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ENVIRONMENTAL MICROBIOLOGY - REVIEW

Genetic and structural determinants on iron assimilation pathways in the plant pathogen *Xanthomonas citri* subsp. *citri* and *Xanthomonas* sp.

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Abstract

Iron is a vital nutrient to bacteria, not only in the basal metabolism but also for virulent species in infection and pathogenicity at their hosts. Despite its relevance, the role of iron in *Xanthomonas citri* infection, the etiological agent of citrus canker disease, is poorly understood in contrast to other pathogens, including other members of the *Xanthomonas* genus. In this review, we present iron assimilation pathways in *X. citri* including the ones for siderophore production and siderophore-iron assimilation, proven to be key factors to virulence in many organisms like *Escherichia coli* and *Xanthomonas campestris*. Based on classical iron-related proteins previously characterized in *E. coli*, *Pseudomonas aeruginosa*, and also *Xanthomonadaceae*, we identified orthologs in *X. citri* and evaluated their sequences, structural characteristics such as functional motifs, and residues that support their putative functions. Among the identified proteins are TonB-dependent receptors, periplasmic-binding proteins, active transporters, efflux pumps, and cytoplasmic enzymes. The role of each protein for the bacterium was analyzed and complemented with proteomics data previously reported. The global view of different aspects of iron regulation and nutrition in *X. citri* virulence and pathogenesis may help guide future investigations aiming the development of new drug targets against this important phytopathogen.

Keywords Iron transport · Outer membrane receptors · Citrus canker · Siderophores · *Xanthomonadaceae* · Regulation

Xanthomonas citri is the gram-negative bacteria that causes the citrus canker, a disease that infects several species of the citrus genus, specially oranges, still without treatment. The disease is characterized by necrotic lesions on leaves and fruits, which present brown water-soaked margins, culminating with premature fruit drop [1]. The phenotypic symptoms lead to a decrease of the productivity in the citrus production, juice industry, and exportation resulting in massive economical losses for the countries affected, including the main orange exporters such as Brazil, the USA, and China [2].

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The mechanism of infection and pathogenicity of the *Xanthomonas* genus has being largely studied including the functional characterization of genes direct or indirectly related to the capability of the microorganism to spread the disease in plants such as *rpf*, *avr*, and *hrp* [3]. These genes are organized in clusters and their expression affects the virulence, production of secreted proteins, and many proteins localized in the cell envelope, which is related to pathogenicity and evasion from the host immune system defenses [4].

Iron is an essential nutrient normally found as a prosthetic group or cofactor of enzymes involved in several important cellular processes such as nitrogen fixation, amino acid synthesis, citric acid cycle, and DNA biosynthesis [5]. However, the oxidative stress caused by the excess of iron in the cell can be extremely damaging due to the *Fenton* reaction, in which the reduced form of iron reacts with hydrogen peroxide originating hydroxyl, a compound that can damage proteins and DNA [6]. In order to ensure the organisms' safety against oxidative stress, the iron must be imported through the cell membranes in controlled amounts by the means of siderophores. These small molecules (200–2000 kDa) are synthesized and secreted by bacteria, fungi, and plants, forming

tight and stable complexes with ferric iron. Siderophores are strictly related to the survival of organisms and many times essential for pathogenesis [7].

Despite the relevance of this metal for organisms, there is a lack of information about iron-related genes and their involvement with virulence and pathogenicity of *X. citri*. In this context, this work presents the putative set of proteins responsible for iron uptake and regulation in *X. citri*, showing structural and sequential comparisons with orthologs and highlighting the main functional and structural features that might be useful for further studies. The description of the iron components and possible compounds forming complexes for the metal acquisition is relevant for the understanding of *X. citri* physiology. Moreover, iron-related proteins could be targets for the development of new therapies against the citrus canker.

Iron as a factor of virulence and pathogenicity in different organisms

Given the broad importance of iron for the growth of bacteria, there is no surprise that several animal pathogens have reported siderophore production and internalization as important factor in their virulence and pathogenicity. Examples include pathogenic and uropathogenic *Escherichia coli* serovars [8] and strains [9], *Pseudomonas aeruginosa* [10], and *Legionella pneumophila* [11]. Similarly, siderophores responsible for the iron uptake from the environment are also important for the virulence of the gram positives *Staphylococcus aureus* [12, 13] and *Bacillus anthracis* [14] (Table 1).

In some phytopathogens such as *Agrobacterium tumefaciens* [26], *Pseudomonas syringae* [15], and *Ralstonia solanacearum* [17], iron import systems were correlated to virulence but not necessarily to pathogenesis.

In *Dickeya dadantii*, formerly known as *Erwinia chrysanthemi*, the production of siderophores chrysobactin and achromobactin is highly needed for efficient pathogenesis [18]. Similarly, the necrosis of apple flowers induced by *Erwinia amylovora* infection is dependent of the ferrioxamine siderophore [19].

Iron and siderophores also have been related to virulence in *Xanthomonadaceae* (Table 1). In *X. campestris pv. campestris*, the nutritional dependence of iron and its acquisition was shown to be mediated by cyclic β-(1,2)-glucans in the membrane [27], as well as the import mediated by TonB-dependent receptors [28]. In this bacterium, the transcriptional regulator XibR (*Xanthomonas* iron-binding regulator) controls the expression of several iron-regulated genes and virulence associated functions [20]. Mutants in genes related to siderophore metabolism in *X. oryzae pv. oryzae* (*colS*, XOO1806, *acnB*, *prpR*, and *prpB*) exhibited a deficiency for growth on iron-limiting medium and a decrease in virulence [29].

Proteomic studies of *X. translucens pv. undulosa* identified many sequences of iron-binding proteins. Further, the analysis of *X. translucens* proteome for iron-binding proteins revealed that this metal assists on the regulation and function of several secreted proteins linked to its virulence [21]. In *X. fragariae*, after the disruption of the pyoverdine biosynthetic pathway the bacterium lost its pathogenicity [22].

In *X. citri*, the genomic analysis aiming the identification of nonribosomal peptides revealed two *loci* (respectively, *locus* 1 and *locus* 2) that showed homology with genes involved with siderophore biosynthesis and lipopeptide synthetases, suggesting a further role of iron in the production of phytotoxins in this bacterium [23]. Iron internalization proteins, including TonB-dependent receptors, were also identified in proteomic analysis [30]. It was suggested that these receptors participate indirectly in biofilm formation and probably have important roles in the adaptability of *X. citri* by modulating the transport of carbohydrates [24]. A wide genome microarray analysis set up to characterize the pathogenic relevant regulons HrpG and HrpX from *X. citri* revealed four genes encoding TonB-dependent receptors as part of those regulons (XAC3050, XAC3489, XAC3444, and XAC1143). These genes are mainly known in the transport of iron-siderophore complexes and cobalamin (vitamin B12) into the periplasm [25] (Table 1).

