Characterization of the CrbS/R
Two-Component System in
Pseudomonas fluorescens Reveals a
New Set of Genes under Its Control
and a DNA Motif Required for
CrbR-Mediated Transcriptional
Activation

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The CrbS/R system is a two-component signal transduction system that regulates
acetate utilization in Vibrio cholerae, P. aeruginosa, and P. entomophila. CrbS is a hybrid
histidine kinase that belongs to a recently identified family, in which the signaling domain
is fused to an SLC5 solute symporter domain through aSTAC domain. Upon activation
by CrbS, CrbR activates transcription of the acs gene, which encodes an acetyl-CoA
synthetase (ACS), and the actP gene, which encodes an acetate/solute symporter. In
this work, we characterized the CrbS/R system in Pseudomonas fluorescens SBW25.
Through the quantitative proteome analysis of different mutants, we were able to identify
a new set of genes under its control, which play an important role during growth on
acetate. These results led us to the identification of a conserved DNA motif in the
putative promoter region of acetate-utilization genes in the Gammaproteobacteria that is
essential for the CrbR-mediated transcriptional activation of genes under acetate-utilizing
conditions. Finally, we took advantage of the existence of a second SLC5-containing
two-component signal transduction system in P. fluorescens, CbrA/B, to demonstrate
that the activation of the response regulator by the histidine kinase is not dependent on
substrate transport through the SLC5 domain.

Keywords: two-component signal transduction systems, Pseudomonas fluorescens, crbS, CBRA,
acetyl-coenzyme A synthetase, acetate metabolism, acetyl-CoA, STAC

INTRODUCTION

The ability to sense and respond to subtle environmental changes is a crucial trait of all
organisms. This has led to the evolution of sophisticated systems that recognize environmental
stimuli and subsequently trigger specific physiological responses. In bacteria, the two-component
signal transduction (TCST) systems regulate chemotaxis, sporulation, nutrient acquisition, and
utilization, among other important cellular processes (Hoch, 2000; Stock et al., 2000; Gao and
Stock, 2009; Gotth et al., 2010; Zschiedrich et al., 2016). As stated by their name, Two-component
systems are comprised of two elements, a histidine kinase as the sensing and signal transmission
component (membrane-bound or cytosolic) and a cytosolic response regulator as the output element. In transmembrane TCST systems, signal detection by an extracytoplasmic sensor domain triggers conformational changes, which propagate across the membrane to the histidine kinase. This results in the phosphorylation of a conserved histidine in the dimerization and histidine phosphotransfer domain (DHP), by the catalytic ATP-binding domain (CA). In many kinases, additional cytoplasmic sensor domains, such as PAS and GAF, regulate this process (Taylor and Zhulin, 1999; Martinez et al., 2002; Gao and Stock, 2009). Finally, a conserved aspartate in the receiver domain of the response regulator (REC) receives the phosphate group.

Recently, our group described the STAC domain; a new protein module associated with bacterial signal transduction (Korycinski et al., 2015). Structurally, the domain consists of a four-helical bundle, composed of two α-hairpins connected by a central loop of typically nine residues; it can be found as a stand-alone protein or within the context of multidomain proteins. In the latter case, it is typically coupled N-terminally to a transmembrane domain related to the sodium-solute symporter family 5 (SLC5) and C-terminally to an array of different domains characteristic of signaling systems, like histidine kinases or the diguanylate cyclase GGDEF domain. Although, the members of the SLC5 family have a variable number of transmembrane domains, those associated with STAC always have thirteen. The role of the STAC domain is still unknown. We speculate that it could mediate interactions with other proteins or regulate the flow of substrates through the transport domain. STAC-containing signaling proteins are broadly represented in bacteria but absent from Archaea and Eukaryotes (Korycinski et al., 2015). Two STAC-containing TCST have been experimentally characterized so far.

