Transcriptional reprogramming and phenotypical changes associated with growth of *Xanthomonas campestris* pv. *campestris* in cabbage xylem sap

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**Abstract**

Xylem sap (XS) is the first environment that xylem phytopathogens meet *in planta* during the early infection steps. *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causative agent of *Brassicaceae* black rot, colonizes the plant xylem vessels to ensure its multiplication and dissemination. Besides suppression of plant immunity, *Xcc* has to adapt its metabolism to exploit plant-derived nutrients present in XS. To study *Xcc* behaviour in the early infection steps, we used cabbage XS to analyse bacterial growth. Mineral and organic composition of XS were determined. Significant growth of *Xcc* in XS was allowed by the rapid catabolism of amino acids, sugars and organic acids, and it was accompanied by the formation of biofilm-like structures. Transcriptome analysis of *Xcc* cultivated in XS using cDNA microarrays revealed a XS-specific transcriptional reprogramming compared to minimal or rich media. More specifically, up-regulation of genes encoding transporters such as TonB-dependent transporters (TBDTs), that could be associated with nutrient acquisition and detoxification, was observed. In agreement with the aggregation phenotype, expression of genes important for twitching motility and adhesion was up-regulated in XS. Taken together, our data show specific responses of *Xcc* to colonization of cabbage XS that could be important for the pathogenesis process and establish XS as a model medium to study mechanisms important for the early infection events.

**Introduction**

During the endophytic phase of its infection cycle, *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causal agent of black rot of *Brassicaceae*, initially colonizes xylem vessels of its host plants. It naturally enters this compartment through wounds or, during guttation process, through hydathodes localized at the leaf margins (Ryan et al., 2011). Extensive multiplication of bacterial cells into the xylem is associated with browning of leaf midribs and V-shaped necroses (Vicente & Holub, 2013). Successful infection relies on multiple pathogenicity determinants important for motility, adhesion (Dow et al., 2003), nutrition, stress tolerance and suppression of plant immunity (Büttner & Bonas, 2010). *In planta* nutrition relies on the ability of *Xcc* to degrade complex plant macromolecules using plant cell-wall degrading enzymes (PCWDE, e.g. cellulases, xylanases or polygalacturonases) and to assimilate the resulting degradation products. Transporters such as TonB-dependent transporters...
(TBDT) allow high affinity scavenging of plant-derived carbohydrates, such as sucrose (Blanvillain et al., 2007), N-acetylglucosamine containing molecules (Boulanger et al., 2010) or xylan (Déjean et al., 2013), the first one being required for full virulence in planta (Blanvillain et al., 2007). In addition, type III effector (T3E) proteins injected inside plant cells with the hrp (hypersensitivity response and susceptibility) type III secretion (T3S) system (Dow et al., 1987; Katzen et al., 1998; Büttner & Bo-"nas, 2010) are essential for disease development and host immunity suppression. In Xcc, hrp gene expression can be achieved in minimal medium (MM) and is in general not expressed in rich medium (RM) (Arlat et al., 1991). On the contrary, the expression of genes involved in nutrients uptake and catabolism is relieved by the presence of the specific substrate (Blanvillain et al., 2007; Boulanger et al., 2010; Déjean et al., 2013).

Several analyses of bacterial adaptation to plant environment using in vivo expression technology were previously performed. Such process was associated in the bacteria with the expression of genes encoding virulence proteins (including T3E), transporters and stress response proteins during the infection process as observed for Pseudomonas syringae pv. tomato (Boch et al., 2002), Dickeya chrysanthemi (Yang et al., 2004),Ralstonia solanacearum (Brown & Allen, 2004) and Erwina amylovora (Zhao et al., 2005). More recently, DNA microarrays have been used to characterize transcriptomes of bacterial phytopathogens responding to plant extracts or conditions thought to mimic the plant or from in planta recovered bacterial cells. These studies revealed the importance of sucrose metabolism in R. solanacearum (Jacobs et al., 2013), modifications in the expression patterns of insertion sequences (IS) in X. oryzae pv. oryzae (Soto-Suárez et al., 2010) and specific transcriptional rearrangements associated with osmotic stress during epiphytic and endophytic phases of P. syringae pv. syringae (Yu et al., 2013). Such approach gives valuable information on how a bacterial pathogen adapts its gene expression pattern to the plant environment. Concerning Xcc, responses to its host plant were first reported using proteome analyses (Andrade et al., 2008; Villeth et al., 2009). When infiltrated inside young cabbage leaves, several proteins related to polysaccharide synthesis, resistance to oxidative stress and outer membrane proteins were found to accumulate in Xcc.

For vascular pathogens such as Xcc, xylem sap (XS) constitutes the first primary medium encountered by Xcc in the early infection process. XS is however generally considered as a low-nutrient medium (Buhtz et al., 2004; Ligat et al., 2011). Therefore, adaptation to this medium by the bacterium could be important to trigger epiphytic/parasitic transition to successfully colonize the plant leaf. Hence, using isolated XS as a growth medium might provide information concerning the transcriptional reprogramming associated with an early infection phase where T3S-dependent suppression of plant innate immunity occurs. We report here on the characterization of the phenotypic, physiologic and transcriptomic adaptation of Xcc strain 8004 during growth in cabbage XS relative to minimal and rich media. Colonization of the cabbage XS by Xcc was accompanied by an increase in bacterial cell aggregation and type-IV motility. Growth was allowed by the presence of organic acids that are readily used by the bacteria. An important rearrangement in the expression pattern of transporters was observed together with up-regulation of motility processes, indicating physiological remodelling in response to this particular medium. Expression of T3S system-related genes was only weakly induced in XS as compared to RM suggesting that other nonmetabolic stimuli such as plant cell wall contact (Marena et al., 1998; Aldon et al., 2000) might also contribute to maximal T3S system expression in planta.