Regulatory mechanisms of iron import in *Xanthomonas citri*

Despite its importance as a cofactor in the catalysis of several enzymes, high iron concentrations can be deleterious to the cell. The redox activity of iron generating oxidizing molecules and free radicals is extremely damaging to the organism [31]. Thus, the maintenance of the intracellular concentrations of Fe(II) and Fe(III) is critical, and since neither is secreted, the regulation is mainly held by controlling their import [32]. One important regulator of iron uptake is the Fur (Ferric Uptake Regulator) protein, which was first described in *Salmonella enterica subsp. enterica* ser. Typhimurium [33] and to date is the most known global regulator for iron uptake in several gram-negative microorganisms.

Structurally, the C-terminus of *S.* Typhimurium Fur binds to Zn(II) on the residues His^{86}, Asp^{88}, Glu^{107}, and His^{124}, which enables the physiological dimer conformation [34–36]. The second metal-binding site is formed by the residues His^{32}, Glu^{80}, His^{89}, and Glu^{100} and binds both Zn(II) and Fe(II). Iron acts as a corepressor in high concentration, allowing Fur to bind to Fur boxes (Fur-binding site or FBS), conserved sequences at the promoter region of key genes related to iron import and siderophore synthesis [20, 37].

The amino acid sequence of XAC1517 protein, the corresponding Fur ortholog in *X. citri*, was used for searching proteins with structural similarity in the Protein Data Bank. The

Table 1 List of pathogens and their relationship with iron and pathogenesis. All pathogens described on the text are followed by the disease they cause or symptoms at the host. Evidences of the relationship between iron and virulence or pathogenesis due to the specific effect of siderophores are shown

Species	Host/disease	Iron-related proteins/ siderophores/experimental analysis	Phenotype	Reference
Animal pathogens				
Escherichia coli	Enteropathogenic	Proteomic analysis	Increasing of siderophores receptors and ABC transporters in pathogenic strains; Siderophores involved in biofilm formation	[8]
Pseudomonas aeruginosa	Opportunistic pathogen	Pyochelin and pyoverdine	Absence of siderophores reduced the lethality in mice	[10]
Staphylococcus aureus	Nosocomial infections	Staphyloferrin A and staphyloferrin B	Knockout of staphyloferrin A and staphyloferrin B resulted in severe growth deficiency	[12]
Bacillus anthracis	Anthrax	Anthrachelin and anthrabactin	Attenuated virulence in mice	[14]
Legionella pneumophila	Lung infection	Legiobactin	Decreasing of infection in mice lungs tissues	[11]
Phytopathogenic species				
Pseudomonas syringae	Cherry fruit	Siderophore production during infection	Not involved in pathogenesis	[15]
Pseudomonas syringae pv. tabaci 6605	Tobacco	Pyoverdine	Attenuated virulence	[16]
Agrobacterium tumefaciens	Tomato and tobacco	Agrobactin	Not required for virulence	[17]
Ralstonia solanacearum	Different plants/bacterial wilt	Staphyloferrin B	Not involved in pathogenesis	[15]
Dickeya dadantii (*Erwinia chrysanthemi*)	Different plants/soft rotting disease	Chrysobactin and achromobactin	Highly needed for pathogenesis	[18]
Erwinia amylovora	Fire blight/rosaceous plants	Ferrioxamine	Needed for pathogenesis	[19]
Xanthomonads				
Xanthomonas campestris pv. campestris	Blight of rice	*XibR*	Regulates the expression of several iron-regulated genes and virulence Deficiency for growth on iron-limiting medium and a decrease in virulence	[20]
Xanthomonas translucens pv. undulosa	Staple food crops	Proteomic-iron-binding motifs	Bacterial leaf streak disease	[21]
Xanthomonas fragariae	Angular leaf spot/strawberry	Pyoverdine	Resulted in complete loss of *X. fragariae* virulence	[22]
Xanthomonas citri subsp. citri	Citrus	Siderophore biosynthesis	Up-regulated genes responsible for proteins involved in siderophore biosynthesis	[23]
		TonB receptors	Biofilm formation and carbohydrate transport	[24]
		Genome-wide microarray	Iron-siderophore complexes and vitamin B12	[25]

result revealed a set of metal regulators that shared from 35 up to 58% of amino acid sequence identity, including the *P. aeruginosa* ortholog (PDB accession code 1MZB) [36] that was used for building the three-dimensional model of *X. citri* Fur (Table S1, Supplementary material). The amino acid sequence alignment of Fur proteins of *E. coli*, *P. aeruginosa*, and *X. citri* shows the high conservation of the residues that form the two essential metal-binding sites (Fig. 1a, pink and green) and the DNA-binding helix (in yellow). This data can suggest that *X. citri* Fur has a similar mechanism of transcriptional activation as described in *E. coli* [32] and *P. aeruginosa* [36]. The positioning of the residues and the putative DNA-binding helix are presented in the *X. citri* Fur three-dimensional model (Fig. 1b).

Along with Fur, XibR is another transcriptional regulator that was recently characterized in *X. campestris* [20]. XibR is a transcriptional factor (TF) from the NtrC family, a well-known class of TFs involved in regulation of several processes by binding to alternative σ factors such as σ-54 [38]. It was demonstrated that *X. campestris* XibR responds to iron

