Role of QseG membrane protein in beneficial enterobacterial interactions with plants and Mesorhizobium

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ABSTRACT
Homologs of qseG gene (coding for the membrane protein QseG), along with the qseEF genes, are present in many Enterobacteriaceae; however, its role in non-pathogenic strains is still unknown. To fill this knowledge gap, we investigated the role of QseG protein of a plant-associated enterobacterium in the interactions with its legume host and in the benefits induced by this enterobacterium in the Mesorhizobium–chickpea symbiosis. Here, we showed that QseG of Kosakonia sp. MH5 is involved in the following processes: (i) the evasion of the plant immune system and (ii) the efficient colonization of chickpea root cells. Furthermore, these features are essential for the beneficial effects of this strain on the Mesorhizobium–chickpea symbiosis. This study demonstrates that the role of QseG is transversal to pathogenic and non-pathogenic enterobacteria and is a step forward to better understanding the molecular bases of plant–bacteria interactions established between legume and beneficial endophytic enterobacteria.

Introduction

Enterobacteriaceae is the largest family of the order Enterobacterales and contains many relevant genera and species, including the model animal pathogenic bacteria Escherichia coli (Adeolu et al. 2016) and also, species described as plant-associated bacteria, such as the ones within the genera Enterobacter, Phytobacter, and Kosakonia (Brígido, Menéndez et al. 2019; Brígido, Singh et al. 2019; Jha et al. 2011; Madhaiyan et al. 2020), amongst others. However, these genera also harbor strains with pathogenic potential for plants (García-González et al. 2018) and for humans (Mertschning et al. 2020; Pillonetto et al. 2018; Salimiyan Rizi et al. 2020), revealing the thin line between pathogenic and mutualistic relationships within this family.

Regardless of the taxonomic and phenotypic diversity within the Enterobacteriaceae family, most of our knowledge of the quorum-sensing (QS) systems in this family comes from the E. coli model. QS is the ability of bacterial cells to regulate their gene expression according to population growth. This mechanism affects the behavior of bacterial groups, and it is involved in the regulation of traits relevant for the outcome of the interactions between bacteria, either pathogenic or beneficial, and their plant host cells. For example, the QS system of different plant-associated bacteria coordinate biofilm formation, virulence factors expression, and bacteria motility (Kai 2018; Loh et al. 2002; van Loon et al. 2008). QS is subject to regulatory cascades, especially two-component systems that often respond to environmental stimulation. In E. coli, the QS regulator proteins E and F (QseEF, also known as GlrKR or YfhKA) are a two-component system involved in the regulation of virulence, metabolism, and cell envelope homeostasis of this bacteria (Reading et al. 2010; Reading et al. 2007; Reichenbach et al. 2009).

Interestingly, genes coding for the two-component system QseEF are co-transcribed in an operon with QseG (also known as YfhG), an outer membrane protein showing homology with α-helical proteins (Reading et al. 2009; Reading et al. 2007). Recently, it was shown that QseG is indispensable for QseF activity in E. coli K-12. QseE was incompetent to phosphorylate/activate the response regulator QseF in the E. coli K-12 ΔqseG mutants (Göpel and Görke 2018). Like the co-transcribed proteins QseE and QseF, QseG also plays a role in regulating the virulence of enteric pathogens (Cameron et al. 2018; Reading et al. 2009; Xiao et al. 2012). For instance, mutation in this gene severely affects the survival of the fish pathogen Edwardsiella tarda in murine macrophages as well as its virulence towards zebra fish (Xiao et al. 2012). Similarly, enterohemorrhagic E. coli (EHEC) ΔqseG mutants show lower capacity to colonize the rabbit intestinal cells comparing with the wild-type (WT) strain (Cameron et al. 2018). Although QseG is important for the virulence of different intestinal pathogenic bacteria, its mechanisms of action differ according to bacteria species. For instance, studies point out a role of QseG in the regulation of the type III secretion system function in EHEC (Cameron et al. 2018; Reading et al. 2009). On the other hand, in Salmonella enterica serovar Typhimurium, QseG have been associated to flagella phase variation and, consequently, to regulate flagellin recognition. Moreover, its deletion confers less virulence and reduced intestinal colonization in an infant rabbit model than the WT strain (Cameron et al. 2018).

Despite the functional characterization of the qseEGF operon in a few genera of intestinal pathogenic bacteria
belonging to the Enterobacteriaceae and Hafniaeae families (Cameron et al. 2018; Göpel and Görke 2018; Reading et al. 2010; Reading et al. 2009; Xiao et al. 2012), its role in non-pathogenic strains, including plant-associated enterobacteria, whose genome contains putative qseEGF operon orthologs (Göpel et al. 2011), is still unknown. Therefore, the characterization of the QseG protein of a plant-associated enterobacterial bacterium presents an opportunity to probe the molecular bases of the plant–bacteria interaction and to better understand the functionally of this protein within Enterobacteriaceae family, whose interaction with their hosts varies from pathogenic to beneficial.