Materials and methods

Bacterial strains and growth conditions

Xcc strain 8004 was cultivated at 30 °C using either the MOKA RM [yeast extract 4 g L⁻¹, casamino acids 8 g L⁻¹, K₂HPO₄ 2 g L⁻¹, MgSO₄ 0.3 g L⁻¹; (Blanvillain et al., 2007)] or the MME MM [casamino acids 0.15 g L⁻¹, K₂HPO₄ 10.5 g L⁻¹, KH₂PO₄ 4.5 g L⁻¹, MgSO₄ 0.12 g L⁻¹, (NH₄)₂SO₄ 1 g L⁻¹; (Arlat et al., 1991)]. Before use, bacteria were pre-cultured in liquid RM under shaking at 200 r.p.m.

Phenotypic characterization of the growth of Xcc in XS

Adhesion assays were performed as described (Darsonval et al., 2009). Motility assays were conducted in semi-solid agar (0.3%) media using 12-well plates in 0.9x strength medium. Plates were then incubated for 48 h at 30 °C.

Plant inoculation procedure

Plant infections were performed by piercing young leaves of Brassica oleracea cv. Bartolo F1 in the midrib as described (Shaw & Kado, 1988; Meyer et al., 2005) using a needle dipped in a bacterial suspension at optical density (OD) 0.4. To measure gene expression levels in planta, three leaf midribs were sampled at 4, 8 or 12 h postinoculation, frozen, grinded and resuspended in 500 μL Trizol. The extract was subsequently treated to prepare RNA as described below. The sampling time t₀ consisted of 10 μL of the bacterial suspension used for inoculation.
Collection of root-pressure exudates from cabbage

XS from *B. oleracea* cv. Bartolo F1 cabbages was collected as previously described (Dugé de Bernonville et al., 2014). Briefly, XS was collected after stem decapitation above the first true leaf pair. After rinsing thoroughly the section with distilled water, root-pressure exudates (hereafter called XS) were collected throughout the day by manual pipetting. The two first harvests were discarded to avoid contamination by the remaining cell wall debris and phloem contamination (Dugé de Bernonville et al., 2014). Fractions were frozen immediately after collection and stored at −20 °C. For bacterial cultivation, XS was sterilized by filtration using 0.22 μM polytetrafluoroethylene filters.

Serial cultivation of *Xcc* in RM, MM and XS for microarrays and metabolite consumption analyses

Three successive growth cycles were performed in each medium. Briefly, each medium was inoculated at an OD at 600 nm of 0.1 using bacterial pellets washed with sterile demineralized water, after overnight growth in RM. For the culture in XS, we pooled several XS samples to have a sufficient culture volume for each experiment. Cultures were performed in 12-well plates in a final volume of 1 mL (metabolite consumption analysis) or 2 mL (transcriptomics), using a microplate spectrophotometer to continuously monitor the bacterial growth at OD₆₀₀ nm (FLUOStar Omega, BMG Labtech, Offenburg, Germany). The incubation temperature was fixed at 28 °C, and the microplates were shaken continuously at 700 r.p.m using the double-orbital-shaking mode. At the beginning of the stationary plateau, bacterial cells were recovered by centrifugation at 12,000 g for 1 min, and then resuspended in fresh medium at OD₆₀₀ nm 0.1. After completion of the second growth cycle, a third one was reinitiated as previously and stopped at mid-exponential growth phase. For RNA extraction, bacterial pellets were immediately frozen in liquid nitrogen and stored at −80 °C. For nutrient consumption analysis, the supernatants (SNs) collected after a centrifugation step, were stored at −20 °C.

Determination of XS composition

The XS composition was analysed in three independent samples. For the consumption analysis, SNs from four independent cultures were analysed. Samples were frostedlyophilized and subsequently derivatized before GC-MS analysis. For amino acid analysis, the samples were dissolved with 50 μL of acetonitrile and 50 μL of N-methyl-N-tert-butylmethylsilyl-trifluoroacetamide (MTBSTFA) was added. Vials were then heated at 70 °C for 4 h and allowed to cool to room temperature prior to analysis. For organic acid and sugar analysis, the samples were dissolved in 50 μL of pyridine and 50 μL of N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) was added. Vials were then heated at 70 °C for 30 min and allowed to cool to room temperature prior to analysis. The derivatized metabolite samples were analysed by GC-MS using a Thermo GC-TSQ Quantum. A 1 μL aliquot of the sample was injected at a split ratio of 1 : 10 and separated on a capillary ZB-5MS column (30 m × 0.25 mm, 0.25 μm; Zebron Phenomenex). Helium was applied as a carrier gas at a flow rate of 1.0 mL min⁻¹. The GC oven was programmed for 1 min at 60 °C and then increased to 120 °C at 30 °C min⁻¹ and to 350 °C at 8 °C min⁻¹. The GC injection temperature was 230 °C, and the interface temperature was 250 °C. Mass spectra of eluting compounds were generated using an electron impact (EI) positive ion source at 250 °C. Peaks were identified using authentic standards and using their MS fragmentation patterns.

Consumption of metabolites was expressed as percentage of metabolites used after culture relative to the initial metabolites concentration in XS.

Inductively coupled plasma (ICP) analysis of mineral content

Mineralization was performed after adding 1 mL of 70% nitric acid to 1 mL of XS and heating at 60 °C for 16 h. The resulting solution was filtered through a 150 mm diameter ashless pleated filter and subsequently diluted to lower nitric acid concentration in a final volume of 6 mL. ICP-optical emission spectroscopy (ICP-OES, Varian Vista MPX) was used to analyse mineral content. Calibration was achieved with serial dilutions of standard micro and macro elements from 5 μg L⁻¹ to 5 mg L⁻¹ and fixing the optimal emission lines.