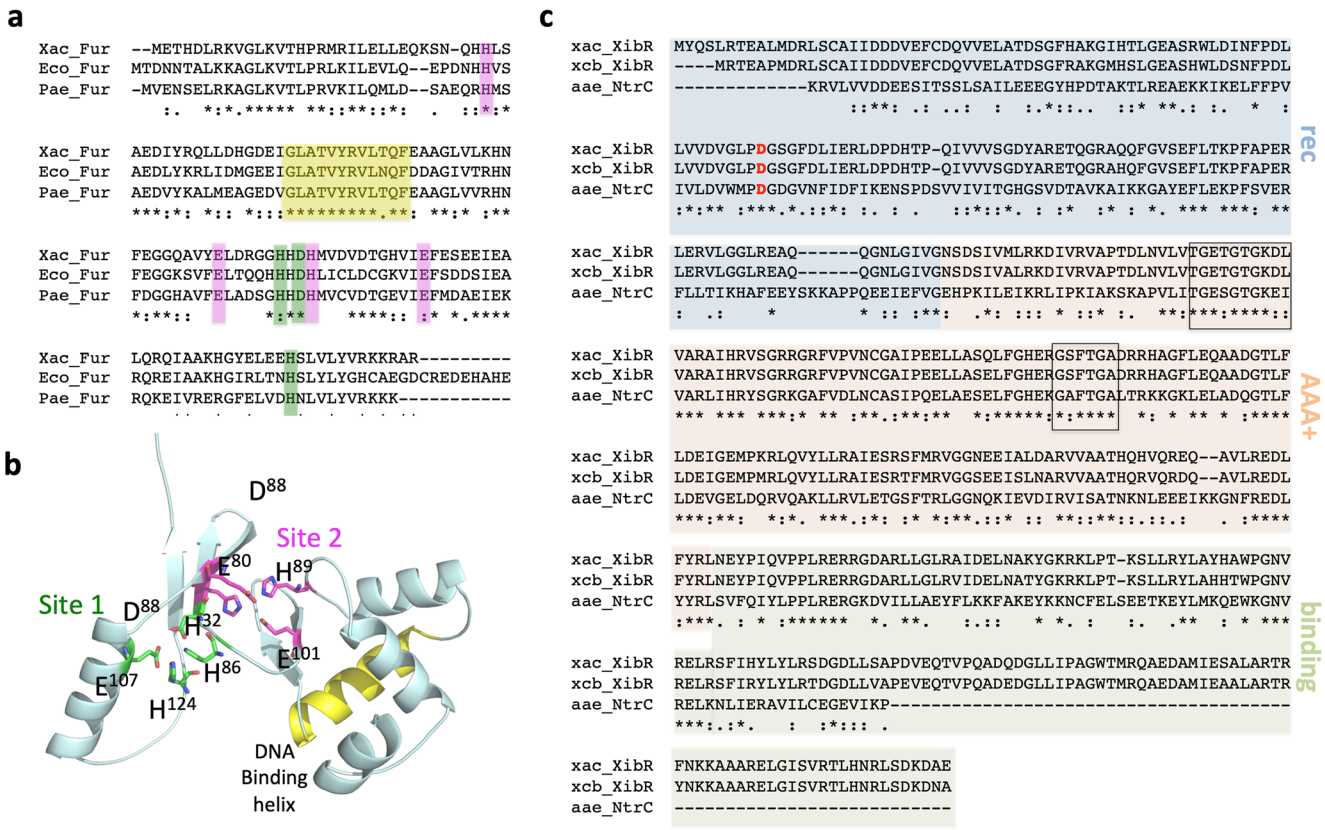

Fig. 1 The iron regulatory proteins Fur and XibR identified in *Xanthomonas citri*. **a** Amino acid sequence alignment of Fur protein from *X. citri* (XAC1517) (Xac_Fur), *Escherichia coli* (b0683) (Eco_Fur), and *P. aeruginosa* (PA4764) (Pae_Fur). The metal-interacting residues and DNA-binding helix are shown in pink/green and yellow, respectively. **b** Three-dimensional model of *X. citri* Fur built from the structural coordinates of the ortholog *P. aeruginosa* (PDB code 1MZB). The model is colored in the same fashion as the sequential alignment. **c** Amino acid sequence alignment of *X. citri* XibR (XAC3733) (xac_XibR), *X. campestris* XibR (xcb_XibR), and *Aquifex aeolicus* NTRC4 TF (aae_NtrC). Domains Rec, AAA+, and DNA binding are colored in blue, orange, and green, respectively; *X. citri* Asp[64] conserved in the two other proteins is shown in red bold and the GESGTGK and GAFTGA motifs are evidenced inside the boxes

depletion and positively regulates motility and chemotaxis, two key processes involved in pathogenesis. Moreover, this protein has a Fur-like function by negatively repressing siderophore synthesis and iron uptake systems [20]. A XibR-like protein with 94% of amino acid sequence identity was identified in *X. citri* (XAC3733). In addition, according to Blastp X PDB, XAC3733 is structurally similar to the structure of the σ-54 activator NtcC4 from the extremophile *Aquifex aeolicus* (PDB accession code 3DZD) [39]. The three-dimensional structure of this protein shows three domains: "*rec*," with a conserved aspartate residue, the "AAA+" domain that has two motifs for interaction with σ factors (GESGTGK and GAFTGA), and the DNA-binding domain [39]. Based on the amino acid sequence alignment of XAC3733, *X. campestris* XibR, and *A. aeolicus* NtrC4 TF, we identified that *X. citri* XAC3733 conserves the aspartate (Asp[64]) and the two functional motifs (Fig. 1c, showed respectively, in red and boxes). The high amino acid sequence identity of XAC3733 and *X. campestris* XibR and the conservation of the functional motifs strongly suggest that it may play a similar function in *X. citri*. However, despite of the presence and conservation of these proteins, transcriptional regulation involves more important factors, as the *cis*-elements of each gene belonging to the Fur regulon and also in which processes those genes are involved. In this sense, the understanding of the iron regulation in *X. citri* is an interesting field to be explored with further functional experiments.

The *Xanthomonas citri xss* cluster

It is well established that *Xanthomonadaceae* such as *X. campestris* [40], *X. oryzae*, and *X. citri* [41] share a similar siderophore biosynthesis cluster (named *xss*, *Xanthomonas* siderophore synthesis) that is homolog to the operon *pvsABCDE* of *Vibrio parahaemolyticus*, responsible for vibrioferrin synthesis [42]. The vibrioferrin-like siderophore produced by *Xanthomonadaceae* was named xanthoferrin [23]. The *xss* cluster present in *X. citri* genome (XAC3175 to XAC3181) and its genome organization is compared with *X. oryzae* (Fig. 2a). The first gene on *X. citri xss* is XAC3175 (*mphE*) and encodes for a 4-hydroxy-2-oxovalerate aldolase

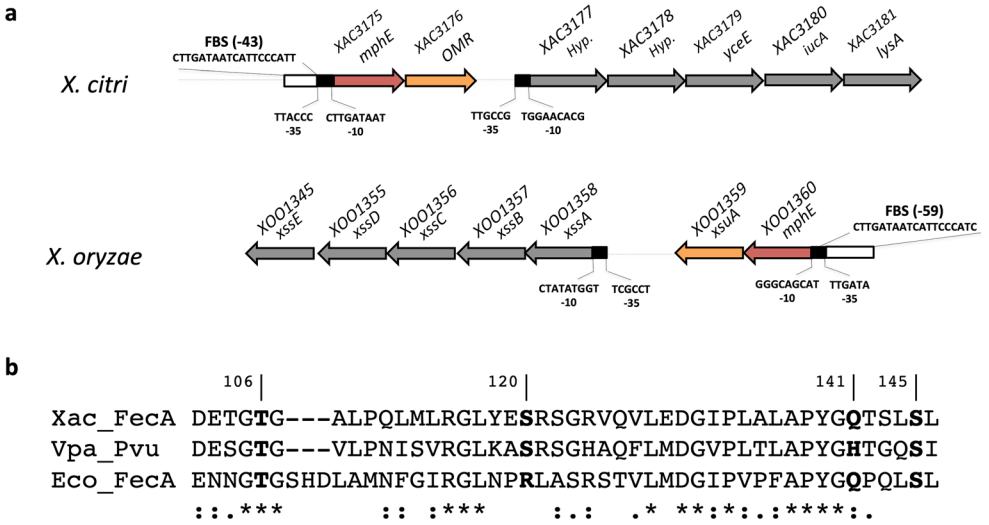

Fig. 2 The *xss* cluster of *Xanthomonas citri*. **a** Gene organization of *xss* operons localized in *X. citri* (+) strand and *X. oryzae* (−) strand. Promoter regions − 10 and − 35 and Fur-binding sites (FBS) are depicted in black and white boxes, respectively. **b** Amino acid sequence alignment of the plug regions of putative FecA orthologs from *X. citri*, *V. parahaemolyticus* in comparison with *E. coli* FecA plug region. The residues that aligned with *E. coli* sequence are detached in bold. The numbers correspond to the residues in *X. citri* sequence