CbrA is a histidine kinase that functions as a global regulator of metabolism, virulence, and antibiotic resistance in the *Pseudomonadaceae* (Nishijyo et al., 2001; Zhang and Rainey, 2007; Yeung et al., 2011, 2014; Quiroz-Rocha et al., 2017). Its cognate response regulator, CbrB, possesses a σ54-interacting domain, which regulates expression of different metabolic genes. For example, the CbrAB system governs the utilization of histidine and other amino acids in *P. fluorescens* (Zhang and Rainey, 2007, 2008; Zhang et al., 2015) and glucose uptake in *Azotobacter vinelandii* (Quiroz-Rocha et al., 2017). The CbrAB system also participates in carbon catabolite repression control (Görke and Stülke, 2008). For example, when *P. aeruginosa* and *P. putida* grow on less-favored compounds, the activity of the CbrAB system is enhanced. This leads to an increase in the abundance of the non-coding small RNAs crcZ and crcY, that inhibit the activity of the Crc global regulator, resulting in an increased expression of its targets which participate in catabolism, pathogenesis, resistance to antibiotics, and biofilm formation (O’Toole et al., 2000; Linares et al., 2010; Abdou et al., 2011). Metabolomic studies suggest that the CbrAB system senses the carbon/nitrogen ratio as a way to read carbon limitation, but the actual signaling molecules and the molecular mechanisms of action remain unknown (Valentini et al., 2014; Zhang et al., 2015).

CbrS is a hybrid histidine kinase, which regulates acetate utilization in *Vibrio cholerae, P. aeruginosa*, and *P. entomophila* (Hang et al., 2014; Jacob et al., 2017). CbrR, the cognate response regulator, is a LuxR-family transcriptional activator, which induces expression of the *acs* gene, which encodes an acetyl-CoA synthase (ACS). This enzyme catalyzes the ligation of acetate and coenzyme A (CoA) to produce acetyl-CoA, which subsequently can be fed into the TCA cycle to produce energy and electron carriers. It is proposed that in pathogenic bacteria, the resulting switch from secreting acetate to assimilating it leads to the depletion of intestinal acetate in the host (Hang et al., 2014; Jacob et al., 2017). A RNAseq analysis of *V. cholerae* highlighted several genes involved in diverse pathways, including pathogenesis, as being influenced by this system (Hang et al., 2014). Nevertheless, besides the acetyl-CoA synthase and the acetate transporter ActP no direct link with other genes has been established (Zaoui et al., 2012; Jacob et al., 2017).

Just as CbrA, CbrS is composed of an SLC5 domain with thirteen transmembrane segments, linked to a histidine kinase by a STAC domain. CbrS differs structurally from CbrA through the REC domain at its C-terminal end of the histidine kinase and through its coiled-coil segments. In particular, the STAC and DHP domains of CbrS are linked by an S-helix, a specific type of dimeric parallel coiled-coil involved in signal transduction (Anantharaman et al., 2006; Stewart and Chen, 2010), which is absent in CbrA (Figure 1).

In this work we use *P. fluorescens* SBW25 as a model to identify new genes in the CbrS/R regulon. Further, using transcriptional fusions, we describe an imperfect palindrome in the promoter region of CbrR-regulated genes that is necessary for transcriptional activation. Finally, we begin the functional characterization of the STAC domain and the STAC-containing signal transduction systems through the phenotypic analysis of deletion mutants and chimeric proteins.

**METHODS**

**Growth Conditions**

*P. fluorescens* and *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 28 and 37°C, respectively. M9 medium was supplemented with acetate, glucose, or histidine at final concentrations of 2.5–20 mM. When required, antibiotics were added at the following concentrations: ampicillin (Ap), 100 µg ml–1; gentamicin (Gm), 25 µg ml–1; kanamycin (Km), 30 µg ml–1. For selection against pK18mobSacB, LB agar plates were supplemented with 10% sucrose. Genes cloned in pSRKgm were induced by the addition of 100 µM of isopropyl-β-D-thiogalactopyranoside (IPTG).

Pre-cultures for assays were prepared in a volume of 5 ml as described previously (Zhang et al., 2015). Growth curves were performed using a Synergy H4 Hybrid microplate reader with the Gen5 software (BioTek, Winooski, VT) as described previously (Zhang et al., 2015).