RNA extraction and cDNA synthesis

Total RNA were extracted using the Trizol reagent and were treated with DNase as described (Darsonval et al., 2009). To prepare bacterial RNA from liquid culture, frozen bacterial pellets were directly resuspended into 600 μL hot Trizol. To prepare total RNA from infected cabbage leaves, leaf discs were ground with a mixer mill and RNase-free glass beads, prior to the addition of Trizol. Concentration of RNA was determined using a nanodrop spectrophotometer, and absence of genomic DNA was checked by PCR with primers targeted to genomic DNA.
Microarrays

cDNA synthesis and hybridization

Bacterial total RNA were extracted from three independent biological repeats for each condition. Five micrograms of total RNA was reverse transcribed with superscript II (Invitrogen) and a dNTP mix containing amino-allyl-dUTP (Sigma). The cDNA obtained was post-labelled using the Cy3 and Cy5 Monoreactive Dye Pack (Amersham Biosciences) and were purified using MinElute Kit (Qiagen) according to the manufacturer’s instructions. All samples were labelled with Cy3 and hybridized against a common pool reference (mix RNA from the three culture conditions) labelled with Cy5. One microgram of labelled cDNA was used per hybridization. Glass slides DNA arrays covering the Xcc strain B100 predicted coding sequences (Vorhöller et al., 2008) [Xcc5KOL1 microarray of 50–70 long oligonucleotides representing the 4553 predicted genes in the genomic sequence of Xcc strain B100 (Serrania et al., 2008)] were pretreated as recommended (Transcriptomic Facility, CeBiTec, Bielefeld University) and hybridized to the GeT-Biopuces Platform (Toulouse-Genopole). After prehybridization with a solution of 2X SSC, 0.2% SDS and 1% BSA at 42 °C for 30 min, 200 µL of the hybridization solution, containing the 20 µL of labelled cDNA mix (Cy5 and Cy3, 1 µg of each) and 180 µL of hybridization buffer (Chyp Hybe buffer from Ventana Medical System) were added on the printed side of the slide. After 8 h of hybridization at 42 °C, slides were washed twice with 2X SSC/0.2% SDS, once with 0.1X SSC and dried by centrifugation.

Data acquisition and pretreatment

Slides were scanned with a 2-laser scanner (Innoscans 900 from Innopsys), and signal was integrated with the IMAGE-GENE v7.0 software (Biodiscovery, Hawthorne, CA) as described (Serrania et al., 2008). Raw microarray data were deposited in the Array Express database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2290. Microarray data normalization and statistical treatments were performed using r v2.15.1 (R Development Core Team, 2011). Intensities from red and green channel were read with the package LIMMA, converted to a marrayRaw class object and then normalized using the optimized scaled intensity-dependent procedure [OSLIN package v1.34; (Futschik & Crompton, 2005)] after applying a model-based background adjustment (normexp algorithm) using local median background estimates.

Data analysis

To lower an heteroscedasticity effect identified in a first differential analysis, spots were ranked according to their estimated residual variance, and groups of 20 spots of similar residual variance were formed. Differential analysis was performed for each group (hence gaining degrees of freedom in the estimation of the residual variance) to assess the significance of the culture condition effect in an ANOVA model with 2 factors (spot and culture condition), with interaction. As the histogram of the 4525 P-values was acceptable with this procedure, the multiple testing procedure of Benjamini and Hochberg (Benjamini & Hochberg, 1995) (MULTTEST package v2.12) was applied to identify differentially expressed probes. A total of 580 spots corresponding to a false discovery rate lower than 5% was retained for further analysis. Post hoc comparisons were then performed using the Student’s t-test on differentially expressed genes to compare the responses of each pair of growth medium. All r packages were downloaded from the BIOCONDUCTOR v2.11 website (Gentleman et al., 2004).

Orthologous genes between Xcc strains B100, ATCC33913 and 8004 were obtained from KEGG (Kanehisa et al., 2012) using the KEGGSOAP interface v1.32 available on r using the bget function. Mapping to the BRITE hierarchy of the locus tags in Xcc strain 8004 was performed using the KEGG Mapper available on the KEGG website. This mapping was successful for 1793 unique genes. Among them, more than 500 genes mapped to two or more BRITE terms. However nonmapped but differentially expressed genes were always manually examined. Gene names, protein definitions and clusters of orthologous groups of proteins (COG) assignments were retrieved for Xcc strain 8004 from the integrated microbial genome website (Markowitz et al., 2012). Some useful information on IMG and KEGG modules, as well as Metacyc data, is available on this website (http://img.jgi.doe.gov/). Enrichment P-values were calculated by comparing the observed effective to a theoretical hypergeometric distribution using the phyper function. Clustering of microarray samples according the gene expression levels was performed with r using the ‘pvclust’ package. Bootstrap probabilities were calculated with 1000 permutations (Suzuki & Shimodaira, 2006).

Quantitative RT-PCR

For qRT-PCR experiments, cDNAs were synthesized from 1 µg of total RNA using the transcriptor reverse transcriptase (Roche), following the manufacturer’s recommendations. Quantitative PCRs were carried out using
SYBRGreen® Roche Reagents in a 10 µL final volume, including a final concentration of 125 nM of each primer and 1 M betaine as an enhancer for GC-rich templates [Xcc genome; (Henke et al., 1997)]. cDNAs were used at 10-fold dilution. Runs were performed on a 384-well block LC480 (Roche). Crossing thresholds (Ct) were calculated with the Light Cycler 480 sw 1.5 software. Gene expressions were normalized by a normalized factor calculated as the geometric mean of the raw expression levels of three reference genes (XC_0459, XC_1235, XC_3806) as recommended (Vandesomple et al., 2002). For in planta measurement of gene expression, normalization was achieved with XC_3806 (rpoD) alone because expression of the other reference genes could not be properly detected. Primer sequences for each gene are reported in supporting information, Table S1.