that shares 98% and 25% of amino acid sequence identity with *mphE* from *X. oryzae* and *V. parahaemolyticus*, respectively. *X. citri mphE* is co-transcribed with XAC3176, a TonB-dependent outer membrane receptor (OMR) that shares 44% identity with *V. parahaemolyticus* ferric vibrioferrin outer membrane receptor PvuA (reference VPA1656) [42].

Since the three-dimensional structures of *V. parahaemolyticus* and *X. citri* OMRs are not available, we used the known sequence of *E. coli* FecA OMR to compare the residues in the plug region. In FecA protein, this region contains the key residues that give specificity and perform interactions with the correspondent siderophore: Thr^{138}, Arg^{155}, Gln^{176}, and Ser^{180} [43]. The amino acid sequence alignment of *E. coli* FecA, *X. citri* XAC3176, and *V. parahaemolyticus* PvuA shows that the plug region of XAC3176 conserves three from the four residues that in *E. coli* are involved in the ligand coordination, Thr^{106}, Gln^{141}, and Ser^{145} (Fig. 2b). The FecA Arg^{155} is replaced by a serine in XAC3176 (Ser^{120}) and PvuA. The complete alignment of the proteins XAC3176, PvuA, and FecA is presented in Figure S1 (Supplementary material). The similar genome organization, the putative role of the enzymes, and the conservation of some key residues from the plug region suggest that XAC3176 might be the xanthoferrin outer membrane receptor in *X. citri*. Indeed, more robust experimental data should be performed in order to understand what is the transport function that XAC3176 plays in *X. citri*.

The next operon has 5 genes that encode enzymes involved in siderophore biosynthesis (XAC3177, XAC3178/XssB, XAC3180/IucA, and XAC3181/LysA) and a member of the major facilitator superfamily protein (XAC3179/YceE) (Fig. 2a). A list of all proteins of *X. citri xss* and their correspondent orthologs in *X. oryzae*, *V. parahaemolyticus*, and *E. coli*, including amino acid sequence identities, is presented in Table S2 (Supplementary material).

The FeoABC of *X. citri*: The ferrous iron import system

Due to iron importance for the cell homeostasis, it is not unusual to find more than one route for its import. Despite the well-known systems of metal chelating siderophore internalization, iron can also be internalized in its ferrous form, Fe(II), after reduction of the siderophore-Fe complex in the periplasm or even outside the cell by specific reductases. In the latter case, the ferrous iron crosses the outer membrane through porins [44]. The Feo system (Ferrous iron transport) is the main pathway by which ferrous iron crosses the inner membrane of gram-negative bacteria. It was first described in *E. coli* [45] and regulated by Fur in a similar fashion as the siderophore producing and other related genes [46].

Genetically, the *E. coli* Feo system is encoded by *feoABC* operon consisting of three co-transcribed genes that encode two small proteins for signaling (b3408 and b3410) and the permease for ferrous iron transport (XAC3409). Several studies have shown a correlation between those genes and virulence in different animal pathogens such as *Helicobacter pylori* [47], *Pseudomonas gingivalis* [48], *Clostridium jejuni* [49], and *S. typhimurium* [50]. Similarly, the *feoABC* operon of *X. oryzae pv. oryzae* (XOO2900 to XOO2898) was related to virulence *in planta* [41] and its ortholog is present in *X. citri* (XAC1854-XAC1856), including the Fur-binding site (Fig. 3a).

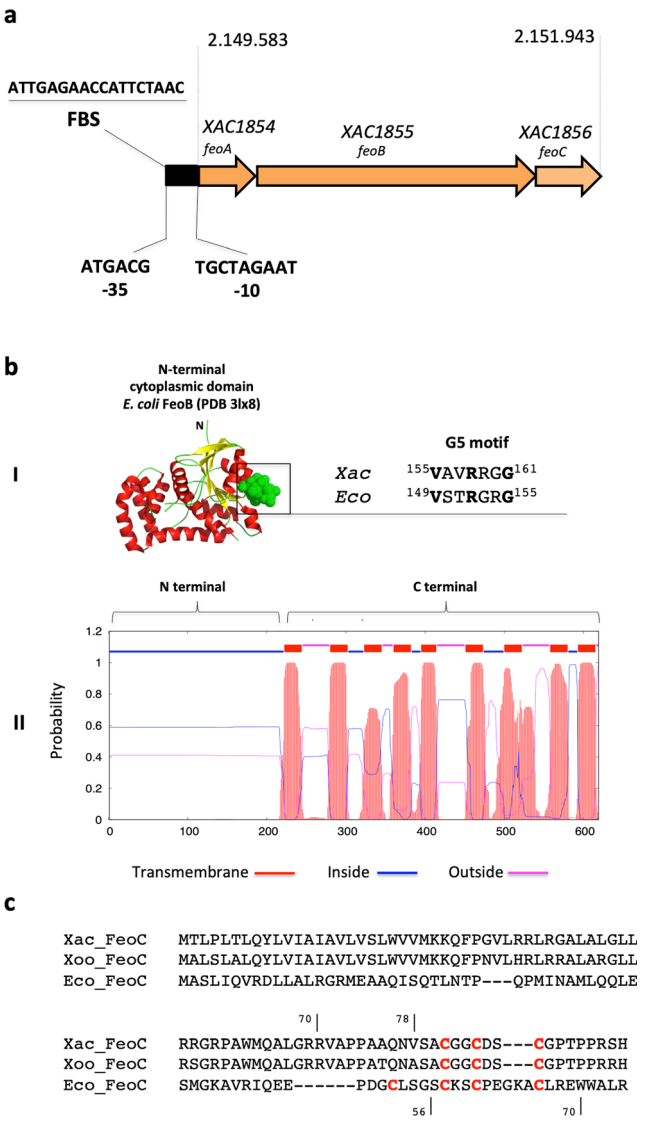

Fig. 3 The *feoABC* operon of *Xanthomonas citri*. **a** Gene organization of the *feoABC* operon in *X. citri*. Promoter regions − 10 and − 35 (black box) are showed as well as the Fur-binding site (FBS). **b** Comparison between *X. citri* and *E. coli* FeoB proteins. (I) Three-dimensional structure of *E. coli* FeoB N-terminal domain (PDB 5FH9) is shown in cartoon representation (helices in red and beta strands in yellow) and highlights the functional G5 motif localized in a coil region (green spheres). A comparison of *E. coli* and *X. citri* G5 regions is shown. (II) Transmembrane prediction of *X. citri* FeoB using the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/) shows a cytoplasmic N-terminal domain (blue line) and 9 alpha-helices (in red), similar to what is described for *E. coli* FeoB. **c** Sequential alignment of FeoC from *X. citri* (XAC1856) (Xac_FeoC), *X. oryzae* (XOO2898) (Xoo_FeoC), and *E. coli* (b3410) (Eco_FeoC), the putative functional cysteine residues are depicted in red bold

