<td>176.45 (+/- 77.7)</td>
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a

*Sucrose uptake is energy-dependent*

Active iron transport through TBDRs depends on the proton motive force (PMF) [56,57]. Thus, we performed sucrose transport experiments in the presence of a PMF inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Addition of 20 μM CCCP inhibited [14C]sucrose transport rate to 1.13% of the transport rate in the absence of CCCP (Table 2), demonstrating that sucrose transport through SuxA and/or SuxC is dependent on the proton motive force and does not occur by facilitated diffusion. These experiments did not permit us to conclude whether SuxA-mediated transport depended on the PMF, since SuxC belongs to the Na+/melibiose cotransporter and H*+* cotransporters family, which require the transmembrane potential for transport across the cytoplasmic membrane. We then tested whether TonB was impaired in the 8 putative *tonB* mutants (Table 2). This result suggests a complete or partial functional redundancy between at least two *Xcc* TonB proteins. Such a redundancy has already been observed in various other bacteria, e.g. *Serratia marcescens* [58], *Vibrio cholerae* [59] and *Pseudomonas aeruginosa* [60,61].

![Figure 9. Concentration-dependent [14C]sucrose transport into wild-type Xanthomonas campestris pv. campestris. Cells were grown in minimal medium with or without sucrose. Calculation of relative expression includes normalization against the 16S rRNA endogenous control gene. SD: standard deviation.](image-url)
Figure 10. Expression of the Xanthomonas campestris pv. campestris suxA gene in the presence of sucrose, fructose or glucose. The pPV-suxA plasmid carrying the promoterless LacZ reporter gene under the suxA promoter region was used to monitor suxA expression in different genetic backgrounds. Expression was measured in minimal medium and cells were harvested at the indicated times. (A) Expression after 6 hours induction in the presence of different sucrose concentrations, in the wild-type background, the suxA and suxB deletion mutant backgrounds (suxA::pVO and suxB::pVO respectively), and the suxA and suxB deletion mutant backgrounds (suxA::pVO and suxB::pVO respectively). (B) Kinetics of LacZ expression in the wild-type background or in the suxA deletion mutant background (suxA::pVO), in presence of 20 mM (triangles) or 100 mM (squares) sucrose. (C) Kinetics of LacZ expression in the wild-type background in the presence of 100 μM sucrose, fructose or glucose.

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in the wild-type background (Figure 10A). As expected, the expression of suxA was highly induced in the suxR mutant, confirming the repressor activity of this gene. However, we observed a significant diminution of the induction level at 2 and 20 mM sucrose in this mutant, suggesting the existence of a second level of control. A high and constitutive induction of suxA expression was also observed in the suxB deletion mutant, suggesting that the amylolucrase gene plays a role in the repression of the suxA::pVO operon. At 2 mM sucrose concentration, a reduction in suxA expression level was also observed in this mutant, but it was smaller than that detected in the suxR mutant. The deregulation of suxA expression in the suxB mutant is in agreement with transport studies which showed a higher sucrose transport rate in this mutant (Figure 8C). No induction of suxA expression was observed in the suxC mutant, whatever sucrose concentration was used. This lack of induction certainly reflects the absence of sucrose entry into the cytoplasm of this mutant, thus preventing the alleviation of SuxR repression. This result clearly shows that the induction of

suxA expression by sucrose requires the entry of this molecule into the cell. The pattern and level of expression of suxA observed in the suxA mutant were very similar to those observed in the wild-type strain, except that this expression was significantly higher in the mutant in the presence of 2 mM sucrose. This difference was reproducibly observed and further investigations are needed to understand this phenomenon.

Expression assays suggested that sucrose transport through SuxA is not high enough to influence the expression of the suxA::pVO operon. Sucrose entry across the outer membrane by slow diffusion seems to be sufficient to promote induction of sux genes after 6 hours growth in the presence of sucrose.

suxA is required for its rapid induction as revealed by using low sucrose concentrations

We performed time-course experiments comparing suxA expression in the wild-type strain and the suxA deletion mutant grown in presence of different sucrose concentrations (0, 20 μM, 50 μM, 100 μM, 200 μM, 500 μM, 1 mM, 2 mM, 5 mM and 20 mM). For clarity, only results obtained with 100 μM and 20 mM are shown in Figure 10B. In the presence of 20 mM sucrose (triangles on Figure 10B), suxA expression was not significantly different after 6 hours growth in the wild-type and the suxA mutant backgrounds. However, a slight difference was observed in the induction level of suxA, from 60 to 120 minutes after sucrose addition. The expression level of suxA was 1.5 to 1.6 fold higher in the wild type strain than in the suxA mutant. Later, this difference tended to decrease and the ratio reached a value of 1.1 after 6 hours. A reproducible and clear difference in curve profiles was noticed in the presence of 100 μM sucrose (squares on Figure 10B). A rapid induction was observed in the wild-type context, from 30 to 120 minutes after sucrose addition, followed by a plateauing, in which expression seems to remain constant. On the other hand, in the suxA deletion mutant background, the induction was linear and less rapid. Between 30 and 120 minutes after sucrose addition, the expression level of suxA was 2 fold higher in the wild-type strain background than in the suxA deletion mutant background, although after 360 minutes, the expression levels were identical in both strains. Similar results were obtained with sucrose concentrations ranging from 20 to 200 μM (data not shown).

We propose that these results could reflect the existence of two pathways controlling suxA expression, which can be clearly differentiated with low sucrose concentrations. One of these pathways is controlled by SuxA. These results can be related to the transport analyses showing the existence of two types of sucrose transport across the outer membrane, one being SuxA-dependent and the other one, slower, being dependent on passive diffusion. We thus postulate that the induction of suxA indirectly reflects these two means of transport. Moreover, this induction is specific to sucrose and is not due to its degradation products glucose and fructose, as no induction was observed following addition of 100 μM of these molecules in the wild-type background (Figure 10C). Therefore, these experiments suggest that SuxA plays a role in sucrose transport which might be crucial at low concentrations of this sugar.