In this work, we carried out several experiments to investigate the role of QseG of Kosakonia sp. MH5 on the symbiotic Mesorhizobium ciceri LMS-1–chickpea (Cicer arietinum L.) association. MH5 strain is an endophytic bacterium isolated from the chickpea roots that exhibits various in vitro plant growth-promoting mechanisms (Brígido, Singh et al. 2019) and promotes chickpea growth under salinity when co-inoculated with LMS-1 in planta assays (Brígido, Menédez et al. 2019). Our work presents evidence that the QseG of MH5 strain maintains its function by positively interfering in the host of colonization and infection processes in a similar way to that is observed in Enterobacteriaceae pathogens in animals. Furthermore, invasion and a proper establishment within the roots and/or root nodules are essential for this strain to exert beneficial effects on the symbiotic chickpea–Mesorhizobium association under salinity.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. The rhizobial strain, Mesorhizobium ciceri LMS-1, was grown in tryptone yeast medium (Beringer 1974), at 28°C for routine use as described in (Brígido et al. 2012). The strain Kosakonia sp. MH5 and its derivatives, ΔqseG mutant and complemented strains, were grown in tryptic soy broth (TSB, Liofilchem) or tryptic soy agar (TSA, Liofilchem) medium at 28°C. The Escherichia coli strains were grown in Luria–Bertani (LB) broth (Sambrook and Russell 2001), at 37°C. To allow the growth of the conjugation strain E. coli DH5α-pir competent cells. Then, the plasmids were purified from positive clones and cloned into E. coli WM3064. The pNPTS138-R6KT-ΔqseG was mobilized into the MH5 cells by biparental mating. Double recombinants were selected as previously described (Ekandjo et al. 2017). Gene knockout was confirmed by PCR in kanamycin-sensitive colonies using the flanking primers, as well as an internal primer pair (Table S2).

To complement the in-frame deletion of the qseG gene, replacement of the deletion qseG gene by the complete qseG gene in its original genomic locaton was performed. For this, a DNA fragment, containing the complete qseG gene and part of its flanking regions, was amplified from the genomic DNA of MH5 WT strain using the flanking primers pair (Table S2). The PCR program was as follows: 95°C for 2 min followed by 30 cycles of 95°C for 20 s, 58°C for 10 s, and 70°C for 15 s. To fuse the qseG flanking regions, another PCR reaction using a mixture of upstream and downstream PCR products as DNA template and the external primers set (Table S2) was performed under the following program conditions: 95°C for 2 min, 30 cycles of 95°C for 20 s, 68°C for 10 s, and 70°C for 36 s.

The amplified PCR product was isolated and purified from an agarose gel and cloned in pNZY28-blunt vector (NZYtech, Lisbon, Portugal). The fused-flanking region of the qseG gene was then subcloned into the EcoRV sites of the suicide plasmid pNPTS138-R6KT (Lassak et al. 2010) and transformed into E. coli DH5α-pir competent cells. Then, the plasmids were purified from positive clones and cloned into E. coli WM3064. The pNPTS138-R6KT-ΔqseG was mobilized into the MH5 cells by biparental mating. Double recombinants were selected as previously described (Ekandjo et al. 2017). Gene knockout was confirmed by PCR in kanamycin-sensitive colonies using the flanking primers, as well as an internal primer pair (Table S2).

DNA methods and construction of Kosakonia sp. MH5 derivatives

The extraction of bacterial genomic DNA was conducted using the E.Z.N.A. bacterial DNA kit (Omega Bio-Tek, Norcross, GA, USA), and the plasmid DNA isolation was performed using the Zymplasm Miniprep kit (Zymo Research, Freiburg, Germany). PCR products and gel bands were purified using the kit DNA Clean & Concentrator or Zymoclean™ Gel DNA Recovery kits (Zymo Research, Freiburg, Germany). Unless otherwise specified, molecular techniques were performed using standard protocols (Sambrook and Russell 2001). The primers used in this study are listed in Table S2.

In vitro phenotypic characterization of the ΔqseG mutant strain

Acyl-homoserine lactones production

To determine the range of acyl-homoserine lactones (AHLs) produced by MH5 WT and its derivatives, two biosensor
strains able to detect long or short chain AHL were used. The transformed biosensor strain *A. tumefaciens* NTL4(pZRL4) was used for long side-chain AHLS (C8-HSL-C14-HSL) detection while strain *C. violaceum* CV026 was used for short side-chain AHLS detection (C4-HSL-C8-HSL). The reporter strains and the MH5 WT and derivatives strains were grown overnight at 28°C in their respective culture medium. For short chain AHL assay, 50 μL of the cell suspension containing the endophytic bacterium (OD565nm = 1) were spread onto LB agar plates previously supplemented with 50 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, Sigma-Aldrich). The plates were dried, inoculated with four droplets of 3 μL of overnight culture of NTL4 (pZRL4) and incubated for 48 h at 28°C. For long chain AHL assay, each bacterial endophyte was streaked approximately 1 cm apart from the biosensor strain CV026 onto LB agar plates and incubated at 28°C for 96 h.

Swarming motility assay

To evaluate motility of the ΔqseG mutant strain and compared it with the WT strain, the swarming motility of each strain was evaluated in plates containing TSB supplemented with 0.5% (w/v) agar. Five microliters of a bacterial suspension grown overnight in TSB medium with an OD565nm of 2 were spotted in the center of the plates. Plates were incubated at 28°C for 14 days, and differences in motility were determined by the rate of radial expansion from the inoculation spot. Five replicas per treatment were performed.