In *E. coli*, the main component of *feoABC* operon is FeoB, likely the permease that allows the passage of ferrous iron from the periplasm to the cytoplasm [51]. FeoB contains two domains, a cytoplasmic N-terminal domain that shows a GTPase activity, and a C-terminal domain that consists of transmembrane helices. The three-dimensional structure of *E. coli* FeoB N-terminal domain (PDB code 5FH9) [52] is showed in Fig. 3b-I. The structure is presented in cartoon red (helices) and yellow (beta strands) and the G5 motif that provides hydrophobic and electrostatic interactions with GTP is shown in green spheres [53]. This motif is located in a coil region, consisting of seven residues conserved when compared with *X. citri* FeoB (Fig. 3b-I). The C-terminus of FeoB consists of 8 α-helices that span the cytoplasmic membrane with two gate motifs, each with 4 stretches of residues and a conserved cysteine that is important for metal binding [51, 54]. Similar to the *E. coli* protein, the secondary structure and transmembrane helices prediction of *X. citri* FeoB (XAC1855) revealed a cytoplasmic N-terminal (residues 1–200) that shares 40% of amino acid sequence identity with *E. coli* ortholog, and 9 alpha-helices forming a transmembrane C-terminal (Fig. 3b-II).

E. coli FeoA (b3408), with orthologs in *X. oryzae* (XOO2897) and *X. citri* (XAC1854), is predicted to be a membrane-interacting protein that works along with FeoB [51]. *E. coli* FeoC, a putative TF, is a highly disordered protein containing a large loop region on its C-terminus with four conserved cysteine residues forming the binding site for Fe(II). Similarly to the Fur protein system, Fe(II) is speculated to act as a corepressor in FeoC [51]. Based on the primary sequence of FeoC from *E. coli* (Eco_b3410), an alignment was performed with *X. oryzae* (XOO2898) and *X. citri* (XAC1856) putative FeoC (Fig. 3c). *X. citri* FeoC shares 89% of sequence identity with *X. oryzae* protein, but only 13% with the putative ortholog of *E. coli*. Despite the low identity, both proteins conserve the important functional cysteines present in *E. coli* protein. The importance of FeoC along with FeoB and FeoA for *X. citri* pathogenesis remains to be investigated.

Putative OMRs and ATP-binding cassette transporters involved in siderophore-Fe uptake in *X. citri*

Siderophores of different classes can bind Fe(III) in the extracellular domain and be internalized by specific OMRs of gram-negative bacteria. In *E. coli*, four OMRs are well characterized: the ferrichrome transporter FhuA (b0150) [55], ferrienterobactin transporter FepA (b0584) [56], ferric citrate transporter and signal transducer FecA (b4291) [43], and the cobalamin transporter BtuB (b3966) [57]. Structurally, those proteins share a high conserved folding consisting of two well defined domains: a N-terminal domain (plug domain), which is mainly responsible for the interaction with the siderophore and that mediates its internalization via conformational changes, and a C-terminal consisting of a β-barrel structure that forms the pore where the plug domain is located [58–60]. As previously suggested [61, 62], the rearrangement of the

plug domain allows the passage of the siderophore from the extracellular medium to the periplasm [37]. It has been demonstrated that the binding of the antibiotic rifamycin to *E. coli* FhuA culminated in several allosteric transitions including the helix-to-coil transition in the called N-terminal switch helix [63]. This helix harbors the TonB box motif, responsible for recruiting and binding of TonB, which is important for the energization of the system.

TonB works by mediating the coupling of the chemiosmotic gradient from the inner to the outer membrane transport with the assistance of two proteins: ExbB and ExbD. Although the mode of action of the three proteins together as a complex is not yet fully understood, they compose an apparatus that cooperatively energizes the OMR and mediates the structural rearrangement that will culminate with the siderophore internalization to the periplasmic environment [58, 64, 65].

To search the presence of putative iron-related OMRs orthologs in *X. citri*, amino acid sequences of the classical FhuA, FepA, FecA, and BtuB of *E. coli* were used in Blastp [66] against the *X. citri* databank. Respectively, four *X. citri* sequences were obtained with query coverage higher than 70% and amino acid sequence identity higher than 30%: FhuA ferrichrome-iron receptor (XAC2185), PfeA siderophore receptor (XAC3620), FecA citrate–dependent iron transporter (XAC3176), and BtuB outer membrane receptor for transport of cobalamin (XAC3194) (Table 2, outer membrane receptors). To complement the analysis, the identified amino acid sequences were used for Blastp against the Protein Data Bank. Besides the abovementioned *E. coli* proteins, the search revealed that *X. citri* XAC2185 (FhuA) sequence has structural similarities with the OMRs of pyoverdine FpvA (PDB 1XKH) and pyochelin FptA (PDB 1XKW) from *P. aeruginosa* and the ferric enterobactin OMR PiuA (PDB 5FP1) from *Acinetobacter baumannii* (Table S3, Supplementary material).