Identification of other Xcc TBDRs in loci probably involved in plant carbohydrates utilization

The genome of Xcc was then explored to see whether other TBDRs could belong to loci involved in the utilization of other plant compounds. Xcc has an extensive repertoire of plant cell-wall degrading enzymes, with cellulolytic, pectinolytic and hemicellulolytic activities [14]. The analysis of carbohydrate active enzymes
identified in the predicted proteomes of 209 Gram negative bacteria and referenced in the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/) showed that, after *Bacteroides* sp. and *S. degradans*, which are well known specialists for polysaccharide degradation, *Xcc* has one of the highest number of predicted genes involved in polysaccharide metabolism per megabase (29.9, total 152) (Table S2). These genes encode 82 predicted glycosyl hydrolases, 45 glycosyl transferases, 5 polysaccharide lyases, 18 carbohydrate esterases and 2 carbohydrate binding proteins. Interestingly, 46 of these proteins are encoded in the vicinity of 24 TBDR/Ps-TBDR genes, thus suggesting the existence of 19 new loci putatively involved in carbohydrate utilization (Table S3). Among those loci, 7 also contain an inner membrane transporter coding gene and a regulatory gene. For 3 loci identified in this analysis, the substrate probably utilized and transported could be easily deduced from the nature of the degradative enzymes: the *XCC0120* TBDR gene is localized upstream of genes coding for a pectin methyl esterase and a pectate lyase and might belong to a locus involved in pectin utilization (Figure 11A); the *XCC4120* TBDR gene is found in a cluster of genes probably involved in xylan metabolism (Figure 11B); and the *XCC2469* TBDR gene might be related to maltose/maltodextrin utilization, since it is associated with genes coding for a cyclomaltodextrin glucohydrolase, two α-glucosidases, a sugar transporter and a maltose transport gene repressor (Figure 11C).

To verify whether these TBDRs are really associated with carbohydrate utilization, we studied their regulation by plant compounds. For this purpose, we performed β-glucuronidase expression assays using all *Xcc* TBDR pVO155 insertion mutants. Cells were grown in rich medium or in minimal medium supplemented or not with polygalacturonic acid (PGA), arabinose, glucose, maltose, sucrose, xylose or xylan. Most TBDR (or Ps-TBDR) genes (44 out of 74) were repressed in rich medium, compared to minimal medium (Table S4). We also observed that in minimal medium, 40 *Xcc* TBDR genes are repressed in the presence of sucrose, xylose, arabinose and/or glucose, suggesting that these genes are submitted to catabolic repression (Table S4). However, the repression patterns were variable, suggesting the existence of several repression pathways. It is worth noting that most Fur-regulated TBDR genes were submitted to catabolic repression.

Three TBDR/Ps-TBDR genes were induced by PGA (*XCC0120, XCC1749 and XCC1750-1751*), one by maltose (*XCC2469*), seven by arabinose (*XCC0050, XCC1749, XCC1750, XCC1892, XCC2828, XCC4120 and XCC4222*), three by xylan (*XCC2828, XCC4120 and XCC4237*) and finally two by xylose (*XCC2828 and XCC4120*) (Table S4). Interestingly, the 2 TBDRs induced by xylose were also induced by xylan and arabinose, and *XCC1749* and *XCC1750* were both induced by PGA and arabinose. *Xcc* TBDR is the only TBDR gene for which expression is specifically induced by sucrose, and *XCC2469*, the orthologue of *C. crescentus* malA TBDR gene [22], is the only one induced by maltose. It is worth noting that the expression of the *XCC0120*, *XCC4120* and *XCC2469* TBDR genes was specifically induced by the postulated substrate of the associated CAZy referenced enzymes (see Figure 11). Altogether, these data suggest that several TBDRs are part of loci which seem to be involved in carbohydrate utilization. We thus propose the existence of 6 putative loci named carbohydrate utilization containing TBDRs (CUT) loci, which were defined by the presence of genes coding for carbohydrate degradative enzymes, inner membrane transporters and sugar related regulators beside TBDR genes. We also identified 15 putative partial CUT loci in the *Xc* genome (see Table S3).

**Conservation and distribution of TBDRs and CUT loci in Xanthomonads**

When compared with proteins in the databases using BlastP and the “distance tree of results option” displayed on the BlastP result page, the best homologous genes of *Xcc* TBDRs/Ps-TBDRs were putative TBDRs of other *Xanthomonas* species and in some cases of *Xylella* strains. These conserved genes might be considered as orthologs. We compared the distribution of *Xcc* TBDRs among Xanthomonads strains for which genome sequences are available, i.e. *Xc* strain 8004, *Xc* (strain 85–10), *Xcc* strain 306), *Xoo* (strains KACC10331 and MAFF311018) and *Xf* (strains 9a5c and PD). A

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**Figure 11.** Genetic organization of putative *Xanthomonas campestris* pv. *campestris* CUT loci and specific induction of the TonB-dependent receptor gene by plant carbohydrates. Putative pectin (A), xylan (B) and maltodextrin (C) utilization CUT loci and specific induction of the corresponding TBDR gene in the presence of PGA, xylose and maltose, respectively. β-glucuronidase assays were performed in at least two independent experiments with TBDR insertion mutants cultivated in minimal medium supplemented with glutamate 20 mM (GT) or specific carbohydrates (PGA: polygalacturonic acid 0.125%; XYL: xylose 20 mM; MAL: maltose 20 mM). TBDR: TonB-dependent receptor; PME: pectin methyl esterase; PL: pectate lyase; CGTase: cyclomaltodextrin glucanotransferase.

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search similar to that used for \textit{Xcc} allowed us to identify 72 TBDR/Ps-TBDR in the proteome of \textit{Xcc} strain 8004, 74 in that of \textit{Xaq}, 61 in \textit{Xev}, 41 and 42 in \textit{Xoo} strains KACC10331 and MAFF311018 respectively, and finally 10 in each \textit{Xf} strain. To further study the relationship between these TBDRs and \textit{Xcc} TBDRs, we performed a comparative study using alignments generated by ClustalW [64]. To carry out this comparative analysis, we only used TBDRs which seemed to be complete and Ps-TBDRs over 600 amino acids, in order to avoid bias in alignments. Examination of the phylogenetic tree, coupled with Blast results analysis, clearly confirmed that most \textit{Xcc} TBDRs are well conserved in other \textit{Xanthomonas} strains (Figure S1 and Table S3). All TBDR or Ps-TBDR genes detected in \textit{Xcc} strain ATCC39313 are present in strain 8004; 55 seem to have orthologs in \textit{Xaq}; 49 are conserved in \textit{Xev} and 34 only are conserved in \textit{Xoo} strains which possess significantly less TBDRs. In most instances, the grouping and distribution pattern of orthologous TBDRs matched the phylogenetic classification of \textit{Xanthomonas} species [65–67]. This observation suggests that this protein family is ancient in the \textit{Xanthomonas} genus. It is worth noting that branch lengths were variable, suggesting differential evolution rates. Moreover, we noticed that some truncated \textit{Xcc} Ps-TBDRs could be functional in other strains: the Ps-TBDR XCC1750-1751 doublet corresponds to unique and complete TBDR proteins in \textit{Xcc}, \textit{Xaq} and \textit{Xoo} strain 8004. Similarly, the 2 Ps-TBDRs XCC3215-3216 and XCC3270-3271, which are also truncated in \textit{Xcc} strain 8004, correspond to unique proteins in both \textit{Xcc} and \textit{Xev}. Interestingly, \textit{Xcc}, \textit{Xaq} or \textit{Xoo} TBDR orthologs of \textit{Xcc} Ps-TBDR having non-canonical C-terminal regions (\beta-sheet ended by an aromatic amino acid followed by a short extension of 1 to 4 amino acids), also displayed this feature (data not shown). In most cases, these non canonical C-terminal domains were identical in all strains. This conservation might reflect a specific functional feature of \textit{Xanthomonas} TBDRs.