Results and discussion
Composition of XS of B. oleracea cv. Bartolo F1
Xs from 6- to 8-week-old cabbage B. oleracea cv. Bartolo F1 plants was recovered through root pressure-induced exudation after shoot decapitation (Ligat et al., 2011; Dugé de Bernonville et al., 2014). Average pH value of freshly sampled XS was 5.6 ± 0.2 (n = 3). GC-MS analysis indicated low concentrations of organic compounds (Fig. 1a). Total sugar content, including myo-inositol, arabinose, mannitol, glucose, fructose and xylose, did not exceed 1 mM. These values were comparable with glucose concentration of B. napus XS [c. 3 mM; (Ratzinger et al., 2009)]. Xylose (0.2 ± 0.1 mM) and mannose (0.24 ± 0.20 mM) were the most abundant sugars. Three carboxylic acids (succinic acid, citric acid and malic acid) were detected at a concentration of 1.2 ± 0.5 mM with malic acid being the most predominant (c. 1 mM). Similar carboxylate concentrations were reported in B. napus (Ratzinger et al., 2009). Free amino acids were quantitatively the most important compounds. Fourteen proteinogenous amino acids and one nonproteinogenous amino acid (norvaline) were detected. Glutamine concentration was the highest (19.5 ± 8.4 mM) as reported in the XS of various other plant genera (Andersen & Brodbbeck, 1989; Brodbbeck et al., 1990; Nakamura et al., 2008). Lysine, cysteine and aspartic acid concentrations were c. 2 mM and norvaline, isoleucine and leucine between 1 and 0.5 mM, and that of other detected amino acids were below 1 mM (threonine, tyrosine, asparagine, serine, methionine, histidine, glutamic acid and alanine). Considering the low protein abundance in the cabbage XS (Ligat et al., 2011), the other nonfree amino acids might only be present in the XS at very low concentrations.

Concerning XS mineral content, K, P and Ca were quantitatively the most important minerals, with concentrations ranging from 1.8 to 3 mM (Fig. 1a). Na and Mg concentrations were 0.5 and 1.1 mM respectively. In addition to these macro-elements, six micro-elements (Zn, Fe, Mo, Sr, Al and Mn) were identified at c. 5 µM in the cabbage XS (Fig. 1a).

Significant qualitative and quantitative differences of the amino acid composition and mineral content of XS, hrp-inducing MM (casamino acids 0.15 g L⁻¹, K₂HPO₄ 10.5 g L⁻¹, KH₂PO₄ 4.5 g L⁻¹, MgSO₄ 0.12 g L⁻¹, (NH₄)₂SO₄ 1 g L⁻¹) and MOKA RM (yeast extract 4 g L⁻¹, casamino acids 8 g L⁻¹, K₂HPO₄ 2 g L⁻¹, MgSO₄ 0.3 g L⁻¹) were observed. Glutamine is absent from MM because of its degradation from caseins during casamino acids production. In this MM, concentrations of aspartic acid, lysine and proline, were estimated about 50 µM (D’Huys et al., 2011) whereas the same compounds were abundant in XS. On the contrary, the yeast peptone used in the RM provides all of the proteinogenic amino acids, as well as some carbohydrates. Calcium concentration is expected to be more than 10 mM in the RM, according to the Ca content of yeast peptone. Potassium and phosphorus were much more concentrated in the two synthetic media as compared to XS, with more than 3 M of K and 100 mM of PO₄ in the RM, and c. 100 mM of K and PO₄ in the MM. Taken together, these data indicate that mineral and organic composition of cabbage XS display clear specificities compared to the two synthetic media, which might impact on Xcc fitness and virulence.

Growth of Xcc in cabbage XS
The growth of Xcc strain 8004 was comparable in the MM and XS media (Fig. 2a and b). Maximal growth rates (μmax) calculated using the colony-forming units (CFU) growth curves were of 0.35 and 0.30 generation h⁻¹, respectively. When cultivated in the RM, Xcc reached higher population densities more quickly (μmax = 0.47 generation h⁻¹). Interestingly, determination of cell density by absorbance showed a higher variability in XS than in the two other media (Fig. 2a). These measurements might be affected by the formation of precipitates as bacterial counts indicated that the cell population cultivated in the MM reached higher level than in XS (Fig. 2b compared to Fig. 2a). Importantly, XS did not flocculate on its own (Fig. 2a). Bacterial cell aggregation in XS could explain these observations.

To test for cell aggregation, biofilm formation in the different media without agitation was tested (Fig. 2c). Islands of aggregated bacteria were observed after 24 h of growth only at the surface of XS (Fig. 2c, see asterisks).
Similar observations were previously reported for the xylem-restricted bacterium *Xylella fastidiosa* (*Xf*). This bacterium grew well in the XS of its natural host (Bi *et al.*, 2007). Its cultivation into grapevine XS was associated with increased biofilm formation and attachment (Zaini *et al.*, 2009). This was probably due to the presence of calcium in grapevine XS ([c. 3 mM; (Andersen *et al.*, 2007)], whose concentration above 1 mM is able to trigger biofilm formation and cell adhesion of *Xf* in an *in vitro* medium (Cruz *et al.*, 2012). In our experiment, total calcium concentration is above 1 mM in both XS and RM. It is therefore unlikely that calcium alone is sufficient to trigger *Xcc* aggregation in XS.

To determine which nutrients are used preferentially to support *Xcc* growth in XS, XS composition was determined after 4 h of culture, at mid-exponential growth phase. On the whole, organic acids and sugars were metabolized the most efficiently. Concentrations in organic acids (succinate, malate and citrate) strongly decreased by 80% of the initial concentration (Fig. 1b). Malate utilization rate (0.2 ± 0.007 mM h⁻¹) was the highest among organic acids. Important utilization of organic acids such as malate was also observed for *Xcc* strain B100 using low molecular weight extracts of cabbage leaf homogenates as a growth medium (Watt *et al.*, 2009). Fructose, glucose and myo-inositol were found at low concentrations in XS (< 50 lM) and could not be detected after 4 h of *Xcc* growth, indicating they were fully assimilated. Content of other sugars was decreased to 60% of their initial concentrations. A differential assimilation of amino acids by *Xcc* was observed. Glutamine, cysteine, lysine, threonine and alanine were the most used by *Xcc* as their content decreased from more than 70% of their initial concentration and up to 100% for alanine. Glutamine was by far the quantitatively most assimilated amino acid with a consumption speed of...