Biofilm formation

Biofilm formation on microtiter PVC plates was performed according to (O’Toole 2011), with few modifications. Briefly, strains were grown in TSB medium at 28°C. After overnight incubation, the OD600nm of the cultures were adjusted to 0.1 in M9 broth medium (Duan et al. 2009). One hundred microliters of bacterial suspension grown overnight in TSB medium with an OD565nm of 2 were spotted in the center of the plates. Plates were incubated at 28°C for 2797, Massachusetts, USA). The plates were sealed and incubated at 28°C for 96 h. After growth, MH5 WT and derivatives were prepared as described by Brígido, Menéndez et al. (2019). Surface sterilization and pre-germination of the chickpea seeds were carried out as earlier described (Paço et al. 2016). The setup of the pots trial was performed as following a similar protocol to that described by Brígido, Menéndez et al. (2019) with the exception that the bacterial suspensions used to inoculate each germinated seedling contained a final OD565nm of 1 of each bacterium. A nitrogen-free nutrient solution (Broughton and Dilworth 1971) was used to water the plants three times a week. To induce salinity stress, 0.075% of NaCl was added to nitrogen-free nutrient solution after the second week of plants growth until the end of the experiment. Four treatment combinations were prepared as follows: (i) chickpea single inoculated with LMS-1; (ii) chickpea co-inoculated with LMS-1 and MH5 WT; (iii) chickpea co-inoculated with LMS-1 and ΔqseG mutant strain; and (iv) chickpea co-inoculated with LMS-1 and ΔqseG-KI strain. Five pots per treatment were used. The plants were harvested after 6 weeks, and the number of nodules, nodules dry weight, root dry weight, and shoot dry weight (SDW) were registered.

Nodule histology

Chickpea nodules were excised from roots of 6-week-old plants and processed for microscopy as previously described (da-Silva et al. 2019). A pool of nodules from at least three representative plants from each treatment (chickpea single inoculated with LMS-1 or co-inoculated with LMS-1 and MH5 WT or co-inoculated with LMS-1 and ΔqseG mutant strains) were processed for microtome sectioning. Toluidine blue-stained cross sections of nodules were examined by brightfield microscopy under a Leica DM6000B microscope.

Gene expression analyses by qRT-PCR

RNA extraction

Expression levels of specific genes in the MH5 WT and ΔqseG mutant strain were evaluated during the early stages of interaction with the legume host. The strains were measured by gene expression analyses as follows: (i) chickpea single inoculated with LMS-1; (ii) chickpea co-inoculated with LMS-1 and MH5 WT; (iii) chickpea co-inoculated with LMS-1 and ΔqseG mutant strain; and (iv) chickpea co-inoculated with LMS-1 and ΔqseG-KI strain. Five pots per treatment were used. The plants were harvested after 6 weeks, and the number of nodules, nodules dry weight, root dry weight, and shoot dry weight (SDW) were registered.

Tolerance to salinity and manganese toxicity

To evaluate the ΔqseG mutant strain tolerance to salinity and manganese and compared it with the WT strain, bacterial growth was determined by measuring the OD565nm during 28 h of growth in the liquid medium. For all treatments, bacterial cultures with an initial OD565nm of 0.1 were prepared after an overnight growth in TSB. After growth, MH5 WT and derivative strains were grown in M9 broth medium supplemented with 1% (w/v) NaCl or 1 mM manganese as stress conditions and grown in regular M9 broth medium as control condition. Three replicas per treatment were performed.

Effect of the ΔqseG mutant strain on the mesorhizobia–legume symbiosis

Pot experiment setup

To evaluate the effects of ΔqseG mutant strain on the rhizobium–legume symbiosis, a plant growth assay was conducted using the symbiotic chickpea–Mesorhizobium model under salinity conditions in a growth chamber. The well-characterized *M. ciceri* LMS-1 was used as rhizobial microsymbiont and was prepared as previously described by Brígido et al. (2013) while the MH5 WT and derivatives were prepared as described by Brígido, Menéndez et al. (2019).

Surface sterilization and pre-germination of the chickpea seeds were carried out as earlier described (Paço et al. 2016). The setup of the pots trial was performed as following a similar protocol to that described by Brígido, Menéndez et al. (2019) with the exception that the bacterial suspensions used to inoculate each germinated seedling contained a final OD565nm of 1 of each bacterium. A nitrogen-free nutrient solution (Broughton and Dilworth 1971) was used to water the plants three times a week. To induce salinity stress, 0.075% of NaCl was added to nitrogen-free nutrient solution after the second week of plants growth until the end of the experiment. Four treatment combinations were prepared as follows: (i) chickpea single inoculated with LMS-1; (ii) chickpea co-inoculated with LMS-1 and MH5 WT; (iii) chickpea co-inoculated with LMS-1 and ΔqseG mutant strain; and (iv) chickpea co-inoculated with LMS-1 and ΔqseG-KI strain. Five pots per treatment were used. The plants were harvested after 6 weeks, and the number of nodules, nodules dry weight, root dry weight, and shoot dry weight (SDW) were registered.