The analysis of the phylogenetic tree also showed that \textit{Xanthomonads} TBDRs can be divided into three main groups, with most TBDRs belonging to group 1. This group can be divided into 8 subgroups (1A to 1H). We noticed that (i) \textit{Xcc} Oar-TBDRs are clustered in subgroup 1D, (ii) most plant carbohydrate induced TBDRs (8 out of 11) are grouped in subgroup 1C, and (iii) most \textit{Xcc} Fur-regulated TBDRs (6 out of 8) are grouped in subgroup 1F. Among these Fur-regulated TBDR genes, XCC3518, XCC3595 and XCC4162, which belong to subgroup 1F, seem specific to \textit{Xcc} strains.

The comparison of genes located adjacent to orthologous TBDR genes in the different \textit{Xanthomonas} strains showed that in most cases, these regions are syntenic (Table S3). Thus, all putative CUT loci identified in \textit{Xcc} genome are well conserved in \textit{Xaq} and \textit{Xev} but only 2 out of 6 were found in \textit{Xoo}. Similarly, almost all putative partial CUT loci are present in both \textit{Xcc} and \textit{Xev}, but only 9 out of 15 are conserved in \textit{Xoo} strains. The putative polygalacturonate utilization locus (XCC09120-XCC09122) seems unique to \textit{Xcc}. The genes bordering this locus are conserved and contiguous in \textit{Xcc} and \textit{Xev}, thus suggesting insertion or deletion events (Table S3).

Finally, among the 10 TBDRs identified in \textit{Xf} strains, 6 are conserved in \textit{Xcc}, \textit{Xaq}, \textit{Xev} and \textit{Xoo}, 1 is conserved in \textit{Xcc}, \textit{Xaq} and \textit{Xev}, whereas only 1 is present in \textit{Xoo}. The 2 remaining \textit{Xf} TBDRs (XF0339/PD1711; XF0599/PD1552) seem more divergent and thus specific to \textit{Xf} strains. However, XF0599 displays weak similarities with XCC2772, a Fur-regulated TBDR of \textit{Xcc}. None of the other \textit{Xf} TBDRs are related to \textit{Xcc} Fur-regulated TBDRs nor to \textit{Xcc} TBDRs belonging to putative CUT loci identified in this study. Only 2 \textit{Xf} TBDR genes (XF2713 and XF1036) correspond to \textit{Xcc} TBDRs present in partial CUT loci.

\textbf{Xcc} TBDRs are conserved in aquatic bacteria and/or phytopathogenic bacteria.

For each \textit{Xcc} TBDR included in the phylogenetic study, we analyzed the BlastP results obtained on the nr databank, to characterize the next best homologous genes after \textit{Xanthomonads} orthologs. This analysis allowed us to cluster \textit{Xcc} TBDRs on the basis of the bacterial origin of the homologous TBDR. Thus, \textit{Xcc} TBDRs could be divided into 4 main groups (Table S5). The first group corresponds to TBDRs showing their next best homologies (after \textit{Xanthomonads} orthologs) with other \textit{Xanthomonas} TBDRs rather than with TBDRs from other genera. This observation mainly concerned TBDRs found in subgroups 1B, 1C and 1D of the phylogenetic tree. Their positions in the tree suggested that they could be generated by successive duplication events that occurred before \textit{Xanthomonas} speciation (Figure S1). The second group corresponds to TBDRs which display high similarities with TBDRs of \beta-Proteobacteria and/or \textit{Pseudomonas} species. Interestingly most of these TBDRs are clustered in subgroup 3 of the phylogenetic tree. Moreover, some of these TBDRs showed high similarities with TBDRs of phytopathogenic bacteria such as \textit{Acidovorax avenae} subsp. \textit{citulli}, \textit{Pseudomonas syringae} pathovars or \textit{Ralstonia solanacearum}. The third group contains two \textit{Xcc} TBDRs proposed to be involved in iron uptake and showing significant similarities with putative TBDRs from \textit{Cyano bacteria}. The fourth group, which is the largest one (with 35 TBDRs out of 70), corresponds to TBDRs which displayed very significant homologies with putative TBDRs of a wide range of aquatic bacteria belonging to \textit{Xcc} or \textit{Xev} classes of \textit{Proteobacteria}. It is worth noting that in most cases, \textit{Xcc} TBDRs were not affiliated to TBDRs of a specific bacterial class, but there was rather a variety of origins. Thus, for 16 \textit{Xcc} TBDRs the best similarities (after \textit{Xanthomonads} similarities) were obtained with putative TBDRs of \textit{C. crescentus} CB15 and \textit{Caulobacter} sp. K31 strains, which are aquatic oligotrophs belonging to the Caulobacterales order of the \beta-Proteobacteria. Homologies were also observed with TBDRs of other \textit{\alpha-Proteobacteria} living in aquatic habitats, such as \textit{Oceanacalculs alexandri}, \textit{Marineulis maris} or \textit{Paracurlarea bernudensis} HTCC2503, or found in multiple environments like \textit{Sphingomonas} sp. SK435, \textit{Sphingopsis aldekenis} or \textit{Novosphingobium aromacitovorans}. Significant similarities were also obtained with putative TBDRs of aquatic \gamma-Proteobacteria classified in the \textit{Altemonadaceae}, including \textit{Alteromonas macrolides}, \textit{Saccharophagus degradans} 2-40, \textit{Pseudalomonas} and \textit{Shewanella} species. One common trait of most of these bacteria is that they show TBDRs overrepresentation with values of TBDR number per megabase ranging from 7.4 to 15.7 (Table S2). This raised the question of whether homologies between these TBDRs were fortuitous and a consequence of their large number or whether they reflect common biological functions.