![Fig. 1.](image-url) Organic and mineral content of *Brassica oleracea* cv. Bartolo F1 cabbage XS. (a). Average concentration of organic and mineral compounds of XS. (b). Organic compound utilization in cabbage XS after 4 h of growth of *Xcc* strain 8004. Data correspond to the percentage of the initial substrate amounts consumed by *Xcc*. Values are calculated as the mean of 3 (a) and 4 (b) independent biological repeats. Bars correspond to 95% confidence interval.

- **Fig. 1.** Organic and mineral content of *Brassica oleracea* cv. Bartolo F1 cabbage XS. (a). Average concentration of organic and mineral compounds of XS. (b). Organic compound utilization in cabbage XS after 4 h of growth of *Xcc* strain 8004. Data correspond to the percentage of the initial substrate amounts consumed by *Xcc*. Values are calculated as the mean of 3 (a) and 4 (b) independent biological repeats. Bars correspond to 95% confidence interval.
3.8 ± 0.4 mM h⁻¹ (Fig. 1b). Aspartic acid, cysteine, asparagine, tyrosine, glutamic acid, serine, methionine and histidine were moderately consumed (20–60% of their initial concentrations). Concentrations of norvaline, leucine and isoleucine remained unchanged during this period.

This analysis shows that XS is a suitable growth medium for Xcc which can rapidly capture most organic compounds of XS. Furthermore, in planta, XS is expected to be continuously renewed so that the growth of Xcc might be better supported than observed in XS in vitro.

Transcriptional reprogramming during growth of Xcc in XS

To gain insights into the physiological adaptation of Xcc to XS colonization, the transcriptome of Xcc strain 8004 grown in XS was monitored using cDNA microarrays based on the annotated open reading frames of Xcc strain B100 (Serrania et al., 2008) and compared to Xcc grown in RM or MM. These Xcc5kOLI microarrays were previously used to hybridize cDNA prepared from Xcc strain ATCC33913 (Bordes et al., 2011), a close relative of strain 8004 (Qian et al., 2005; Guy et al., 2013) justifying the use of these arrays for other strains than B100. Firstly, hierarchical clustering constructed over the 4525 probes indicated a good reproducibility as all the biological repeats inside one condition clustered together (Fig. S1a). Interestingly, the MM transcriptome (MMt) clustered with the RM transcriptome (RMt) rather than with the XS transcriptome (XSt), indicating that original responses are associated with the growth of Xcc in XS (Fig. S1a).

Differentially expressed genes were detected by a linear model approach (580 probes differentially expressed in comparison to the common reference) and post hoc mean comparison with a FDR-corrected P-value cut-off of 0.05. Of these 580 probes, 419 were differentially expressed between the XSt and the MMt, 443 between the XSt and the RMt and 294 between the MMt and the RMt (Tables 1 and S2). Validation of selected differentially expressed genes was performed by quantitative RT-PCR (qRT-PCR, Fig. S1b). A good correlation between the qRT-PCR and microarray dataset was obtained (0.67 < r² < 0.92). As often reported, some quantitative differences in gene expression levels between the two methods can be observed and likely account for the lower r² values of the comparisons XSt versus MMt and XSt versus RMt (Fig. S1b). Indeed, linearity between qPCR and microarray data is not always perfect as described [(Dallas et al., 2005) and reference herein], and the weaker the log ratio, the lower the correlation values. In addition, log ratios are frequently reported to be greater than those obtained by microarrays (Yuen et al., 2002),
Total differentially expressed 419 443 294 301
Log ratio
Log ratio
hierarchies were significantly enriched with genes weakly ‘lipopolysaccharide biosynthesis’ and ‘lipid biosynthesis’ proposed by the KEGG resource (http://www.kegg.jp/kegg/ries according to the BRITE hierarchical classification fold change threshold; Fig. 3, Tables 1 and S2). Among them, 204 genes could be assigned to functional categories according to the BRITE hierarchical classification proposed by the KEGG resource (http://www.kegg.jp/kegg/brite.html) which described ontology of gene function (Kanehisa et al., 2006) (Table 2). The 97 other genes encoded putative proteins or proteins of unknown function. Most of the differentially expressed genes in XS were grouped in the ‘transporters’ (39 genes) and ‘enzymes’ BRITE hierarchies (58 genes). We performed gene set enrichment tests to determine over-represented specific functions. The ‘transporters’, ‘amino acid-related enzymes’, ‘lipopolysaccharide biosynthesis’ and ‘transcription factors’ hierarchies were significantly up-regulated (hypergeometric testing, P < 0.05). Contrastingly, the ‘lipopolysaccharide biosynthesis’ and ‘lipid biosynthesis’ hierarchies were significantly enriched with genes weakly expressed in XS. The BRITE hierarchy ‘enzymes’ corresponds to broad protein functions, including an ‘enzyme’ term. We further annotated the 57 differentially expressed genes falling into this category using the classification of the prokaryotes COG (Tatusov et al., 2003) (Table 2). This classification is based on groups of orthologous genes thought to be involved in a same process and allowed a more precise identification of molecular functions represented in our dataset. The [G] (Carbohydrate transport and metabolism) and [C] (Energy production and conversion) classes were the most quantitatively represented in the XS-specific genes. There was a significant enrichment of the class [Q] (Secondary metabolites biosynthesis, transport and catabolism) among the more expressed genes in XS and of the class [V] (Defence mechanisms) among the genes less expressed in XS.

In the following sections, we will focus successively on several functional categories which may contribute to the physiological adaptation of Xcc to XS and the regulation of virulence.