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grown in a mixture of TSB medium and chickpea root exudates (1:1, V/V) at 28°C for 24 h. Chickpea root exudates were previously obtained as described by Srivastava et al. (1999). To ensure a complete break of bacterial cell walls, the cells were mechanically lysed, and the total bacterial RNA was extracted using the Absolutely RNA MiniPrep Kit (Agilent Technologies, Santa Clara, USA). Three biological replicates of each strain were used.

To evaluate changes in chickpea response to inoculation with MH5 WT or ΔqseG mutant strain, a plant growth assay was performed as described previously. Chickpea seedlings were co-inoculated with LMS-1 and MH5 WT or co-inoculated with LMS-1 and ΔqseG mutant strain. Chickpea seedlings single inoculated with LMS-1 were used as control. The expression of plant defense-response-related genes in roots was assessed 24 h after bacterial inoculation. The entire root system was gently and thoroughly washed with distilled water. Then, roots were separated from stem, frozen in liquid nitrogen, and ground to a fine powder using mortar and pestle and stored at −80°C for further analysis.

Three seedlings were pooled per replicate and three biological replicates were used per treatment. Total RNA of chickpea seedlings was extracted using the kit Maxwell® 16 LEV simplyRNA Tissue Kit (Promega, Madison, USA).

**RNA analysis and cDNA synthesis**

The concentration and purity of the RNA extracted from both bacteria and plants were evaluated using a NanoDrop 800 UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The RNA integrity was checked on 1% agarose gel electrophoresis. Approximately, 2 μg of total RNA from bacteria and 1 μg of total RNA from chickpea seedlings were subjected to reverse transcription for cDNA synthesis using the NZYtech First-strand cDNA synthesis kit (NZYtech, Lisbon, Portugal).

**qRT-PCR**

To evaluate changes in the expression levels of genes related to plant–microbe interactions, the expression of ompA, tagI, and virK genes were evaluated in the MH5 WT and ΔqseG mutant strains after exposition to chickpea root exudates. On the other hand, gene expression profile of genes related to plant defense-response, namely CaRBOH-like, CaPR1, and CaFLS2, were assessed in chickpea seedlings co-inoculated with LMS-1 and either with MH5 WT or ΔqseG mutant strain. The primers for these target genes were designed using the Primer-Blast NCBI tool (Ye et al. 2012). The 16S rRNA gene was used to normalize the expression of the bacterial target genes while Ca18SrRNA and CaGAPDH genes were used to normalize the expression of the chickpea target genes. All primers used herein are listed in Table S2. Primer pairs were tested on cDNA samples to ensure amplification of the target region. The specificity of amplification reactions was verified by a melting curve analysis performed at 60–95°C. qRT-PCR was conducted in a 96-well plate format on a 7500 real-time PCR detection system (Applied Biosystems) using the Green Master Mix (2×) ROX kit (Nzytech). The cDNA used was diluted 5× with Dnase/Rnase free water and used 800 ng in a reaction volume of 20 μL and performed as outlined by the manufacturer (Applied Biosystems). For each gene, three technical replicates of three biological replicates were performed. No template controls to detect DNA contamination or primer dimers were also performed. Expression levels for the target were determined by the 2^ΔΔCt method, where ΔCt is the difference between the Ct of a target gene and the Ct of the reference gene, and transcript levels were calculated and normalized according to Willems et al. (2008).

**Evaluation of root colonization ability by MH5 WT and ΔqseG mutant strain**

**Colony-forming unit quantification of bacterial cells in different rhizocompartments**

The standard dilution plating method was used to evaluate the ability of root colonizing by MH5 WT and ΔqseG mutant strain. For that purpose, a plant growth assay with chickpea seedlings co-inoculated with LMS-1 and MH5 WT or ΔqseG mutant strain was performed as described previously. The roots sampling was carried out at 24, 48, and 72 h after seedlings bacterial inoculation. After thoroughly washing the roots with sterile water, they were cut transversally into two sections of equal size and the seed-proximal section was kept. For each treatment, roots from a pool of three plants were harvested. To obtain the bacteria attached to rhizoplane, the root sections were submerged in 3X Ringer’s solution (Surette et al. 2003) and vortexed for 2 min at max velocity, and then bacterial cells were recovered by centrifugation and then suspended in 3X Ringer’s solution. To obtain the bacteria within root cells, the root sections were surface-disinfected as described in Rashid et al. (2012) and crushed with a sterile pestle in 3X Ringer’s solution. Serial dilutions of the bacteria suspension were prepared and 100 μL of each dilution was plated in TSA plates supplemented with 20 μg/mL of ampicillin. Plates were incubated at 28°C for 48 h. Three biological replicates with three plants each were performed.

**Fluorescence microscopy**

To visualize chickpea root colonization, GFP-tagged MH5 WT and GFP-tagged ΔqseG mutant strains were used to inoculated surface-disinfected and pregerminated chickpea seeds. Briefly, surface-disinfected and pregerminated chickpea seedlings were transferred to a 0.75% water-agar plate (square petri dish, 140 × 140 mm) and inoculated with 400 μL/plant of each of the bacterial suspensions at OD600 of 0.4 prepared as described above. Plants were overlaid with double filter paper and incubated in a growth chamber for 5 days. Then, plant roots were excised and carefully removed from the plates to a microscope slide. Roots were visualized using a confocal laser scanning microscope (Leica TCS SPE). For each treatment, at least 2 plates of 3 seedlings/each were observed plus an uninoculated control (chickpea seeds without bacterial inoculum).