\textbf{Conservation of TBDR regions and CUT loci in Gram-negative bacteria}

Some regions surrounding TBDR genes were found to be conserved in closely related bacteria such as \textit{Vibrio parahaemolyticus} or \textit{P. aeruginosa}. Thus, the XCC3050 Fur-regulated TBDR belongs to a cluster of 6 genes showing similarities with the \textit{puck-pse}ABCDE gene cluster of \textit{V. parahaemolyticus}, involved in the uptake and biosynthesis of the siderophore vibrioferrin [68] (Table S5). Similarly, a group of proteins encompassing XCC3067 TBDR and putatively involved in cobalamin uptake and biosynthesis in \textit{Xcc} was conserved in \textit{Pseudomonas} species (Table S3). Preliminary experiments showed that the expression of this \textit{TBDR} gene is repressed by the presence of vitamin B12 in \textit{Xcc}, thus suggesting that this cluster is functional (data not shown).
Several CUT loci identified in Xcc were not conserved in taxonomically related bacteria but rather in the group of aquatic bacteria showing TBDR conservation and in particular in C. crescentus. The sux locus, 3 of the putative CUT loci and 4 of the putative partial CUT loci identified in this study were entirely or partially conserved in this group of bacteria (Table S3). Thus, the putative maltose CUT locus was partially conserved with the mal locus recently identified in C. crescentus, which contains the MalA TBDR proposed to be involved in the uptake of maltodextrins [22] (Figure S2B). Interestingly, MalA shows significant similarities with the Xcc2469 TBDR whose expression is induced by maltose and maps in the maltose CUT locus (Figure 11C).

Similarly, the putative xylose locus, containing the Xcc2828 TBDR gene, is also very well conserved in C. crescentus (Figure S2C). The corresponding CC0999 TBDR gene was shown to be induced by xylose in this bacterium [69]. Moreover, CC2828, which was also shown to be xylose-induced in C. crescentus, displays significant homologies with the xylose-induced TBDR gene Xcc4129 belonging to a xylose CUT locus. Furthermore, the 20-bp palindromic motif conserved upstream of genes of the C. crescentus xylose regulon, was also found upstream of genes putatively involved in xylose metabolism in Xcc, as well as in the promoter region of Xcc2828 and Xcc4120 TBDR genes [69].

The sux CUT locus seems less well conserved and showed some degree of variation (Figure S2D). The amyllosucrase gene (Xcc3359) is only well conserved in the corresponding C. crescentus locus. In the loci conserved in other bacteria, the degradation of sucrose seems to involve more classical pathways [for review see 70]. Moreover, the MFS transporter of the Xcc locus is different from that of the C. crescentus sucrose locus encoded by CCI133, although it is well conserved in both Sphingomonas SK38 and Erthrobacter litoralis. These differences show the existence of some degree of plasticity in the evolution of the putative CUT loci. Further investigations are needed to see whether all these partially conserved loci are involved in the utilization of the same molecule. However, this wide conservation of CUT loci is in favor of their existence. These results also confirm that the similarities observed between TBDRs are probably not fortuitous.

**DISCUSSION**

**Overview**

The outer membrane (OM) of Gram negative bacteria serves as a selective permeation barrier, excluding hydrophilic solutes, including most nutrients. However, OMs contain embedded integral proteins, named outer membrane proteins (OMPs), which allow sensing and entry of nutrients into the cell. A major class of OMP with a certain substrate specificity, called porins, allows the translocation of hydrophilic solutes through the OM. Another class of OMP, the TonB-dependent receptors (TBDRs), is mainly involved in the translocation of hydrophilic solutes through the outer membrane transporter gene and a regulatory gene are also involved in the entry and utilization of plant carbohydrates. Moreover, 15 Xcc TBDR genes belong to partial CUT loci, thus suggesting the existence of CUT systems composed of different parts scattered in the genome. The existence of such multipartite CUT systems was supported by the observation that 11 TBDR/Ps-TBDR genes are specifically induced by plant carbohydrates such as sucrose or plant cell wall derived compounds including arabinose, xylose, peptic-polygalacturonate or maltose. Moreover, in 5 cases, there is a correlation between the inducing carbohydrate and the degradative enzyme(s) present in the CUT locus. This allows us to propose the existence of a sucrose CUT locus as well as loci involved in the utilization of complex carbohydrates such as pectin, xylos or starch.

**Xcc TBDRs belonging to the Fur regulon**

Our global study of Xcc TBDRs showed that only a small fraction of them seems to be involved in iron uptake. Among the 72 TBDR/Ps-TBDRs identified in the Xcc genome, only 9 are up-regulated under iron-limiting conditions. We established that these 9 genes are repressed by the Fur repressor and that they are the only TBDR genes having a Fur-box in their promoter region. Recently, a proteomic approach carried out in P. aeruginosa PAO1, which contains 34 putative TBDR genes, identified a very similar number of TBDR genes regulated by iron-starvation. In fact, in this bacterium, 7 TBDRs are produced under iron-starvation conditions and 4 others are specifically induced by the presence of heterologous siderophores, under iron-restricted conditions [72]. Therefore, although it is possible that some Xcc TBDR genes need specific heterologous siderophores for their expression, as observed in P. aeruginosa, the number of TBDR genes involved in iron uptake seems very comparable in both bacteria. This suggests that the other putative TBDRs/Ps-TBDRs might be involved in different biological functions.

**The identification of CUT loci/systems in Xcc suggests a relationship between TBDRs and carbohydrate utilization**

Our data reveal that several Xcc TBDRs are related to plant carbohydrate utilization. A large proportion of Xcc TBDR genes are coupled with carbohydrate active enzymes. For 6 of them, an inner membrane transporter gene and a regulatory gene are also present in the same region, thus defining the existence of putative CUT loci involved in the uptake and utilization of plant carbohydrates. Moreover, 15 Xcc TBDR genes belong to partial CUT loci, thus suggesting the existence of CUT systems composed of different parts scattered in the genome. The existence of such multipartite CUT systems was supported by the observation that 11 TBDR/Ps-TBDR genes are specifically induced by plant carbohydrates such as sucrose or plant cell wall derived compounds including arabinose, xylose, peptic-polygalacturonate or maltose. Moreover, in 5 cases, there is a correlation between the inducing carbohydrate and the degradative enzyme(s) present in the CUT locus. This allows us to propose the existence of a sucrose CUT locus as well as loci involved in the utilization of complex carbohydrates such as pectin, xylos or starch.