Strong modifications in transporter expression patterns

As indicated above, the ‘transporter’ BRITE hierarchy is significantly enriched among the genes with high expression level in XS (Tables 2 and S3). Strongly expressed transporter genes in the XSt included eight TBDT-related genes, five ABC-like and four putative MFS. Concerning TBDTs, XC_0167 ortholog in Xcc strain ATCC33913 (XCC0158), was previously shown to be induced under iron deprivation conditions (Blanvillain et al., 2007) suggesting a role in iron-siderophore uptake. Although total concentration of iron in XS (4.73 ± 2.84 μM, Fig. 1a) should be sufficient for bacterial growth, the presence of iron chelators such as organic acids could decrease the concentration of free iron. Three of the XS-induced TBDT genes are localized in putative carbohydrate utilization loci containing TBDTs (CUT loci) involved in utilization of pectin/polygalacturonate (XC_2484) and two unknown carbohydrates (XC_1284 and XC_3063) (Blanvillain et al., 2007). Consistently, we observed the concomitant higher expression of genes related to carbohydrate degradation in the XSt: XC_0160 (5-keto-4-deoxyuronate isomerase), XC_3590 (pectate lyase), XC_1642 (α-glucosidase), XC_2481 (β-galactosidase) and

**Table 1.** Total number of differentially expressed genes in Xcc strain 8004 cultivated in cabbage XS, MM and RM (P < 0.05)

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<th>log2exp XSt vs. MMt</th>
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<td>223</td>
<td>229</td>
<td>151</td>
<td>168</td>
</tr>
<tr>
<td>Log ratio &lt; 0</td>
<td>196</td>
<td>214</td>
<td>143</td>
<td>133</td>
</tr>
<tr>
<td>Total differentially expressed</td>
<td>419</td>
<td>443</td>
<td>294</td>
<td>301</td>
</tr>
</tbody>
</table>

[TBDTs](https://www.biology.org/) consisting in putative carbohydrate utilization loci containing a TBDT ortholog in Xcc strain ATCC33913 (XCC0158), was previously shown to be induced under iron deprivation conditions (Blanvillain et al., 2007) suggesting a role in iron-siderophore uptake. Although total concentration of iron in XS (4.73 ± 2.84 μM, Fig. 1a) should be sufficient for bacterial growth, the presence of iron chelators such as organic acids could decrease the concentration of free iron. Three of the XS-induced TBDT genes are localized in putative carbohydrate utilization loci containing TBDTs (CUT loci) involved in utilization of pectin/polygalacturonate (XC_2484) and two unknown carbohydrates (XC_1284 and XC_3063) (Blanvillain et al., 2007). Consistently, we observed the concomitant higher expression of genes related to carbohydrate degradation in the XSt: XC_0160 (5-keto-4-deoxyuronate isomerase), XC_3590 (pectate lyase), XC_1642 (α-glucosidase), XC_2481 (β-galactosidase) and

![Venn diagram representing genes differentially expressed in XS from Brassica oleracea cv. Bartolo F1 as compared to MM and/or RM. Mo: more expressed; Le: less expressed.](https://www.biology.org/)

**Fig. 3.** Venn diagram representing genes differentially expressed in XS from Brassica oleracea cv. Bartolo F1 as compared to MM and/or RM. Mo: more expressed; Le: less expressed.

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This suggests the presence of complex carbohydrate in XS that were not detected here. Early induction of three Xcc TBDT genes, XC_0759, XC_1241 and XC_1619, was also observed in planta in midrib inoculated cabbage seedlings within 8 h postinoculation (Fig. 4a, b and c), reaching similar expression levels than those obtained in XS in vitro. As a control, transcripts of a TBDT gene not induced in XS (XC_0849) were not detected in planta. It is noteworthy that the X. axonopodis pv. citri (Xac) ortholog of XC_1241, XAC3050, was recently shown to accumulate in Xac biofilms (Zimaro et al., 2013). However, the involvement of XC_1241 in biofilm formation or maintenance has still to be elucidated.

Interestingly, expression of the citrate inner membrane transporter CitH (XC_0810) was probably induced by the presence of citrate in the cabbage XS (Fig. 1a). Its induction correlates with rapid metabolization of citrate by Xcc in XS (Fig. 1b).

**Metabolic processes up-regulated in XS**

Our microarray analysis revealed that 19 genes important for key metabolic pathways were highly expressed in the XSt (Table 3). Three pathways were particularly represented in this set, including ROS detoxification, amino acid and phenolic acid metabolisms.

Genes involved in the detoxification of reactive oxygen species (included in the COG functional class [P], see Table 2) encoding a superoxide dismutase (XC_0200) or catalases (XC_3037 and XC_4037) were more expressed in XS than in the two other media. As a control, transcripts of a TBDT gene not induced in XS (XC_0849) were not detected in planta. It is noteworthy that the X. axonopodis pv. citri (Xac) ortholog of XC_1241, XAC3050, was recently shown to accumulate in Xac biofilms (Zimaro et al., 2013). However, the involvement of XC_1241 in biofilm formation or maintenance has still to be elucidated.

Interestingly, expression of the citrate inner membrane transporter CitH (XC_0810) was probably induced by the presence of citrate in the cabbage XS (Fig. 1a). Its induction correlates with rapid metabolization of citrate by Xcc in XS (Fig. 1b).
Brown & Allen, 2004; Yang et al., 2004; Zhao et al., 2005).

Several genes related to phenolic acid metabolism were also found to be more expressed in the XSt than in the two other transcriptomes. Among those genes, pcaG (XC_0380), pcaC (XC_0383) and XC_3642 (fumarylacetoacetate hydrolase) may be involved in the generation of precursors of the tricarboxylate cycle to provide energy. This was in accordance with the important consumption of tyrosine during growth of Xcc in XS (Fig. 1b). Accordingly, the strong accumulation of XC_1753 and XC_3751, both encoding probable isochorismatases, indicated the reinforcement of aromatic compound metabolism in XS. Such particular metabolism has been reported to be activated in the presence of monophenolic compounds (Peters & Verma, 1990), in epiphytic populations of P. syringae pv. syringae colonizing bean leaves (Yu et al., 2013), in R. solanacearum during colonization of tomato xylem vessels (Jacobs et al., 2013) and in P. aeruginosa incubated with sugar beet root exudates (Mark et al., 2005). This ability to metabolize phenolic substrates might be mandatory for bacterial microorganisms to successfully colonize plant niches.