**Bioinformatics analyses**

Analyses of the gene synteny of the qseEGF region in Enterobacteriales were performed using the MicrobesOnline tool (Dehal et al. 2010) accessed in November 2020 and NCBI database. For this, sequences of one representative species of each of the seven Enterobacteriales families (Adeolu et al. 2016), whose genome sequences were available in MicrobesOnline website, were chosen and compared with MH5 WT. The representative species were as follows: E. coli str. K-12 substr. MG1655 (Enterobacteriaceae), Erwinia
amylvora CFBP1430 (Erwiniaceae), Pectobacterium carotovorum subsp. carotovorum PC1 (Pectobacteriaceae), Versinia enterocolitica subsp. enterocollitica 8081 (Versiniaceae), Edwardsiella tarda EIB202 (Hafniaceae), Proteus mirabilis HI4320 (Morganellaceae), and Pragia sp. CF-458 (Budviciaceae).

Statistical analysis

Data were analyzed using either Student’s t-test or one-way ANOVA. The Duncan test was used to detect significant differences between the treatments means (P<0.05). Statistical analysis was carried out using SPSS V.21 software (SPSS Inc.).

Results

qseEGF operon is conserved in Enterobacterales

The qseEGF operon is annotated in the genome of several enterobacterial species (Göpel et al. 2011) and it is relatively well-characterized in a few enteric pathogens. To check whether the gene architecture of this operon is conserved in Enterobacterales, we analyzed the gene synteny of qseEGF region in representative species of the seven families of this order. The genomic analysis revealed that qseEGF operon was conserved in this order (Figure S1). The putative qseEGF operon (Locus tag: DK872_RS06570-RS06580) of MH5 WT was localized downstream the small RNA GlmY (NZ_QODH0100002: 32386-32570) and upstream to the gene glnB (WP_002438074.1), which encodes a nitrogen regulatory protein P-II (Figure 1A). The amino acid identities of the QseE, QseG, and QseF proteins of MH5 WT are of 85%, 58%, and 94% to the respective orthologues in E. coli. The putative qseG gene (DK872_RS06575) presented a sequence of 738 bp, which likely encodes a protein of 245 amino acid peptides with a predicted molecular weight of 27.9 kDa. An alignment of its predicted amino acid sequence with sequences of characterized proteins from Enterobacterales confirmed the identity of QseG in MH5 WT (Figure 1B).

In vitro phenotypic characteristics of ΔqseG mutant strain are not affected by deletion of the qseG gene

To characterize the QseG protein of a plant-associated endophytic enterobacterium, a QseG knockout mutant of the MH5 strain was constructed by in-frame deletion of an internal fragment of 645 bp of its qseG gene sequence. The ΔqseG mutant strain genotype was restored when the in-frame qseG gene deletion was successfully replaced by the complete qseG gene sequence in its original genomic location.

AHLs production in the MH5 WT and ΔqseG mutant strains was detected using the biosensors strains A. tumefaciens NTL4 (pZLR4) and C. violaceum CV026. Both MH5 WT and ΔqseG mutant strains were able to activate the biosensor strain Agrobacterium tumefaciens NTL4 (pZLR4). On the other hand, none of the tested enterobacterial showed positive results for the C. violaceum CV026 bioassay, indicating that MH5 WT is unable to synthesize AHL-type molecules with short acyl chains. Thus, the ability of the MH5 WT to synthetize AHL with medium to long fatty-acid chains did not require the action of the QseG protein. Similarly, neither biofilm formation on abiotic surfaces such as microtiter PVC plates (Figure S2A) or sand (Figure S2B) nor swarming motility (Figure S2C) were affected by the qseG gene deletion in the ΔqseG mutant strain.

Our previous results showed that Kosakonia sp. MH5 is tolerant to high salt concentrations (5% NaCl) and to manganese (1 mM Mn) (Brígido, Menéndez et al. 2019). To investigate whether QseG plays a role in responding to these abiotic stresses, the growth of the MH5 derivative strains was analyzed in M9 broth medium supplemented with NaCl or manganese. There were no significant differences between the growth curves of the MH5 WT, ΔqseG mutant and ΔqseG-KI strains in any of the conditions tested (Figure S2D).

QseG regulates the expression of genes related to plant–microbe interactions

Considering that QseG is implicated in the regulation of the expression of genes critical for the interactions between enterobacterial species and animal hosts (Cameron et al. 2018; Reading et al. 2009; Xiao et al. 2012), we hypothesized that QseG of plant-associated enterobacterial species also plays a role in the evasion of host immune system. To test this hypothesis, the expression levels of homologues of genes well-known for their roles in virulence of pathogenic bacteria were studied in MH5 WT and ΔqseG mutant strains upon exposition to chickpea root exudates for 24 h. Three genes were selected: the ompA (DK872_RS02445), which codes for the outer membrane protein A (OmpA), and contributes to bacterial adherence, biofilm formation, and host defense evasion (Smith et al. 2007); the virK (DK872_RS21215) and tagI (also known as impE; DK872_RS20860) code for effector proteins secreted for the type II and VI secretion system (T6SS), respectively (Förster et al. 2014; Yamazaki et al. 2008).