**Plasmid and Strain Construction**

Bacterial strains and plasmids used in this study are listed in Table 1. All molecular techniques were conducted following to...
## FIGURE 1 | Alignment of CbrA and CrbS from *P. fluorescens*

Relevant features are marked as follows: Red, Transmembrane regions of the SLC5 domain; Green, STAC domain; Light Blue, PAS domain; Brown, DHp domain; Gold, CA domain; Dark Blue, REC Domain. Underlined bases indicate Coiled Coils. Boxed residues indicate the conserved Arginine used as fusion point in the construction of chimeric proteins and deletion points for the STAC domain.

| CbrA       | MPMSFLTQMLLISAAYLAALFGWVISERGMIPRAIRHPLTVTSLGVSAYASAAYFTGVGLAYQGYG |
|------------|---------------------------------------------------------------------|
| CrbS       | --MTLSGLIAAVALAYMAIMFPAIFYGDRRRAPLPVRVANVYSLNAVCTSWTFPGAVGQAEEQILWA |
| CbrA       | FLSSYLGVSAFLPVPVLPLLTPILKTRYQTSSLALDLFAFRF-RSTWAAGALTIFMLLGVPLLAILQQA |
| CrbS       | FLPYILGFPLLLLVPWLVQMKILSQENITSIADFAERYGKSPLAVVUCILVGVPLYIAILQKG |
| CbrA       | VADISILTREP-------VQHRVAFLACALTILFTIFFSRHIATREKHEGLVFIAFESVIKLIAGG |
| CrbS       | IVLGDNLLIGADDTGTRAQDTALIVSLLVLAFTIVFGTNLDATEHRGMFLIAFESVIKLFAFLAV |
| CbrA       | GLYALYGVFDFGPQELWQLWLLQNTQATAALHTLPLQEGPWRLLLTVFFASAVPMIMYHTFNTENLNPRLV |
| CrbS       | GAFVTRYGLDFGDFLSQAILAPRLEYWKETNVNSMVQVTGVMAMICLPRFHVTVVENFDPQDLR |
| CbrA       | SASWGPLPLLMLSLAVPLILWAG--LKLGATTTNPYFTLIGIAANPSAALALLAYVGGLASASGLIVTT |
| CrbS       | LAKWFPVFAYLALAFFVIALGKGMMPLGSVLPSYISLPMAEHPALAVLFIAGGSAATGMIVAS |
| CbrA       | LALSQMAINHLPLLPLY--------QQPAAENIYRWLKWTRRALTVAIMAGYGFYLLGACQDLANLGIQAV |
| CrbS       | IALSTMSNMLLPWLLRRSS-AERPVPRFHWMVSVRVSIMVILLYSVRGLGSTASLATICQIAF |
| CbrA       | VATQFLYPGLVLVYPWTANRRGIFAGLAILGVLTVMLLPVGNLQGFIYPPLNNIMYLVDD------ |
| CrbS       | AAVTQALAPAALGALWQANKRRGIFLQAAGATFLFWYTILVPTAKSLGWSLSLPGTLWMSHPLGLSV |
| CbrA       | TSWHMAAISAASLAMFTLSLFTNASPEETSAABAACAVDNV-RRPQFLSLHAASPQEFATQALKPGA |
| CrbS       | TSLTTLGTVFSLAAGFTLFWVMSLSTRVSEHQQAGRFQIEGIQISQASFLSMVQISDLSSLAFVGE |
| CbrA       | KAAQKEVQALRDLYLPDEEDPARYRLRRLRDEIANLSGGLMPVSVQDMVFETFLIPAGENYVTEDIHF |
| CrbS       | ERAQQSFIRFAYRQKGFQNPNDADNWWIAHTERLLAVGLGASSTRAVKAAEIC----------- |
| CbrA       | IESRLEDYHSLRTLGAEELDALLRRYHRQTLQELPMGVSILAKDQEILMWNKAMEELTGIAAQRV-VGSR |
| CrbS       | -EMQLEDYVRIADEASEVLQFNRALLQGAIENTOQGISVDQSLKLVAWNRYLELFNYFDGLISVGRPI |
| CbrA       | NTL----------GDPWKLLEQLGFLNPDEHLKQLHALDGQT RWLNHLKIAADEPAGNPSGLVLLVVE |
| CrbS       | ADIRYNAERGLCPGAEVHVARLHLWQGRAHTSERELPFPNGRVIEILIGNMPGFG------GFVSFPT |
| CbrA       | DLTETQMLLEDKLVH----------SERLAAGLCAAGVAHEIGNPITGIA |
| CrbS       | DITAFREAQAEALTEANEGLEQRYTERTHELQSNVLNTDAKGVASEASQSKTRFIAVSHDMIQPLNAAR |
| CbrA       | CLAQNLRREEED-GEIEISQG1LQEQTRKRSVQSLMSFAHA--GGHOQNDEACVLAEVAQDIAGLAA |
| CrbS       | LFSAAHSHQDNGLSSEARQIVHLDSSLRSAEDLDLSDLISDIRENEKINGORQPQFVNELFTDLGAEFK |
| CbrA       | LNRNRFQVFQNFLCQDPHDWVDGDSSQRLAQVRLINLLSNARDATPAGAVRKTEVFHVDLIVEDEGSI |
| CrbS       | ALAQEQGLRFRLR-GRSLRVDKLLKLRLTNFAYDVAGG--GPFVGLVVRRKGECLDEVWDGRPGI |
| CbrA       | PQNIMDRLEFEPFTTTK-----PGEQETGLGLALVYSIVEEEHYQITSDAPTSQRTRGIRVTLPRLV |
| CrbS       | PQDKQKVIFEFKRLDQHTRAEGGLGLAIADGLCRVLDHRLSVS----WPGKGSFVSRVPLARNQ |
| CbrA       | TSVN---------- |
| CrbS       | ATPLVKTFQETGLPLSGAQVLCDNEESILIGNMRSRLLTRWRGCEVWTADTAQCAALLAEGVRPQLALV |
| CbrA       | YHLHDHGETGTEIMGWLRAQLAEPICPGVISAADGRPENMVAEVHAAGDYLAKPKVPAALRALLSRHPL |