The sequences of all putative orthologs were used for structural alignment and, when available, for identification of residues involved with siderophores coordination. The gene cluster and operon organization of the identified orthologs in

Table 2 Putative orthologues of the iron uptake systems from *Escherichia coli* identified in *Xanthomonas citri*. (−) not identified or (nr) amino acid sequence identity with coverage and similarity lower to 70% and 30%, respectively

E. coli protein	KEGG entry	Protein predicted function	Protein name in *X. citri*	KEGG entry	Protein predicted function	Query coverage %/sequence identity *E. coli* × *X. citri*
Outer membrane receptors						
FhuA	b0150	Iron complex outer membrane receptor	FhuA	XAC2185	Ferrichrome-iron receptor	91/40
FepA	b0584	Ferrienterobactin outer membrane transporter	PfeA	XAC3620	Siderophore receptor protein	99/47
FecA	b4291	TonB-dependent outer membrane ferric citrate transporter and signal transducer	FecA	XAC3176	Citrate-dependent iron transporter	72/32
BtuB	b3966	Vitamin B12/cobalamin outer membrane transporter	BtuB	XAC3194	Outer membrane receptor for transport of vitamin B12	98/34
ATP-binding cassette transporters						
FhuD	b0152	Iron(3+)-hydroxamate import ABC transporter periplasmic-binding protein	nr	-	-	-
FhuB	b0153	Iron(3+)-hydroxamate import ABC transporter permease	nr	-	-	-
FhuC	b0151	Iron complex transport system ATP-binding protein	ABC	XAC3669	ABC transport ATP-binding protein	85/26
FepB	b0592	Ferrienterobactin ABC transporter periplasmic-binding protein	nr	-	-	-
FepD	b0590	Iron complex transport system permease protein	nr	-	-	-
FepG	b0589	Iron complex transport system permease protein	nr	-	-	-
FepC	b0588	Iron complex transport system ATP-binding protein	PstB	XAC1574	Phosphate ABC transport ATP-binding protein	76/32
FecB	b4290	Iron complex transport system substrate-binding protein	nr	-	-	-
FecC	b4289	Iron complex transport system permease protein	nr	-	-	-
FecD	b4288	Iron complex transport system permease protein	nr	-	-	-
FecE	b4287	Iron complex transport system ATP-binding protein	CysA	XAC1020	Sulfate ABC transporter ATP-binding protein	86/36
BtuC	b1711	Vitamin B12 transport system permease protein	nr	-	-	-
BtuD	b1709	Vitamin B12 transport system ATP-binding protein	YhbG	XAC2971	Lipopolysaccharide export system ATP-binding protein	84/28
BtuE	b1710	Glutathione peroxidase	BtuE	XAC4346	Glutathione peroxidase	99/47

X. citri were compared with *E. coli* (Fig. 4 and Table S4, Supplementary material). Residues that coordinate the ferrichrome in *E. coli* FhuA (PDB 1BY5) and pyoverdine in *P. aeruginosa* FpvA [10, 67] were identified and compared with *X. citri* FhuA sequence showing no sequential similarity (Fig. 4a). Genomewise, *E. coli fhuA* is followed by a co-transcribed operon for the ATP-binding cassette (ABC) transporter *fhuBCD*, which encodes a full importer consisting of permease, ATPase, and a substrate-binding protein, respectively, that are responsible for internalizing ferrichrome through the inner membrane [68]. However, in *X. citri* genome, *fhuA* is located in the same operon as two hypothetic genes (XAC2184 and XAC2186) not being co-transcribed with components of an ABC transporter system like evidenced in *E. coli* genome (Fig. 4a) (Table 2).

The comparison of *X. citri* PfeA with the *E. coli* FepA (47% identity) and *P. aeruginosa* PfeA (50% identity) showed that residues involved in binding of azotochelin, protochelin, and enterobactin in the *P. aeruginosa* protein are present in *X. citri* PfeA, suggesting a possible interaction with these or similar