![Fig. 4. Expression of Xcc strain 8004 genes in vitro and in planta was analysed by qRT-PCR: XC_0759 (a), XC_1241 (b) and XC_1619 (c) encode TBDTs and XC_3021 (d) encodes HrpE. For the in planta analysis, infected tissues were sampled 0, 4, 8 and 12 h postinoculation (hpi). Data are normalized to the expression of rpoD. Asterisks indicate significant differences with the expression level obtained during growth in RM (Wilcoxon rank sum test, *P < 0.05; **P < 0.01; ***P < 0.001).](https://academic.oup.com/femsec/article-abstract/89/3/527/2680435)

Table 3. Metabolic features of Xcc strain 8004 transcriptionally activated in cabbage XS

<table>
<thead>
<tr>
<th>Category</th>
<th>Locus tag</th>
<th>Gene name</th>
<th>log2exp XS vs. MM</th>
<th>log2exp XS vs. RM</th>
<th>Predicted function</th>
<th>Ortholog in B100</th>
<th>Ortholog in ATCC33913</th>
</tr>
</thead>
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<tr>
<td>ROS detox</td>
<td>XC_0200</td>
<td>sod1</td>
<td>0.45</td>
<td>0.38</td>
<td>Exported superoxide dismutase</td>
<td>xccb100_0211</td>
<td>XCC0190</td>
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<tr>
<td></td>
<td>XC_3037</td>
<td>cat1</td>
<td>0.57</td>
<td>0.68</td>
<td>Catalase</td>
<td>xccb100_0314</td>
<td>XCC1205</td>
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<tr>
<td></td>
<td>XC_4037</td>
<td>cat3</td>
<td>1.18</td>
<td>1.52</td>
<td>Exported catalase</td>
<td>xccb100_4136</td>
<td>XCC3949</td>
</tr>
<tr>
<td>Aromatic compound metabolism</td>
<td>XC_0380</td>
<td>pcaG</td>
<td>0.47</td>
<td>0.68</td>
<td>Protocatechuate 3,4-dioxygenase alpha chain</td>
<td>xccb100_0394</td>
<td>XCC0368</td>
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<tr>
<td></td>
<td>XC_0383</td>
<td>pcaC</td>
<td>0.64</td>
<td>0.92</td>
<td>4-carboxymuconolactone decarboxylase</td>
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<tr>
<td></td>
<td>XC_1268</td>
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<td>0.84</td>
<td>0.92</td>
<td>3-dehydroquinate synthase</td>
<td>xccb100_1314</td>
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<tr>
<td></td>
<td>XC_1753</td>
<td></td>
<td>0.47</td>
<td>0.36</td>
<td>Isochorismatase-like protein</td>
<td>xccb100_1809</td>
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<tr>
<td></td>
<td>XC_3751</td>
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<td>0.51</td>
<td>0.71</td>
<td>Putative isochorismatase</td>
<td>xccb100_3866</td>
<td>XCC3680</td>
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<tr>
<td>Cofactor biosynthesis</td>
<td>XC_1098</td>
<td>cobU</td>
<td>0.39</td>
<td>0.41</td>
<td>Bifunctional adenosylcobalamin biosynthesis protein</td>
<td>xccb100_1135</td>
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<td>Cobalamin (5'-phosphate) synthase</td>
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<tr>
<td>Amino acid metabolism</td>
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<td>0.36</td>
<td>0.71</td>
<td>Zinc metalloprotease</td>
<td>xccb100_0084</td>
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<tr>
<td></td>
<td>XC_0636</td>
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<td>XC_1356</td>
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<td>0.7</td>
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<td>1.26</td>
<td>1.13</td>
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<td>XCC0851</td>
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<td></td>
<td>XC_3642</td>
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<td>1.24</td>
<td>0.86</td>
<td>Fumarylacetoacetate hydrolase family protein</td>
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<td>XC_3986</td>
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<td>Protease DO</td>
<td>xccb100_4087</td>
<td>XCC3898</td>
</tr>
</tbody>
</table>

Expression values correspond to means of log2 ratios between expression levels in XS and MMt or XS and RMt.
Regulation of virulence in XS

The XSt was also characterized by specific expression of genes involved in regulating virulence (Table 4). In particular, three different expression profiles of transcription factors were obtained: (1) Six transcription factor encoding genes were specifically more expressed in XS than in RM or MM; (2) three were more expressed in RM than in XS but more in XS than in MM; and (3) six were specifically less expressed in XS than in RM or MM. The probe corresponding to a SIR2-like protein encoding gene (Xccb100_4409) which belongs to the first expression profile described above was strongly expressed in Xcc. This region corresponds to a previously nonannotated gene and was recently renamed Xcc_CDS002 using microarray-based gene re-annotation (Zhou et al., 2011). A mutant strain of Xcc strain 8004 deficient for Xcc_CDS002 displayed a strongly altered virulence phenotype on Chinese cabbage (Zhou et al., 2011). XrvA (Xc_1046) was more expressed in XS than in MM, but less than in RM. The Xoo XrvA protein was reported to regulate the expression of several virulence genes such as hrpG, hrpX and the rpf genes and is required for full virulence on rice (Feng et al., 2009). Interestingly, the growth of Xf in susceptible grape XS was similarly associated with a high expression of XrvA (Shi et al., 2010, 2012). These data suggest that XS could serve as an important signal to induce expression of virulence genes important for optimal colonization of plant xylem vessels in the early infection steps.