**The sux CUT locus is functional**

The existence of functional CUT loci was confirmed by the detailed study of the sux CUT locus, which showed its involvement in the entry and utilization of sucrose in Xcc. This locus comprises four genes, **suxA, suxB, suxC** and **suxR**, coding for a TBDR, an amyllosucrase, a sugar inner membrane transporter and a regulatory protein, respectively. [14C]Sucrose uptake experiments showed that SuxA and SuxC are both required for sucrose entry into the cell. Concentration-dependent sucrose transport experiments showed a biphasic kinetic, with a similar pattern observed for vitamin B12 uptake into *E. coli* through the *BtuB* TBDR [55], and more recently for maltose transport into C. crescentus through the MalA TBDR [22]. In both of these systems, it has been concluded that the first phase reflects the binding of the transported molecule to the OM TBDR, and that the second slower phase reflects the binding to a cytoplasmic membrane transporter. Thus, we presume in our experiments that the low *Kd* value (0.033 μM) mainly reflects binding to SuxA and that the higher *Kd* value (0.59 μM) binding to SuxC. Sucrose transport was significantly lower in the *suxC* mutant than in the *suxC* non polar mutant. This difference might explain the differential phenotype of these two mutants observed for growth on sucrose: the *suxC* non polar mutant was not impaired in growth on media containing sucrose, whereas the *suxC* mutant was unable to grow under these conditions. These observations suggest the existence of an alternative pathway that supports facilitated diffusion of sucrose across the OM, whereas it seems that there is a unique route for
crossing the inner membrane, depending on SuxC. The existence of this alternative pathway was also revealed by expression analyses of the sux locus. The regulation of sux genes seems to follow a classical inducer/repressor control, mediated by the SuxR repressor, sucrose being the inducer. The expression of sux genes is induced by sucrose but not by fructose or glucose. This induction was detected with sucrose concentrations ranging from 20 μM to 20 mM. These experiments showed that SuxA transport influenced sucrose induction of suxA at low sucrose concentrations (20 to 200 μM), whereas this effect is masked at higher concentrations, probably by interference by passive diffusion.

Our data also indicated that sucrose transport through the sux system is active and depends on the proton motive force. However, we could not conclude whether it depends on the TonB-ExbBD energy coupling system. In addition to TonB, the Xcc genome harbours 7 TonB-like proteins, which might substitute one for another. Another Xcc feature is the presence of a second exbD gene, named exbD2, mapping downstream of the tonB-exbBD1 locus. exbD2 is not required for iron uptake but is essential for HR induction, whereas tonB, exbD and exbD1 genes are necessary for both processes [73,74]. This differential behavior might be related to the existence of at least two classes of TBDRs in Xcc, one involved in iron uptake and the other one in transport of plant compounds. Further work is needed to address this hypothesis.

The sux locus represents a new sucrose utilization system

The Xcc sux locus is clearly different from other sucrose utilization loci already found in bacteria (for review see [70]). In particular, it differs from the scr sucrose-utilization system from enteric bacteria, which is also present in the plant pathogenic bacterium Erwinia amylovora, where it plays a major role in plant colonization [75]. The differences concern regulation, sucrose utilization and transport. The scr genes are regulated by a LacI/GalR family repressor, but fructose is the inducer [76]. In this system, the degradation of sucrose uses a sucrose-6-phosphate hydrolase/fructokinase degradation pathway [70]. On the contrary, the Xcc sux locus contains an amylosucrase orthologous to SUH, a unique sucrose hydrolase previously characterized in Aga [54]. This enzyme is responsible for intracellular sucrose hydrolysis. It is active on sucrose but not on sucrose-6-phosphate. In the scr system, sucrose metabolism and uptake are coupled by the phosphoenolpyruvate-dependent carbohydrate-phosphotransferase system (PTS), which controls crossing of the inner membrane and sucrose phosphorylation. The transport across the outer membrane is mediated via a porin, named ScrY [77,78]. Thus, the transport of sucrose is very different in sux and scr systems. Interestingly, the $K_D$ value of sucrose binding to SuxA is 1500- to 3000-fold lower than that of the E. coli ScrY sucrose porin, which varies from 13 mM [79] to 50 mM [80]. Similarly, C. crescentus MalA TBDR transports maltodextrins with $K_D$ values 1000-fold lower than those of the LamB porin, which facilitates the passive diffusion of maltodextrin [22]. It is worth noting that the $K_D$ values of sucrose binding to SuxA and maltodextrins binding to MalA are comparable. Thus, SuxA and MalA represents a new class of outer membrane carbohydrate transporters showing a much higher affinity for their substrate than porins.

The importance of the sux locus in Xcc is highlighted by the fact that it is required for full virulence on Arabidopsis thaliana. The phenotype of susB and susC mutants on plants can be related to their inability to grow on medium containing sucrose (20 mM). However, the non polar susC mutant, which grows as well as the wild type strain on medium containing sucrose, was also affected in pathogenicity. Thus, it appears that the ability to scavenge sucrose with a very high affinity plays a key role during the interaction with host plants.

In conclusion, data obtained on this sucrose CUT locus strongly support the existence of the other CUT loci identified in Xcc, which might have a similar mode of action (see model presented in Figure 12). This suggests the presence of several systems which seem to partially overlap and which are involved in the scavenging of plant molecules. They might form a complex network required for the exploitation of plant resources but which might also participate in signaling.

TBDRs and CUT loci are ancient in the Xanthomonas genus

The importance of TBDRs and CUT loci is not restricted to Xcc since they are well conserved in Xac and Xcc. A phylogenetic study of Xanthomonads TBDRs suggests that these proteins are an ancient class of proteins in the genus. Moreover, the grouping pattern observed in our phylogenetic study seems to correlate with functional features of Xcc TBDRs, thus suggesting the existence of structure/function relationships. However, it seems that there is some degree of variation in the repertoire of TBDR genes and CUT loci among Xanthomonads, as the number of genes and loci was lower in Xoo strains and also in Xylella. This latter species is considered as a minimal pathogen in the Xanthomonads with a restricted habitat and a reduced genome [81]. Xylella possesses a significantly lower number of TBDRs and only one partially conserved CUT locus.

Figure 12. Model of CUT loci functioning based on the Xanthomonas campestris pv. campestris sux locus. This scheme shows sucrose outer membrane transport via the SuxA TBDR or by passive diffusion through a putative porin. After crossing the inner membrane through the SuxC transporter, sucrose is proposed to interact with the SuxR repressor (thus allowing sux gene induction) and also to serve as a substrate for the SuxB amylosucrase. The large double headed arrow below the sux locus represents the balance between metabolic adaptation and virulence control putatively mediated by CUT loci.