FIGURE 1 | Alignment of CbrA and CrbS from *P. fluorescens*. Relevant features are marked as follows: Red, Transmembrane regions of the SLC5 domain; Green, STAC domain; Light Blue, PAS domain; Brown, DHp domain; Gold, CA domain; Dark Blue, REC Domain. Underlined bases indicate Coiled Coils. Boxed residues indicate the conserved Arginine used as fusion point in the construction of chimeric proteins and deletion points for the STAC domain.
TABLE 1 | Strains and Plasmids used in this work.

| Strain          | Relevant phenotype                  | Reference  |
|-----------------|-------------------------------------|------------|
| **Pseudomonas fluorescens**                      |                                      |            |
| SBW25           | Wild-type strain                    | Silby et al., 2009 |
| PBR809          | ΔcrbA                               | Zhang and Rainey, 2007 |
| ES01            | ΔcrbS                               | This work  |
| ES02            | ΔcrbR                               | This work  |
| ES03            | Δacs                                | This work  |
| ES04            | ΔcrbS with Tn7-crbs, Gm<sup>r</sup> | This work  |
| ES05            | ΔcrbR pSRKgmc-crbr, Gm<sup>r</sup>  | This work  |
| ES06            | Δacs pSRKgmc-acs, Gm<sup>r</sup>    | This work  |
| ES07            | Δpflu1813                           | This work  |
| ES08            | Δact<sup>P</sup>                    | This work  |
| ES09            | Δpflu5625                           | This work  |
| ES10            | Δ0110                               | This work  |
| ES11            | Δpflu1813 Δpflu5625                 | This work  |
| ES12            | Δpflu1813 Δ0110                     | This work  |
| ES13            | Δact<sup>P</sup> Δpflu625           | This work  |
| ES14            | Δact<sup>P</sup> Δ0110             | This work  |
| ES15            | Δpflu5625 Δ0110                     | This work  |
| ES16            | Δact<sup>P</sup> Δpflu625 Δ0110     | This work  |
| ES17            | ΔcrbA ΔcrbS                         | This work  |
| ES18            | ΔcrbA ΔcrbS with Tn7-SLC5-CbrS/HK-CbrA, Gm<sup>r</sup> | This work  |
| ES19            | ΔcrbA ΔcrbS with Tn7-SLC5-CbrA/HK-CbrS, Gm<sup>r</sup> | This work  |
| ES20            | ΔcrbA with Tn7-crbrΔSTAC, Gm<sup>r</sup> | This work  |
| ES21            | ΔcrbS with Tn7-crbrΔSTAC, Gm<sup>r</sup> | This work  |
| ES22            | SBW25 pSRKgmc-crbr<sup>R</sup>      | This work  |
| ES23            | SBW25 pSRKgmc-acs                   | This work  |
| **Plasmid**     | Relevant phenotype/usage            | Reference  |
| pSRKgmc         | Broad-Host-Range Expression Vector, Gm<sup>r</sup> | Khan et al., 2008 |
| pSRKgmc-crbr<sup>R</sup> | Expression of crbr in P. fluorescens, Gm<sup>r</sup> | This work  |