Table 4. Xcc strain 8004 genes with differential expression within the cabbage XS involved in regulatory, motility and virulence processes

<table>
<thead>
<tr>
<th>Category</th>
<th>Locus tag</th>
<th>Gene name</th>
<th>log2exp XS vs. MM</th>
<th>log2exp XS vs. RM</th>
<th>Predicted function</th>
<th>Ortholog in XccB100</th>
<th>Ortholog in ATCC3913</th>
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</thead>
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<tr>
<td>Transcription Factors</td>
<td>XC_0044</td>
<td>nppA</td>
<td>−0.55</td>
<td>−0.63</td>
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<td>xccb100_0048</td>
<td>XCC0044</td>
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<tr>
<td></td>
<td>XC_0318</td>
<td>nppA</td>
<td>−0.55</td>
<td>−0.63</td>
<td>NAD-dependent deacetylase</td>
<td>xccb100_0331</td>
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<tr>
<td></td>
<td>XC_0571</td>
<td>hrpG</td>
<td>0.44</td>
<td>0.55</td>
<td>Transcriptional regulator</td>
<td>xccb100_0588</td>
<td>XCC3608</td>
</tr>
<tr>
<td></td>
<td>XC_0808</td>
<td>hrpX</td>
<td>−0.62</td>
<td>−0.19</td>
<td>Sucrose utilization transcriptional regulator</td>
<td>xccb100_0841</td>
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<tr>
<td></td>
<td>XC_1046</td>
<td>xrvA</td>
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<td>xccb100_1076</td>
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<tr>
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<td>xccb100_1326</td>
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<tr>
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<tr>
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<td>XC_2269</td>
<td>xva</td>
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<td></td>
<td>XC_1409</td>
<td>cheB2</td>
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<td></td>
<td>XC_2160</td>
<td>yapH</td>
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<td>0.06</td>
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</table>

Expression values correspond to means of log2 ratios between expression levels in XS and MM or XS and RM. ns, not significant; na, not available.
Concerning the T3S system-related genes, a higher expression of several hrp-related genes in MM than in RM, was observed for \(XC_{3010}\) (hrpB2), \(XC_{3014}\) (hpaC), \(XC_{3021}\) (hrpE) and \(XC_{3177}\) (xopQ) as previously described (Arlat et al., 1991; Wengelnik & Bonas, 1996) (Table 4). In addition, three genes (\(XC_{0077}\), \(XC_{0241}\) and \(XC_{0634}\)) that were shown to be positively regulated by either HrpG and/or HrpX in Xac (Guo et al., 2011), did also display a higher expression level in MM (Fig. S1). Our results suggest that expression of hrp genes is weakly/partially activated during growth of \(Xcc\) in XS, because these genes accumulated more in the MMt than in the XSt (Table 4). However, with the exception of \(XC_{3005}\) (hrpB7) which was much less expressed in XS than in RM (ratio = –0.38), two other T3S-related genes were more expressed in XS than in RM, as indicated by the expression of hrpE (\(XC_{3021}\), Fig. S1), thus suggesting that XS was better to induce the T3S system than RM. hrpE expression was stronger \textit{in planta} than in XS (Fig. 4d). Hence, full hrp gene induction in Xcc cannot be fully explained by the XS composition alone and requires additional plant-derived signals. A recently published RNA-seq study on \textit{in vitro} grown \(Xcc\) identified 21 genes which promoter regions contain active PIP-boxes (Liu et al., 2013). Among these, 4 genes (\(XC_{1241}\), \(XC_{2929}\), \(XC_{3299}\) and \(XC_{3379}\)) were also highly expressed in XS.

**Regulation of motility in XS**

Several motility-related genes were found to have a higher expression in XS (Table 4). These included twitching motility [\(XC_{0939}\) (pilO), \(XC_{1183}\) (pilG) and \(XC_{2317}\) (pilZ)] and flagellar-driven motility [\(XC_{2264}\) (flip), \(XC_{2265}\) (flk) and \(XC_{2266}\) (flh)]. Interestingly, both types of motility are required for biofilm formation in \(P.\ aeruginosa\) (O’Toole & Kolter, 1998), and this could also be the case for \(Xcc\). Culture of Xcc in XS indeed triggered strong adhesion of bacterial cells, leaving the planktonic population largely outnumbered by the aggregated one (Fig. 5a and b). This aggregation was much more important than in the two synthetic media. The YapH gene product (\(XC_{2160}\)), a hemagglutinin previously shown to be an important factor in cell adhesion in \(X.\ fuscans\) ssp. \(fuscans\) (Darsonval et al., 2009), was more expressed in the XSt in comparison with the MMt. Besides, a faster spread of the bacteria inside the semi-solid agar plate was also visible for the XS and RM media (Fig. 5c). Taken together, these data suggest that two bacterial populations might occur during colonization of the cabbage XS, a strongly aggregated one and a planktonic one. This specific behaviour could be due to the ability of \(Xcc\) to sense XS components, as suggested by a higher expression level for three chemosensor genes, \(cheB2\) (\(XC_{1409}\)) and 2 \(cheW\)-like (\(XC_{2318}\) and \(XC_{2300}\)) in the XSt. Regulation of this phenotype may involve \(XC_{2317}\) (pilZ) which positively regulates the production of extracellular enzymes, including proteases and glucanases (McCarthy et al., 2008); this is in good agreement with the higher expression of glucanases (see above) and several proteases in the XSt (Table 3). However, \(Xcc\) mutant strains lacking functional \(XC_{2317}\) are not altered in their pathogenicity (McCarthy et al., 2008). The comprehensive monitoring of formation kinetics of the two \(Xcc\) populations could reveal a role in controlling disease progression as proposed (Torres et al., 2007). Indeed, a balance between formation of microcolonies and
population spread inside the plant vessels may help avoiding plant defences while ensuring local enrichment in plant-derived nutrients.

Conclusions

In this study, we demonstrate that cabbage XS does not only allow similar growth rates as in MM, but also induces specific transcriptional responses not observed in MM. This is probably due to the particular composition of XS, including the presence of a low amount of plant-derived proteins. Our data indicate that several functions such as transporter rearrangement, motility and aggregation are part of the early processes involved in the colonization of the host plant by Xcc. XS thus represents an important tool to study in vitro early infection and may help to identify new determinants in Xcc required for a successful colonization of its hosts.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Fig. S1. Analysis of the transcriptome of Xanthomonas campestris pv. campestris strain 8004 grown in cabbage xylem sap (XS), minimal medium (MM) and rich medium (RM).
Table S1. Primer sequences used for quantitative RT-PCR amplification of selected genes.
Table S2. Differentially expressed genes in Xanthomonas campestris pv. campestris strain 8004 for each comparison.
Table S3. Differentially expressed genes belonging in the ‘transport’ BRITE hierarchy in Xanthomonas campestris pv. campestris strain 8004 for each comparison.