Although there was a trend towards a downregulation of the expression levels of virK in the ΔqseG mutant strain, only the expression of ompA and tagI genes were statistically lower than the MH5 WT strain (Figure 2). In addition, the degree of downregulation was stronger for the effector protein TagI, whose expression levels were less than 0.5-fold changes of the expression detected in the WT strain.

QseG plays an essential role in Mesorhizobium–chickpea–Enterobacteriaceae interactions

Since our previous results showed that MH5 WT was able to enhance the symbiotic LMS-1–chickpea association under salinity (Brígido, Menéndez et al. 2019), the involvement of QseG protein in the beneficial effect promoted by MH5 WT in this tripartite association was investigated.

Although the nodulation ability of LMS-1 microsymbiont was not statistically affected by the presence of any of the MH5 derivative strains (Figure 3A), the nodule dry weight was significantly higher in plants co-inoculated with LMS-1 and any of the strains containing a functional QseG protein compared to the remaining plants. Consequently, plant total biomass was 1.6 times higher in plants co-inoculated with the rhizobia and MH5 strains with a complete qseG gene (Figure 3B). In contrast, the increase in nodule dry weight
was counteracted when the chickpea plants were co-inoculated with the Mesorhizobium strain and the ΔqseG mutant strain. Altogether, these results indicate that the benefits of MH5 WT in the symbiotic Mesorhizobium–chickpea association were dependent on a functional QseG protein.

To determine whether the histology of the nodules formed by LMS-1 was affected by the presence of MH5 WT or ΔqseG mutant strains, we analyzed histological cross sections of the nodules of chickpea plants single inoculated with LMS-1 or co-inoculated with LMS-1 and MH5 WT or ΔqseG mutant strains. The bright field light microscopy (Figure 3(C–H)) showed that nodules of all treatments presented a typical histology of indeterminate effective nodules, having differentiated meristematic, infection, and bacteroid zones. Despite the difference in nodules size, no differences on nodule histology were observed among the different treatments.

QseG plays a role in root infection and colonization

Expression of genes related to plant defense evasion is reduced in the ΔqseG mutant strain, suggesting that the knockout mutant strain elicits broader plant defense responses than the MH5 WT strain. To test this hypothesis,

Figure 1. The qseEGF operon is conserved in Enterobacterales. (A) Schematic representation of the gene synteny of QseEGF operon. Primers used for the knockout are shown. Genes are just approximately drawn to scale. (B) Alignment of QseG protein amino acid sequences from bacteria belonging to the order Enterobacterales. Sequences were retrieved from NCBI and belong to the following genomes (accession numbers are in parentheses): Kosakonia radicicinata DSM 16656 (ARD96161.1), K. radiicum DSM 16656 (KJ220933.1), Edwardsiella tarda EIB202 (ACY83621.1), Salmonella enterica subsp. enterica serovar Typhimurium str. SL1344 (HAD6495497.1), and Citrobacter rodentium (WP_012906675.1).
we evaluate changes in chickpea response to co-inoculation with LMS-1 and MH5 WT or ΔqseG mutant strain, during the early stages of plant–bacteria interactions. Chickpea seedlings single inoculated with LMS-1 were used as control. We studied the expression of a putative respiratory burst oxidase homolog, RBOH (CaRBOH-like; XP_004494602.1), related to the production of reactive oxygen species (ROS) in response to environmental constraints, and a pathogenesis-related protein (PR) 1 (CaPR1; XP_004485656.1). Additionally, the expression of a putative chickpea flagellin receptor, flagellin sensing (FLS) 2 (CaFLS2; XP_012572420.1). Expression levels of CaFLS2 in the roots were upregulated in plants co-inoculated with the Mesorhizobium and any of enterobacterial strains compared with single-inoculated plants at 24 h after inoculation (Figure 4(A)). Even though CaFLS2 expression was 1.3-fold changes higher in plants co-inoculated with the MH5 WT than in plants co-inoculated with the ΔqseG mutant strain. Similarly, CaRBOH-like expression was differentially induced in co-inoculated plants, being its expression much higher in plants co-inoculated with LMS-1 and ΔqseG mutant. Hence, the ΔqseG mutant strain triggered plant defense responses in chickpea roots 3.9-fold changes stronger than the MH5 WT strain. Curiously, compared with roots of single-inoculated plants, CaPR1 expression was only significantly upregulated in plants inoculated with the MH5 WT.

Following, we evaluated if the capacity for colonization and infection of chickpea roots by the enterobacterial endophyte were altered by the deletion of the qseG gene sequence. For that purpose, two different approaches were performed: (i) direct observation of chickpea root colonization under confocal microscope after the inoculation of chickpea seedlings with the RFP-tagged MH5 WT or GFP-tagged ΔqseG mutant strain or the mixture of both at 5 days post-inoculation and (ii) analyzing colonization and infection on roots by counting the number of viable enterobacterial cells on the surface or within root cells at 24, 48 and 72 h post-inoculation. The first approach revealed that both MH5 WT and ΔqseG mutant strains were able to effectively colonize chickpea roots (Figure 4(B–D)). When inoculated together, the MH5 WT showed to be more competitive than the ΔqseG mutant strain. The results of the second approach, which include the strain LMS-1 as compatible chickpea mesorhizobia, suggested a delay in the proliferation of ΔqseG mutant strain on the roots at 24 h post-inoculation (Figure 4(E)). In this time point, the number of ΔqseG cells attached to the chickpea roots was three times higher than with the MH5 WT. But the number of attached bacteria did not differ significantly between these two strains at 48 h post-inoculation. Interestingly, internal root tissue colonization by MH5 WT was higher than by the ΔqseG mutant strain at all tested time points (Figure 4(F)).