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Conservation of Xcc TBDRs and CUT loci reveals carbohydrate scavenging abilities in other bacteria

One of the main surprises of this work was the observation that the large majority of Xcc TBDRs (35 out of 72) display significant similarities with TBDRs of bacteria which are mostly found in aquatic habitats and which are not closely related to Xanthomonads (see Table S5). Phylogenetic studies place Xanthomonads as a deep branch in the γ-Proteobacteria class, close to that of the β-Proteobacteria [66,82]. The bacteria possessing similar TBDRs belong either to the α-Proteobacteria class or to the Alteromonadales order of the γ-Proteobacteria. Most bacteria in this latter order have been collected from diverse aquatic environments and belong mainly to 6 different genera: Alteromonas, Calitellia, Idiomarina, Pseudoalteromonas, Saccharophagus and Shewanella. The α-Proteobacteria mentioned in this study are also found mostly in aquatic habitats and are members of several orders, including Caulobacteriales, Sphingomonadales, Rhodospirillales and Rhodobacterales. Similarities were not restricted to TBDRs genes, and genome context analyses showed that at least 8 putative (partial) CUT loci identified in Xanthomonas species were conserved among members of these aquatic bacteria. Several of them are found in association with abiotic or biotic surfaces, such as the surface of alga or shellfish. Some are adapted to live in oligotrophic environments, while others are abundant in nutrient-rich habitats or in association with particulate of organic matter. Some others are associated with decaying tissues of plant or algae. Although Xanthomonas seems to be very different from these bacteria in terms of habitat, lifestyle and taxonomy, we identified several common traits. All these bacteria belong to the class showing a TBDR overrepresentation (with TBDRs/Mbp ratios>7.4). Most of them are able to degrade a wide variety of plant molecules or other complex carbohydrates such as chitin, alginate, as well as various aromatic compounds. This combination of characters and the similarities with Xcc suggest the existence of a shared biology which might be related to the ability to scavenge carbohydrates. If the situation in these bacteria is similar to that observed in Xcc, we can speculate that there is a significant proportion of TBDRs involved in the uptake of molecules other than iron-siderophores complexes. Moreover, as previously noticed [33], we observed that bacteria having a restricted habitat, such as obligate parasites or symbionts, have no or only a very small number of TBDRs. Thus, these proteins might be considered as good indicators of bacterial lifestyle.

Association between TBDRs and carbohydrate utilization in other Proteobacteria

A specific role for TBDRs has already been attributed to bacteria displaying an overrepresentation of this protein family. Sphingomonads are widely distributed in nature and are mostly found in soils and aquatic environments. Some strains are associated with plants [83]. Strains like Sphingomonas sp. A1 are able to take up and metabolize macromolecules such as alginate produced by brown seaweed and certain bacteria. This uptake is mediated by large superchannels, which form a pit in the outer membrane, and which function as a funnel or concentrator (for review, see [84]). Four TBDRs seem to be part of this superstructure and are thus proposed to participate in alginate transport [85]. Recently, the manipulation of these superchannels revealed the importance of these structures in biofilm development [86].

In C. crescentus, beside the prediction of 67 TBDRs, a genome analysis revealed the presence of several genes for the breakdown of plant polysaccharides as well as transport systems, suggesting that plant polymers are a significant source of nutrient for this organism [87]. Interestingly, several C. crescentus TBDR genes have been shown or proposed to be associated with the uptake of plant compounds. As described above, Neugebauer and colleagues showed that C. crescentus can grow on maltodextrins and that the transport of these molecules is mediated by the MalA TBDR [22]. Proteomic and transcriptomic studies have shown that TBDR genes are expressed at higher levels in minimal medium than in rich medium [69,88–90], as we observed in Xcc. Moreover, several TBDR genes are specifically induced by the presence of xylose [69]. Interestingly, 2 TBDRs induced by xylose are conserved between C. crescentus and Xcc. Strikingly, putative regulatory cis-element motifs are conserved in the promoters of these homologous genes in both species. Moreover, one of these 2 Xcc TBDR genes (XCC2828) belongs to a putative partial CUT locus which is conserved in C. crescentus (Figure S2C). This suggests a possible lineage between these loci. Moreover, 3 other Xcc CUT loci are entirely or partially conserved in the C. crescentus oligotrophic bacterium, including the maltose and sucrose CUT loci. TBDRs in oligotrophs like C. crescentus might play a very important role by allowing the foraging of carbohydrates in nutrient poor environments.

Common themes between Xanthomonas and aquatic bacteria

It is clear that the life cycle of Xanthomonas spp. presents common features with the different lifestyles of bacteria described above. The leaf surfaces encountered by Xanthomonas during their epiphytic development might correspond to an oligotrophic environment. Plant leaves release secondary metabolites which can be used by epiphytic microorganisms [83]. However, carbon resources have been shown to be the most limiting resource on plants [91]. Simple sugars, like glucose, fructose and sucrose, are the most dominant carbon sources on plants that have been examined [92]. Studies with bacterial biosensors in situ on plants revealed a high heterogeneity of sucrose availability, with an average accessibility of only about 20 μM on moist bean leaves [93]. Interestingly, this sucrose concentration allowed the induction of suxA gene in our expression assays, thus suggesting that the suxA uptake system might function on plant surfaces. The involvement of suxA locus in epiphytic life is now being investigated. Furthermore, TBDRs might facilitate the exploitation of plant debris generated during disease development, thus resembling bacteria living on dead tissues. This phenomenon might have an impact on Xanthomonas life cycle by increasing Xanthomonas population size and thus facilitating new infections.

TBDRs and virulence on plants

It appears that in Xanthomonas, some TBDRs such as SuxA play an additional and specific role by controlling virulence. Therefore, it is tempting to propose that a strategy shared with non-pathogenic bacteria and based on the active uptake of plant-derived nutrients, could have been diverted by Xanthomonas for the control of pathogenicity. It is worth noting that several phytopathogenic bacteria such as P. syringae or E. carotovora subsp. Atroseptica, as well as Pseudomonas species associated with plants, have an intermediate overrepresentation of TBDRs, suggesting that this feature could be shared by other bacteria interacting with plants. In R. solanacearum, the expression of kep genes coding for a type III secretion system (TTSS) which controls disease and HR development, is specifically induced upon contact with plant cells [94]. This signaling is mediated by PhrA, a TBDR belonging to the transducer subclass. This receptor senses contact with plant cells and transduces this signal into the cytoplasm via PhrI and PhrR, which are FecI/FecR.
homologs [94–96]. Apparently, this regulatory system does not require the transport of plant molecules [94]. Although Xcc, Xcc, Xoo or Xac do not carry any TBDR showing a high homology with PhoA, recently a TBDR controlling both HR and pathogenicity was described in X. oceae pv. oceae, the causal agent of bacterial leaf streak of rice. This TBDR does not belong to the transducer subclass and only shows a weak similarity with PhoA [97]. This TBDR gene is highly conserved in Xoo strain MAFF 311018 (XCC0672), Xcc (XCV3654) and Xcc (XCC0674). Interestingly, in Xoo it is located close to trh (XOO0073), a regulatory gene controlling the expression of hph genes [53]. The orthologue of this regulatory gene is also conserved in Xcc (XCC0672), in the vicinity of the XCC0674 TBDR gene. Thus, it is possible to speculate that both genes might be involved in a common circuit controlling the expression of hph genes in Xanthomonas spp. In our study, a mutation in XCC0674 did not affect the pathogenicity of Xcc. However, as there are more TBDRs in Xcc than in Xoo strains, we can speculate that functional redundancy might have masked the effect of the mutation. Further work is needed to see whether these genes regulate hph genes in Xcc. Nevertheless, we established a link between hph genes and TBDRs in Xcc. Indeed, we observed that 2 Xcc TBDR genes are regulated by the hphGC and hphX regulatory genes. Therefore, it seems that there is an overlap between hph genes and at least 2 TBDR genes. What is the function of these two TBDRs? Are they specifically required during the infection of plants, through the action of the hph regulon, to exploit specific released plant molecules?