| pSRKgmc-acs     | Expression of acs in P. fluorescens, Gm<sup>r</sup> | This work  |
| pET30<sup>b</sup> | High-level expression of target proteins. Km<sup>r</sup> | Novagen |
| pET30<sup>c</sup>-crbr<sup>R</sup> | Heterologous expression of CrbR, Km<sup>r</sup> | This work  |
| pK18mobSacB-crbS | Allelic exchange vector, Km<sup>r</sup>, Sac<sup>α</sup> | Schäfer |
| pK18mobSacB-crbS | Deletion of crbs, Km<sup>r</sup>, Sac<sup>α</sup> | This work  |
| pK18mobSacB-crbr<sup>R</sup> | Deletion of crbr, Km<sup>r</sup>, SacB | This work  |
| pK18mobSacB-acs | Deletion of acs, Km<sup>r</sup>, SacB | This work  |
| pK18mobSacB-pflu1813 | Deletion of pflu1813, Km<sup>r</sup>, Sac<sup>α</sup> | This work  |
| pK18mobSacB-act<sup>P</sup> | Deletion of act<sup>P</sup>, Km<sup>r</sup>, SacB | This work  |
| pK18mobSacB-pflu5625 | Deletion of pflu5625, Km<sup>r</sup>, SacB | This work  |
| pK18mobSacB-pflu0110 | Deletion of pflu0110, Km<sup>r</sup>, SacB | This work  |

(Continued)

standard protocols (Sambrook and Russell, 2001). *Escherichia coli* DH5α was used for general cloning. *E. coli* DH5α::pir was used for cloning in pK18mobSacB, and *E. coli* B21 DE3 was used for the heterologous expression of proteins. *P. fluorescens* was transformed by electroporation as described elsewhere.
All constructs were verified by sequencing. Primers used in this study are listed in Table 2.

Plasmids pSRKgm-acs, pSRKgm-cbrR, and pET30-cbrR were built by cloning the ORF of each gene into the NdeI/BamHI sites of plasmids pSRKgm and pET30.

Derivatives of pk18mobSacB used for allelic exchange were obtained by cloning in the plasmid a PCR product containing the gene to be deleted, together with 500 bp to 1 kb of upstream and downstream sequence. Then the targeted gene was amplified out by total PCR of the plasmid with phosphorylated divergent primers. The resulting PCR product was ligated, and the religated plasmid was recovered by transformation.

To construct plasmid pUC18T-mini-Tn7T-Gm-GW-crbS, the crbS coding region with its putative promoter region was PCR-amplified and cloned into the pEntR221 vector from Invitrogen using the Gateway BP Clonase II enzyme mix (Invitrogen). The crbS region was then transferred to vector pUC18T-mini-Tn7T-Gm-GW, using the Gateway LR Clonase II enzyme mix (Invitrogen). To construct plasmids pUC18T-mini-Tn7T-Gm-GW-crbS ΔSTAC and pUC18T-mini-Tn7T-Gm-GW-cbrA ΔSTAC, the STAC domain was amplified from plasmids pEntR221-crbS and pCR8-cbrA respectively, using phosphorylated divergent primers. The resulting PCR product was ligated, and the religated plasmid was recovered by transformation. The crbS ΔSTAC and cbrA ΔSTAC regions were then transferred to vector pUC18T-mini-Tn7T-Gm-GW, using the Gateway LR Clonase II enzyme mix (Invitrogen).