To check if internal root colonization by the ΔqseG strain was compromised even at later points of this multipartite interaction, a hydroponic assay with chickpea plants grown under salinity stress conditions was performed. The results showed that the growth of chickpea plants co-inoculated with LMS-1 and ΔqseG were restricted (Figure 4(G)) and consistent with the results from the pot experiment. The colony-forming unit (CFU) counting showed that MH5 WT population inside the chickpea roots was 10-fold superior to the ΔqseG mutant strain at 28 days post-inoculation (Figure 4(H)). Together, these results show that the internal root infection and colonization ability of the MH5 WT was compromised by the qseG gene deletion, indicating that QseG influenced the onset of plant–bacteria associations, especially the endophytic colonization, nevertheless are not very important for epiphytic growth.

**Discussion**

We observed the involvement of QseG protein in the early stages of the plant-endophytic bacteria interactions, particularly in the internal root tissue colonization and plant’s immune system evasion. The involvement of QseG of the endophytic enterobacterium Kosakonia sp. MH5 strain in the regulation of expression genes related to host defense evasion is supported by (i) the downregulation of the expression of ompA and tagJ genes in the ΔqseG mutant strain and (ii) the defective colonization of the internal root cells from an early to a later stage of the plant–ΔqseG mutant strain interaction. OmpA is a virulence gene that seems to be crucial to mitigation of host defenses by animal pathogens (Confer and Ayalew 2013; Smith et al. 2007) and to the motility and efficient plant root colonization (Cole et al. 2017). TagJ (type VI accessory genes J) is an accessory protein present in sub-families 3 and 5 of type VI secretion systems, T6SSs (Boyer et al. 2009), which, in plant-associated bacteria is involved in plant manipulation and interbacterial competition (Bernal et al. 2018). These observations agree with the fact that beneficial microbes are initially recognized as potential invaders, but they are able to short-circuit plant defense responses to enable successful colonization of host roots (Zamioudis and Pieterse 2012). This is particularly apparent for endophytes living within the plant and, therefore, are directly exposed to the host’s immune system (Liu et al. 2017). Therefore, it seems plausible that plant-beneficial bacteria, as MH5 strain, need to escape root immune responses in order to establish an effective colonization (Yu et al. 2019).

With the ΔqseG mutant strain’s ability to evade the plant’s defense reduced, it is expected that chickpea inoculation with this strain elicits broader plant defense responses than the MH5 WT strain. Consistent with this hypothesis, CarBOH-like expression was higher in chickpea roots inoculated with MH5 ΔqseG mutant strain. RBOH’s is among the major
class of enzymes involved in the oxidative burst, one of the earliest events following the recognition of microbes by the plant immune system (Torres and Dangl 2005). Transcriptional upregulation of RBOH’s genes in response to the presence of phytopathogens and beneficial bacteria is described in several plants, including *Arabidopsis*, barley, maize, and chickpea (Arthikala et al. 2017; Chen et al. 2017; do Amaral et al. 2014; Gupta et al. 2013; Morales et al. 2016; Torres et al. 2017). Moreover, CaRBOH-like expression was higher in roots of double-inoculated plants. This result is congruent with studies that suggest rhizobia have evolved to reduce or actively suppress host defence responses (Zamioudis and Pieterse 2012). For instance, inoculation of *Ensifer (Sinorhizobium) meliloti* Sm2011 does not trigger expression of MtFLS2, while inoculation of the phytopathogen *Pseudomonas syringae* pv. *Tomato DC3000* or co-inoculation of both bacteria does (Chen et al. 2017).

Lower expression of the salicylic acid (SA)-dependent marker gene CaPR1 (Breen et al. 2017) in plants co-inoculated with *Mesorhizobium* and ΔqseG mutant strain may suggest a role of QseG in inducting disease resistance, but it can also be the result of its inability to activate PR expression for not reaching the required population density within plant cells. Either way, the induction of disease resistance by triggering the expression of PR genes, as observed by some plant growth-promoting bacteria, is reduced in plants...
inoculated with the ΔqseG mutant. For instance, inoculation of tobacco plants with beneficial Pseudomonas spp. strains triggers the expression of PR3 and induces systemic resistance against the bacterial pathogen Erwinia carotovora (van Loon et al. 2008). High PR gene expression levels were also associated with induction of resistance against the fungal pathogen Verticillium dahliae in Arabidopsis plants inoculated with Paenibacillus alvei K165 (Tjamos et al. 2013). Similarly, inoculation of Arabidopsis plants with Kosakonia radicincitans DSM 16656 leads to activation of SA and jasmonate (JA)/ethylene (ET) defense-related genes and increased resistance to chewing and phloem-feeding insects (Brock et al. 2013; Brock et al. 2018). Thus, it can be speculated that priming for chickpea enhanced defense is improved by MH5 WT inoculation. On the other hand, induction of systemic resistance is dependent of the initial population density of the plant growth-promoting bacteria as was observed in radish (Raaijmakers et al. 1995). For instance, Gluconacetobacter diazotrophicus ROS-detoxifying mutants, which are also deficient in root colonization, fail to induce PR10 expression in rice (Alquéres et al. 2013; Raaijmakers et al. 1995). Nevertheless, induction of resistance in chickpea by this beneficial endophytic bacterium needs to be further investigated.