What other functions for Xcc TBDRs?
We have been able to define a putative role for 21 TBDRs out of 72 identified in Xcc. The nature of the molecules putatively transported by the other TBDRs remains to be discovered. It is probable that TBDRs are not restricted to the transport of carbohydrates and that they can take up various other molecules produced by plants. Azospirillum irakensis, a plant associated bacterium, is able to metabolize salcin, a phenolic glycoside produced by plants. Interestingly, the ad operon, which controls the degradation of salcin, contains a TBDR gene which was proposed to be involved in salcin uptake [98]. Thus, secondary metabolites including phenolic compounds might be assimilated through TBDRs. We are now trying to characterize which new molecules may be transported by Xcc TBDRs, to better understand the adaptation of this pathogen to host plants.

TBDRs, CUT loci and evolution in Gram-negative bacteria
This work has identified the existence of an ensemble of bacteria that have an overrepresentation of TBDR genes, and that share specific loci for the scavenging and utilization of carbohydrates. They belong to very different lineages in Proteobacteria and this raises the question of the origin of these TBDRs and CUT loci. Did they arise by convergent evolution or were they transferred from species to species by lateral gene transfer? Our data suggest that this latter hypothesis is most likely. Recently, a genomic comparative study established that Xcc and Xac have close to 40% of their genes showing highest similarities to genes from non γ-Proteobacteria, especially from z-Proteobacteria (20%). These genes seem to belong to genomic islands, denominated “unusual best-match islands” (UBIs) [82]. Interestingly, among 33 UBIs thus identified in Xcc genome, 14 contain TBDRs and/or CUT loci. However, there was no significant difference between the GC content of each of these loci and the overall content of the genome. It is therefore possible that these loci were acquired very early in the evolution of Xanthomonads and thus played a key role in their adaptation to plants. Most bacteria showing a TBDR overrepresentation possess TBDRs or CUT loci conserved with Xcc. However, there is a main exception with members of the Bacteroidetes phylum, as none of the TBDRs characterized in this phylum showed very significant similarities with those identified in Xcc. Bacteroidetes can be encountered in two very different niches, the marine environment and the human intestine [99,100]. Marine Bacteroidetes such as Gemella foetida are associated with particulate of organic matter [99], whereas those found in the intestine are assembled on partially digested food particles [101]. In both cases, these bacteria are able to consume biopolymers. Bacteroides thetaiotaomicron, which is a prominent mutualist in the distal intestine of adult humans, has the largest ensemble of TBDR genes and glycolobiome yet reported (Table S2). Studies have shown that this bacterium has a carbohydrate foraging behavior [100–102]. It is well known to bind starch through a protein complex of the outer membrane, which comprises SusD and the SusC TBDR protein [103]. One hundred and six paralogs of SusC and fifty three paralogs of SusD were predicted in the B. thetaiotaomicron genome [104]. In our study, we did not detect in Xanthomonas genomes any protein displaying similarities with SusD. Moreover, none of the TBDRs identified in Xcc showed strong similarities with SusC. A Blast analysis suggested that the SusC TBDR and the SusD OMP are specifically conserved in Bacteroidetes (data not shown). These results suggest that TBDRs involved in carbohydrate uptake evolved independently in Proteobacteria and Bacteroidetes. The analysis of the function and evolution of TBDRs in these phyla will certainly help us to better understand the adaptation of bacteria to their environment. This knowledge, which concerns the utilization of plant molecules that are widespread in the environment, will have a major impact not only in plant pathology, but also in human health as well as in the cycling of carbon and geobiology in marine environments.

MATERIALS AND METHODS
Bacterial strains, plasmids and growth conditions
The Xanthomonas campestris pv. campestris (Xcc) strains and plasmids used in this study are listed in Table S6. Xcc cells were grown at 30°C in MOKA rich medium (Yeast Extract 4 g/l, Casamino acids 8 g/l, K2HPO4 2 g/l, MgSO4.7H2O 0.3 g/l) or in MME minimal medium [12]. E. coli cells were grown on LB medium [105]. Antibiotics were used at the following concentrations for Xcc: rifampicin, 50 μg/ml; kanamycin: 50 μg/ml; tetracycline: 5 μg/ml. Antibiotics were used at the following concentrations for E. coli: ampicillin, 50 μg/ml; kanamycin: 50 μg/ml; tetracycline: 10 μg/ml.

Growth curves were generated using the Bioscreen C instrument (Labsystems, Helsinki, Finland) in three independent experiments. Growth measurements were realized in 200-well microtiter plates on 350 μl volumes of a minimal medium containing 20 mM sucrose, inoculated at an OD600 = 0.15 from a washed starter culture. Non-inoculated wells were used as asepsis controls. Optical densities at 600 nm values were measured every 30 min over a period of 2 to 3 days at 28°C. The microplates were shaken for 5 sec before each measurement.

Construction of Xanthomonas campestris pv. campestris mutants.
Insertion mutants were constructed using the suicide plasmid pVO155 [36]. Oligonucleotide primers used for PCR amplification will be provided upon request. Amplicons were 300 bp in
average. Location of insertions are indicated in Table S6. Deletion mutants in XCC3358, XCC3359, XCC1390, XCC3209 and XCC2927 were constructed using the cre-lox system adapted from Marx and colleagues [37,38]. Deleted regions are indicated in Table S6.

A fur mutant strain was obtained using the manganese mutagenesis method [106] in LB medium containing 5 mM MnCl2. After incubation for 40 h at 30°C, surviving colonies were harvested for siderophores over-expression on CAS agar plates [107] adapted for Xanthomonas (K2HPO4 1 g/l; MgSO4 1 mM; Casamino acids 0.15 g/l; (NH4)2SO4 1 g/l; sucrose 20 mM; CAS 60.5 mg/l; HDTMA 72.9 mg/l; FeCl3 10 μM). A resistant strain over-expressing siderophores was selected; the fur gene was sequenced and contains a point mutation (T212A leading to L71Q).