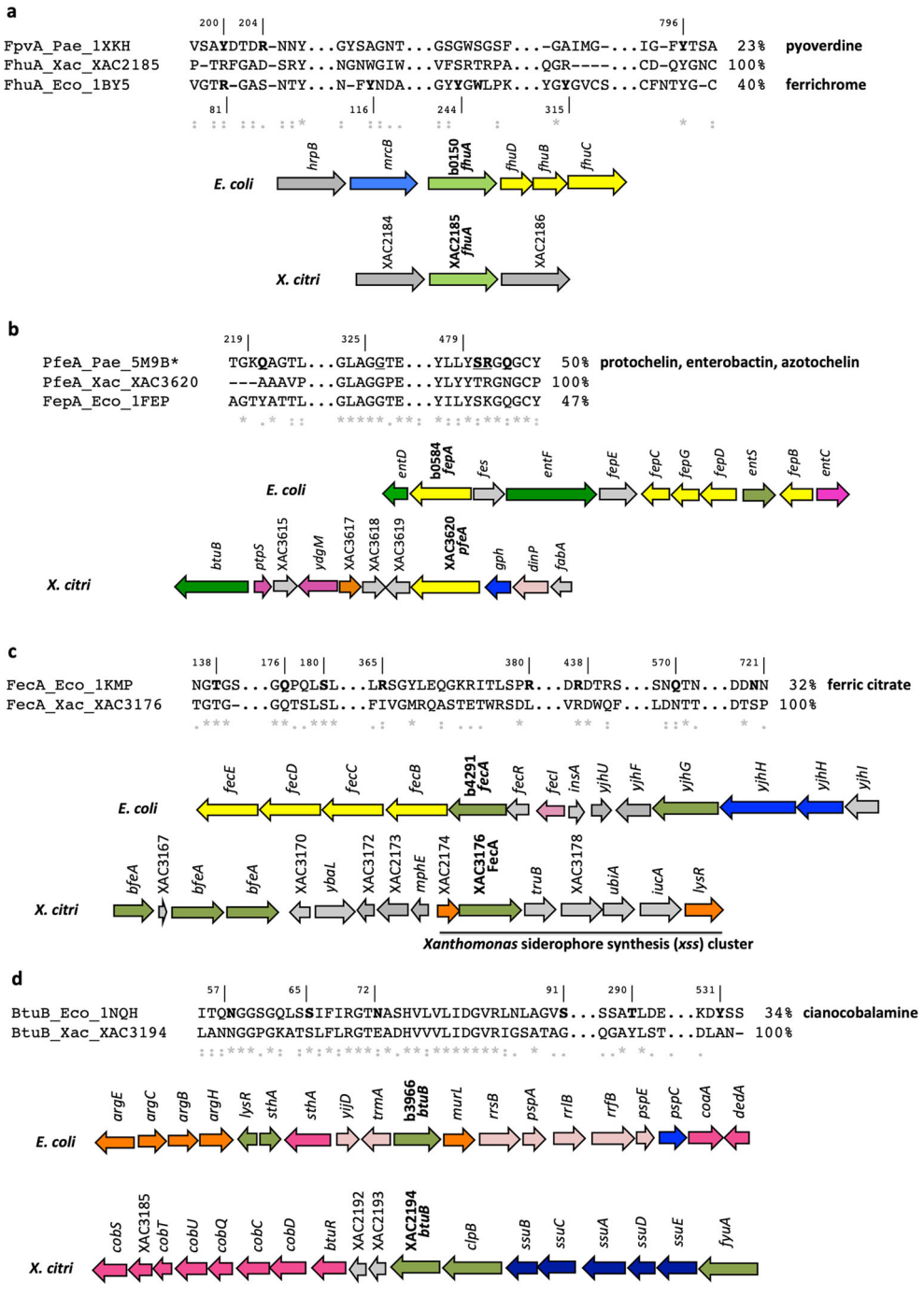

Fig. 4 Partial amino acid sequence alignment and cluster organization of *E. coli* OMRs and their orthologs in *X. citri*. The percentage of amino acid sequence identity between *X. citri* proteins and orthologs is shown. Gene references, names, and colors follow the KEGG code. Residues involved in the coordination of the ligands in the available structures are presented in bold. The gene cluster organization in *X. citri* is also compared with *E. coli*. **a** Partial alignment of *E. coli* FhuA bound to ferrichrome (PDB 1BY5) and its orthologs in *X. citri* (XAC2185) and *P. aeruginosa* FpvA bound to pyoverdine (PDB 1XKH). The localization of *fhuA* gene organization in *X. citri* is compared with *E. coli*. **b** *E. coli* FepA (PDB 1BY5) and its orthologs in *X. citri* (PfeA, XAC3620) and *P. aeruginosa* (PfeA) bound to azotochelin, protochelin, and enterobactin (PDB references 5NR2, 5NC4, and 6Q5E, respectively). **c** Amino acid sequence alignment of *E. coli* FecA and its ortholog in *X. citri*. Residues that interact with ferric citrate in *E. coli* protein (PDB 1KMP) are shown in bold. The *xss* cluster is compared in the two strains. **d** *E. coli* BtuB amino acid sequence and residues that interact with cobalamin (PDB 1NQH, in bold) aligned with the amino acid sequence of the *X. citri* ortholog

siderophores that could be experimentally investigated. As it was shown for *fhuA*, in the gene cluster to which *pfeA* belongs, there are no genes encoding the corresponding ABC transporter components (Fig. 4b).

X. citri FecA, the OMR found as part of *xss*, presented five from seven residues involved in the ferric citrate binding when compared with *E. coli* FecA (PDB 1KMO) (30% identity). *E. coli* FecA–binding site is composed mainly of positively charged residues to interact with negatively charged ferric citrate, and the sequential similarity of *X. citri* FecA might indicate that it could import a similar siderophore. Still, the higher identity with the vibrioferrin receptor from *V. parahaemolyticus* (44%) that has no structure resolved and the presence of *fecA* in *xss* cluster strongly suggests that xanthoferrin is imported by this OMR, not citrate.

E. coli fecA is preceded genomically by a σ-19 factor (*fecI*) and an anti-σ factor (*fecR*), which is an already described alternative regulation system along with Fur [69] and followed by the complete ABC transporter for dicitrate import *fecBCDE*. In *X. citri fecA* gene cluster, at least three genes named *bfeA* encoding putative outer membrane receptors for enterobactin (XAC3166 to XAC3168) are found upstream from the *xss* operons, but again, no ABC transport genes were identified (Fig. 4c, Table 2).

Regarding the cobalamin import, in *X. citri* more than one ortholog of BtuB were identified but only XAC3194 showed 34% of amino acid sequence identity with the *E. coli* putative ortholog. Still, the comparison with the classical BtuB from *E. coli* did not give any clues that it could be functional for the vitamin binding. On the opposite, an operon dedicated to cobalamin synthesis and assimilation, including the transcriptional regulator *btuR* (XAC3191 to XAC3184), is located just downstream of the *btuB* gene (XAC3194) (Fig. 4d).

The identification of the four putative OMRs orthologs in *X. citri* indicates that this bacterium has distinct systems for uptake of siderophores, including some very interesting candidates for experimental approaches such as PfeA (XAC3620) and FhuA (XAC2185). Interestingly, no corresponding ABC transporters were identified at least in the same operons or clusters that encode the OMR genes. This fact suggests that the mechanisms for siderophore uptake and iron assimilation in *X. citri* need to be further explored.

Iron-related genes in different proteomics analyses of *X. citri*

Key proteomic analyses have been performed in the past 5 years in order to access *X. citri* protein profile in different growing conditions. The first analysis was based on the comparison of the proteins that were expressed during infection of *X. citri* in *Citrus sinensis* leaves (*in planta*) and in nutrient broth (NB) rich media (in vitro) [70] (Table 3). The effect of the phosphate in *X. citri* proteome was also evaluated in A medium, known as the Pho regulon inducer, and A medium added of inorganic phosphate (Pi) [71]. We observed that significant amount of proteins identified in these proteomes belong to the cellular envelope such as OMRs that were up- or down-regulated. Different orthologs of the *E. coli* cobalamin transporter BtuB (XAC3050, XAC3444, and XAC2531), ferric enterobactin receptors (XAC3166, XAC3168, XAC3207), and citrate/ferrin transporters (XAC3334, XAC4368, XAC3176 XAC1023) were regulated by phosphate levels and during plant infection. *X. citri* XAC0823 (PhuR), another putative ortholog of *E. coli* FecA (b0150), is present in phosphate depletion in A medium and it is down-regulated in presence of Pi. Besides the membrane receptors and transporters, Fur regulator (XAC1517) and proteins from *xss* cluster (XssA and XssB) were up-regulated in *Citrus sinensis* leaves and A medium + Pi showing the importance of xanthoferrin biosynthesis during infection and stress conditions [70, 71].

TonB receptors also were identified in biofilm proteome when compared with planktonic cells but none of those described in this work [24] (Table 3). Many of the proteins are described with similar functions indicating that *X. citri* has redundancy of systems for iron acquisition during *X. citri* growth, infection, and pathogenesis, which detaches the relevance of the element for the bacterium.

Discussion

Iron assimilation system in *X. citri*, as in many organisms, is a complex network of transcription regulators, siderophore synthesizing proteins, efflux pumps, and inner and outer membrane proteins that together promote the fine homeostasis needed for maintenance of the micronutrients in appropriate levels inside the cell. Despite some similarities, specially regarding the regulators, most of the systems identified in *X. citri* lack the complementary proteins for full import of the siderophores, such as known for *E. coli*, or showed low identity with the most studied proteins in other microorganisms. In the literature, it is not unusual that a great number of siderophore importers are found in organisms that do not possess the machinery for their synthesis or export; those are commonly known as xenosiderophores. Examples of siderophores produced by one species but used by another are described in the literature [72] and represent an adaptative advantage found in several gram-negative bacteria when competing with other organisms for scarce sources of metal [37].