The hypothesis that QseG is important for the host plant–bacteria interaction is also strengthened by the observation that, despite co-inoculated chickpea seedlings displayed the highest transcript levels of the putative CaFLS, its expression decreased in plants inoculated with the ΔqseG mutant strain. FLS2 is one of the best characterized plant recognition receptor of the model plant Arabidopsis thaliana (Zipfel and Oldroyd 2017). Although FLS2 orthologue in chickpea was not
yet characterized, its sequence shows 82.5% of identity to MtFLS2 (XP_013457339.1) and conserved domains. FLS2 recognize the flagellin epitope flg22, a highly conserved part of the N terminus of bacterial flagella (Boller and Felix 2009). Kosakonia sp. MH5 encodes two flagellin homologues with identical flg22 motifs and 100% identity to flg22 of Salmonella enterica (Figure S3), which has recently been shown to triggers FLS2-dependent defence responses in alfalfa (Wahlig et al. 2019). Therefore, the mutation in the qseG gene may alter the flagellin recognition as earlier observed in S. enterica serovar Typhimurium (Cameron et al. 2018). Despite that flagellar phase variation was not before reported in Kosakonia spp., a study revealed that flagellin glycosylation islands are common in the genome of bacteria belonging to this genus (De Maayer and Cowan 2016). Flagellin post-transcriptional modifications may interfere with the recognition of the immunogenic epitopes of the flagellin protein and as consequence helps to evade host defence responses (Verma et al. 2005). Although this is not consistent with a reduced chickpea defence response, the intensity of the immune responses does not always correlate with the expression level of the FLS2 receptor in the root system but rather depends on the expressing tissue (Wyrsch et al. 2015). The FLS2 promoter activity is mainly present in the Arabidopsis root stele and expands to the cortex and epidermal region (Beck et al. 2014; Wyrsch et al. 2015). From this perspective, lower levels of expression of FL2 in chickpea roots inoculated with ΔqseG mutant may be due to its inability to reach those root tissues that priming to a proper balance of defense responses according to the expected exposure to elicitors.

Although the importance of a functional QseG for the benefits of the MH5 in the chickpea–Mesorhizobium symbiosis is evident, we do not fully identify the mechanisms of action behind the benefits of this enterobacterium. Looking to the gene synten of the qseEGF operon region of MH5 strain, its genomic context is typical of bacteria assigned to the families Enterobacteriaceae, Erwiniaeae, and Pectobacteriaceae (Göpel et al. 2011). In these families, the gene encoding the PII protein GlnB is localized just downstream of qseEGF genes while in Yersiniaceae, Hafniaeae, Morganellaeeae, and Budviciaceae, the gene nitrE (NH3-dependent NAD+ synthetase) is inserted between qseF and glnB (Figure S1). In E. coli, the genes encoding QseEGF and GlnB are co-transcribed in an operon. PII proteins are widely distributed small homotrimeric signal transduction proteins that bind to different targets, including NifA in nitrogen fixing bacteria (Sotomaior et al. 2012). It was shown that Azospirillum brasilense NifA is only active in the presence of GlnB (Arsene et al. 1996) and the uridylylated form of GlnB is essential for NifA activity, and potentially for interaction of GlnB with the N terminal domain of NifA (Inaba et al. 2015). Curiously, transcription of glnB increases in E. coli qseG mutant, suggesting that QseG act through the promoter upstream of glnB to repress its transcription (Reading et al. 2009). It can be speculated that qseG mutant changes the transcription of glnB, which indirectly may have altered the activation of NifA in the nitrogen fixing MH5 strain. Nevertheless, further characterization of the ΔqseG mutant through transcriptomic analysis and analysis of the QseG protein–dependent changes in the plant transcriptome will be important future steps toward determining the role QseG membrane protein and their targets play in bacteria–plant interactions.

Conclusion

Our work shows that QseG of Kosakonia sp. MH5 is involved in the following processes: (i) the evasion of the plant immune system and (ii) the efficient colonization of chickpea root cells. Furthermore, the invasion and a proper establishment within the roots and/or root nodules are essential for this strain to be able to exert beneficial effects on the symbiotic chickpea–Mesorhizobium association under salinity. Our present evidence that the QseG of an endophytic enterobacterium associated with plants maintains its function by positively interfering in the process of colonization and infection of the host similarly to what is observed in Enterobacteriaceae pathogens in animals. This corresponds, as far as we know, to the first study regarding the role of QseG protein of an endophytic enterobacterium in the plant–enterobacterium interaction showing that its role is similar and transversal to enterobacteria regardless their interaction with the host. Our findings provide insights into the molecular bases of beneficial legume endophytic–enterobacteria interactions and explore the fine line between beneficial and pathogenic enterobacteria.

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Disclosure statement

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