Plasmid constructions
The XCC3358, XCC3359 and XCC1470 genes (suxA, suxB and fur respectively, see Figure 6 and Table S6) were amplified by PCR using appropriately designed primers (Oligonucleotide primers used for PCR amplification will be provided upon request). PCR products corresponding to suxA and suxB genes were cloned into pCZ525, a derivative of pSC154 [108], without cydA' coding sequence. The obtained plasmids were cloned into pLAFR6 [109] to give pL-XCC3338 and pl-L-XCC3359 respectively. The PCR product corresponding to the fur gene with its promoter and terminator sequences was cloned into pFAJ1700 [110] to give pL-XCC31470.

The XCC3358, XCC1390 and XCC3209 promoter regions (see Table S6) were PCR amplified with appropriately designed primers. These promoter regions were cloned as HindIII-XhoI fragments, into the pCZ750 plasmid, a pFAJ1700 [110] derivative containing the KpnI-AscI lacZ' gene from the pCZ367 plasmid [108].

Expression studies
β-galactosidase and β-glucuronidase assays; bacterial cultures in the appropriate medium were harvested at different time points and β-galactosidase and β-glucuronidase assays were performed as previously described [111,112].

Quantitative RT-PCR (qRT-PCR): a 6 hour bacterial culture in the appropriate medium was harvested at an OD600 = 0.4 to previously described [111,112].

TBDRs and Carbohydrate Uptake

Pathogenicity tests
Pathogenicity tests were conducted on Arabidopsis thaliana Sf-2 ecotype as previously described [114]. Each strain was tested on sets of 4 plants with 4 leaves per plant. Disease development was scored at days 5, 7 and 9 post-inoculation using a disease index ranging from 0 (no symptom), to 4 (leaf death).

[14C]sucrose transport experiments
Overnight cultures in minimal medium (MME) without carbon source (uninduced) or with 20 mM sucrose (induced) were centrifuged. Pellets were resuspended in MME and the OD600 was adjusted to 1. [14C]sucrose (PerkinElmer, specific activity of 21.8 GBq/mmol) was added to a final concentration of 0.5 μM. For competition experiments, sucrose, fructose or glucose was added to [14C]sucrose at a final concentration ranging from 0.5 to 100 μM. After different times, from 20 sec to 2 hours, samples of 0.2 ml were collected on cellulose nitrate filters, washed with 10 ml water, dried, and finally, the radioactivity was determined in a liquid scintillation counter.

The concentration-dependent initial sucrose transport was determined using the rapid dilution method as described [22]. Cells were precultured in minimal medium without sugar. After centrifugation and adjustment to an OD600 of 1, cells were incubated for 15 sec in presence of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 μM [14C]sucrose and 0.2 ml samples were diluted into 5 ml MME supplemented with 0.1 mM sucrose. Cells were collected by filtration, washed with 10 ml MME supplemented with 10 mM sucrose, dried and the radioactivity was determined.

For inhibition of the proton motive force (PMF) with carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP), cells were incubated for 10 minutes at 30°C with 20 μM CCCP prior to the addition of [14C]sucrose.

In silico analysis
Location of the signal sequence responsible for the outer membrane localization was determined using the SignalP 3.0 server [115] (http://www.cbs.dtu.dk/services/SignalP/) with default parameters for Gram-negative bacteria.

Comparison alignments used to identify the TonB-box were realized using ClustalW [64] (http://www.ebi.ac.uk/clustalw/) or Multalin [116] (http://prodes.toulouse.inra.fr/multalin/multalin.html) softwares.

β-sheets in the last 50 amino-acids of Xcc TBDRs were located using the secondary structure prediction method [117] on the PSIPRED Protein Structure Prediction Server [118,119] (http://bioinf.cs.ucl.ac.uk/psipred/).

The MotifSampler program [41,42] was used to identify a motif corresponding to the Xcc Fux-box upstream of the 9 Fur-repressed TBDR and Ps-TBDR genes. MotifScanner program [42] or the PatScan pattern matcher software [47] were used with the identified motif to locate all the Fur-boxes in the Xcc genome.

Pip boxes (TTCGGC)n7(TTCCG) [120] and hprq boxes (TTCCG)n6(TTTC) [121] were identified in the Xcc genome using the PatScan software [47] (http://wwwunix.ms.cas.cz/compbio/PatScan/HTML/patscan.html).

For phylogenetic analysis, amino acid sequences were aligned and phylogenetic trees were reconstructed by the neighbor-joining method as implemented in ClustalX [122].

SUPPORTING INFORMATION

Figure S1 Phylogenetic tree of the family of TonB-dependent receptor proteins from Xanthomonas campestris pv. campestris strains ATCC33913 and 8004, Xanthomonas axonopodis pv. citri strain 306, Xanthomonas campestris pv. vesicatoria strain 85–10, Xanthomonas oryzae pv. oryzae strains KACC10331 and MAFF310108 and Xylella fastidiosa strains 9a5c and PD.
Figure S2 Genome context of genes associated with TonB-dependent receptors present in putative (partial) CUT loci of Xanthomonas campestris pv. campestris (Xcc) and conservation in non related bacteria. Homologous genes are marked by matching colors. White color indicates non conserved genes. For conserved genes, percentages of identity and similarity to the corresponding Xcc gene are indicated.

Table S1 Regions and domains of Xanthomonas campestris pv. campestris putative TonB-dependent receptors (TBDRs).

Table S2 Distribution of TonB-dependent receptors in 226 sequenced Gram negative bacterial genomes. The EMBL-EBI, Integro web portal (http://www.ebi.ac.uk/integro/EBI-Integro-HomePage.do) and the NCBI ENTREZ Genome Project database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj) were used to select sequenced bacterial genomes. TonB-dependent receptors (TBDRs) were detected by screening the Pfam database using the two Pfam domains (PF07715, Plug; PF00593, TonB_dep_Rec). Only proteins displaying the two Pfam domains were considered.

Table S3 Genome context of Xanthomonas campestris pv. campestris (Xcc) TonB-dependent receptor (TBDR) genes and definition of putative CUT loci, conservation in Xanthomonads or in other genera, and TBDR conservation in Pseudomonas aeruginosa PA01.

Table S4 Differential expression ratios of Xanthomonas campestris pv. campestris TonB-dependent receptor (TBDR) pVO155 insertion mutants.

Table S5 Conservation of Xanthomonas campestris pv. campestris (Xcc) TonB-dependent receptors (TBDRs) beside Xanthomonads orthologies.

Table S6 List of plasmids and Xanthomonas campestris pv. campestris strains used or generated in this study.

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Author Contributions

Conceived and designed the experiments: EL SB DM MA. Performed the experiments: EL SB DM AB ML CG ND JV. Analyzed the data: EL SB DM MA. Contributed reagents/materials/analysis tools: EL SB DM AB ML CG ND JV. Wrote the paper: EL MA.