Chimeric constructs PcrbS-SLC5(CrbS)/HK(CbrA) and PcrbR-SLC5(CbrA)/HK(CrbS) were obtained by overlapping PCR of a synthetic DNA fragment and two PCR products as illustrated in Figure S1 and in Supplementary File 1. The resulting product was cloned in pUC18T-mini-Tn7T-Gm-GW with pEntR221 as an intermediary using gateway technology as described earlier in this section.

Plasmid pBBGFP was constructed by amplifying out the uidA gene of pBB53Gus with divergent primers. The resulting PCR was ligated with a phosphorylated PCR product of the GFP gene from pET3α [GFP(LVA); Ferris et al., 2012]. Transcriptional fusions were constructed by cloning a PCR product of each putative promoter region in the EcoRI/KpnI sites of plasmid pBB53Gus or pBB53GFP. Mutagenesis of the acs promoter transcriptional fusions was achieved by the quick-change strategy (Agilent) using plasmid pBB53PracsGUS or pBB53acsGFP as a template. Transcriptional fusions used to map the acs promoter were built by amplifying plasmid pBB53PracsGUS with divergent primers carrying an EcoRI site or a KpnI site. The resulting PCR product was digested with the corresponding restriction enzyme, ligated, and transformed for the recovery of the reconstituted plasmid.

Strains ES04 and ES21 were built through Tn7 mediated integration by transforming strain ES01 with pUX-BF13 and the corresponding derivative of vector pUC18T-mini-Tn7T-Gm-GW as describe elsewhere (Choi et al., 2005; Choi and Schweizer, 2006). The same strategy was used for strains ES18 and ES19, which were derived from strain ES17, and for strains ES20 and ES21 derived from strain PBR809 and strain ES01, respectively.

### Proteomic Analysis

Five-milliliter cultures of strains sbw25, ES01, and ES03 were inoculated from pre-cultures to an A620 of 0.5 in M9 medium supplemented with acetate and incubated at 30°C. After 8 h cultures were pelleted, frozen with liquid nitrogen and delivered to the Proteome Center of the University of Tübingen for processing and analysis. After tryptic digestion and methylation, samples were examined by liquid chromatography-tandem mass spectrometry-LC-MS/MS analysis on a Proxeon Easy-nLC1200 coupled to a QExactive HF (130 min gradient, HCD, Top12). Processing of data was done using MaxQuant software (vs. 1.5.2.8). The spectra were searched against a UniProt Pseudomonas fluorescens SBW25 database (7,063 entries). Downstream analysis was done using Perseus software (vs. 1.5.0.15) to perform significanceB calculation of normalized protein group ratios (P-value threshold 0.01). Dataset is available as Supplementary File 2.

### β-Glucuronidase Assays

Five-milliliter cultures of the appropriate strains were inoculated from pre-cultures to an A620 of 0.5 in M9 medium supplemented with glucose or acetate and incubated at 30°C for 8 h. β-Glucuronidase activity expressed in modified Miller units was quantified as described previously (Jefferson et al., 1986). Photometric measurements were performed with the Synergy H4 Hybrid microplate reader.

### GFP Detection Assays

Five-milliliter cultures of the appropriate strains were inoculated from pre-cultures to an A620 of 0.5 in LB medium, and heterologous expression of CrbR was induced with 500 µM IPTG (isopropyl-β-D-thiogalactopyranoside). After 4 h of incubation at 37°C, 200 µl of each culture were transferred to a 96-well plate, and GFP fluorescence (485/530 nm), and cell density A620 were measured using the Synergy H4 Hybrid microplate reader.

### 5′Race

RNA was isolated using the Rneasy Mini kit (Qiagen) from a 5 ml culture of P. fluorescens SBW25 (O.D.600 0.5) incubated under acetate-utilizing conditions for 8 h. cDNA synthesis was performed with the RevertAid First-Strand cDNA Synthesis Kit (Thermoscientific) using primers ACSIRACE1, ActPRACE1, 0110RACE1, and 5625RACE1 for acs, pflu1813/actP, pflu0110, and pflu5625, respectively. A poly-ATP tail was added to the 5′ end of cDNAs using Terminal Transferase (ThermoScientific) as instructed by the manufacturer and the tailed cDNA sample was subjected to PCR amplification using kit’s oligo(dT)18.
### TABLE 2 | Primers used in this work.