Considering the ability of xenosiderophores import and the putative OMRs presented in this study, it is plausible that *X. citri* can transport siderophores that it does not produce. That would also explain the absence of Fur boxes in the promoter regions of many genes described in this work. On the contrast, the presence of a Fur box at the promoter of the *xss*

Table 3 Iron-related proteins identified in different experimental proteomic studies of *Xanthomonas citri*. Conditions analyzed phosphate levels [71], rich medium and infection conditions [70], and biofilm [24]. A medium: Tris 120 mM, NaCl 80 mM, KCl 20 mM, Na_4Cl 20 mM, Na_2SO_4 3 mM, $MgCl_2$ 1 mM, $ZnCl_2$ 51 μM, bacto peptone 0.5%, glucose 0.5%; $CaCl_2$ 0.2 mM; A medium + Pi (10 mM NH_2PO_4); NB, nutrient rich broth; *Citrus sinensis* (sweet orange) leaves. The name of the proteins is followed by the KEGG reference in parenthesis. Some proteins were identified in more than one reference

Conditions	Group function/reference codes
A medium [71]	Ferric enterobactin receptors BfeA (XAC3166, XAC3168, XAC3207) TonB-dependent receptors BtuB (XAC3050, XAC3444) CirA (XAC0074) FecA (XAC0693, XAC0823, XAC3176*, XAC3334, XAC4368) FhuA (XAC1435, XAC2185, XAC2830, XAC3158, XAC3756) FyuA (XAC3201) Ferric iron uptake outer membrane FhuE (XAC3370, XAC3498) Ferripyoverdine receptor FpvA (XAC0176)* Oar protein (XAC2343) Outer membrane hemin receptor PhuR (XAC0823)* Iron complex outer membrane receptor YncD (XAC0126)
A medium + Pi [71]	Ferric enterobactin receptors BfeA (XAC3166, XAC3168, XAC3207) TonB-dependent receptors (XAC0008, XAC4052) BtuB (XAC2531, XAC2742, XAC3050, XAC3444, XAC3613) CirA (XAC0074) FecA (XAC1023, XAC3176*, XAC3334) FhuA (XAC1435, XAC2185, XAC2830) FyuA (XAC0716, XAC1276, XAC3201) IroN (XAC4048) Ferric iron uptake outer membrane FhuE (XAC3370, XAC3498) Fur regulator (XAC1517)* Ferripyoverdine receptor FpvA (XAC0176)* Oar protein (XAC2343) Outer membrane hemin receptor PhuR (XAC0823) Iron complex outer membrane receptor YncD (XAC0126)
NB (nutrient broth) [70]	Ferric enterobactin receptors BfeA (XAC1149, XAC3168, XAC3207) TonB-dependent receptors BtuB (XAC2531, XAC2600, XAC2742, XAC3444) FecA (XAC3334, XAC4368, XAC3176) FhuA (XAC2830) IroN (XAC3311) Ferric iron uptake outer membrane FhuE (XAC3370) Fur regulator (XAC1517) Iron complex outer membrane receptor YncD (XAC0126) XssA (XAC3177)*
Citrus sinensis leaves [70]	Ferric enterobactin receptors BfeA (XAC3166, XAC3168, XAC3207) TonB-dependent receptors (XAC0008, XAC4052, XAC3489) FhuA (XAC3756) Fur regulator (XAC1517) XssB (XAC3178)*
Biofilm [24]	TonB-dependent receptors (XAC3050, XAC3071, XAC3489) BtuB (XAC3444) BfeA (XAC3168)

*Proteins described in this work

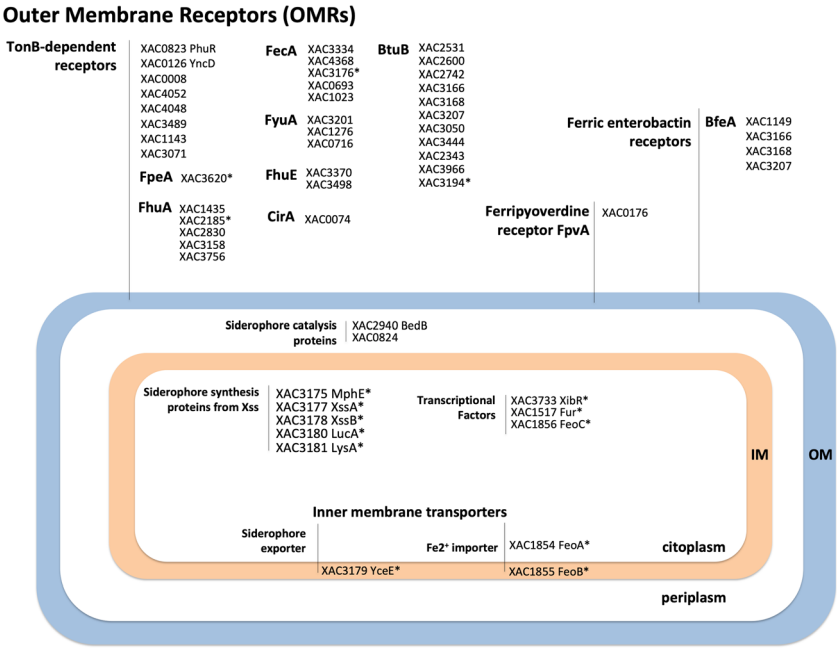

Fig. 5 Schematic localization of the *Xanthomonas citri* iron-related proteins that were previously described in the literature and the ones identified in this work (evidenced with an asterisk). Proteins were separated according to the defined categories of KEGG database

OMR XAC3176 strengthens the hypothesis that this protein is responsible for the import of a *X. citri* siderophore xanthoferrin, the homolog to *X. oryzae* siderophore produced by its own *xss* [41]. Indeed, XAC3176, XAC3177, and XAC3178, all belonging to the same operon that has Fur-binding site, were identified in all proteomic analyses corroborating their relevance for the bacterium.

As for the inner membrane protein FeoB (XAC1855), sequential- and structural-based analyses suggest that the ferrous form of iron could be internalized as described in other species. Besides the OMR XAC3176, most likely the xanthoferrin importer in *X. citri*, other proteins from *xss* cluster that are localized in the cytoplasm were also identified along with the inner membrane exporter XAC3179. Further analyses on the structure and function of each individual protein in *xss* might help in addressing the production of xanthoferrin in *X. citri*.

Finally, the two putative transcriptional factors Fur and XibR from *X. citri* were recognized in the bacterium genome and showed high sequential identity to the extensively characterized *P. aeruginosa* Fur. On the other hand, *X. citri* FeoC is closely related to its ortholog in *X. oryzae*, but poorly comparable with the well-characterized *E. coli* FeoC. Based on the set of results, all the proteins described in this study and those evidenced in proteomics analyses were organized along with their proposed cellular localization (Fig. 5).

Altogether, the findings of this study that were based on the compilation of bioinformatics data and comparisons with proteins described in the literature give functional insights to the *X. citri* pathways for iron internalization and assimilation. Further studies are still needed to address the relationship among the many proteins described and their function in iron uptake, virulence, and pathogenesis of *X. citri*. Moreover, these proteins might become suitable as targets for development of effective growth inhibitors against this phytopathogen in the future.

Authors' contributions GSG wrote the manuscript and prepared the figures. AB helped with organization and data analysis and made the final corrections of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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