| Name               | Sequence                               | Use                                                                 |
|--------------------|----------------------------------------|----------------------------------------------------------------------|
| CrbS-800f          | CTGCCTTACAGCAGGCGAGGATTGACGG          | Cloning of the crbS region in pK18mobSacB                            |
| CrbS-800R          | CTGCCTTACAGCAGGCGAGGATTGACGG          | Cloning of the crbS region in pK18mobSacB                            |
| CrbSdel1           | GGTAACTCGAGCATAGGAGGAC                | Deletion of crbS                                                     |
| CrbSdel2           | GGTAACTCGAGCATAGGAGGAC                | Deletion of crbS                                                     |
| CrbSGWF            | GGGGACCACTTTGCAAAAGGCGGTTGCAAGG     | Gateway cloning of the crbS region                                  |
| CrbSGWR            | GGGGACCACTTTGCAAAAGGCGGTTGCAAGG     | Gateway cloning of the crbS region                                  |
| pflu1195-600f      | TACTGTCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbR region in pK18mobSacB                            |
| pflu1195-500r      | TACTGTCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbR region in pK18mobSacB                            |
| pflu1195del1       | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| pflu1195del2       | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| pflu1195NdeIF      | TACTGTCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbR region in pK18mobSacB                            |
| pflu1195BamHI      | TACTGTCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbR region in pK18mobSacB                            |
| 4766-600f          | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| 4766-500r          | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| 4766del1           | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| 4766del2           | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| 4766Ndel           | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| 4766NdeI           | GGTAACTCCGAGCATAGGAACAC              | Deletion of crbS                                                     |
| 5625-750f          | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| 5625-700r          | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| 5625del1           | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| 5625del2           | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| 0110-700f          | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| 0110-700r          | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| 0110del1           | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| 0110del2           | CATGCTGCAAGCTTGCAAGGCGGTTGCAAGG     | Cloning of the crbS region in pK18mobSacB                            |
| Pracs1EcoF         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Pracs1kpnR         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Pr5625ecoF         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Pr5625kpnR         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Pr1813ecoF         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Pr1813kpnR         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Pr0110ecoF         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Pr0110kpnR         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Acs1PrMapF50       | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Acs1PrMapF100      | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Acs1PrMapR         | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| −76                | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| −62                | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| MapfrontF          | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Map+1R             | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Map+17R            | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |
| Map-18R            | TGGGATCCGAGCATAGGAACAC              | Cloning of the promoter region of acs in pSRKgm                      |

(Continued)
primer and primers ACS1RACE2, ActPRACE2, 0110RACE2, and 5625RACE2. Only PCR reactions for \textit{acs} and \textit{pflu1813/actP} generated products of the expected size. Finally, the PCR products were sequenced using primers ACS1RACEseq and ActPRACEseq.

\section*{Bioinformatics and Statistical Analysis}

The Microbial Genomic Context Viewer (Overmars et al., 2013) was used to search for homologs of the CrbS/R regulon in Gammaproteobacteria and to retrieve their putative promoter region. The search was performed in all the genomes available in the platform, but when several strains of the same species were available, only one was used. MEME (Bailey et al., 2009) and FIMO (Grant et al., 2011) searches were run with the default options searching for a motif between 6 and 30 nucleotides long. Sequence analyses were performed in the MPI bioinformatics toolkit (Alva et al., 2016; https://toolkit.tuebingen.mpg.de) using Quick2D, HHpred (Hildebrand et al., 2009), PCOILS (Lupas et al., 1991), and MARCOIL (Delorenzi and Speed, 2002). Transmembrane segments were predicted with FMHMM (Sonnhammer et al., 1998) and Phobius (Käll et al., 2004). For transcripational fusions, comparisons between samples were performed by ANOVA and Tukey’s multiple comparison test using SPSS version 23 (IBM).

\section*{RESULTS}

\subsection*{Identification of Proteins Regulated by CrbS/R in \textit{P. fluorescens} sbw25}

In \textit{P. fluorescens} sbw25, CrbS is encoded by the gene \textit{pflu4471}, CrbR by the gene \textit{pflu1195}, and the acetyl-CoA synthase by the gene \textit{pflu4766}. Although, regulation of \textit{acs} by CrbR has been shown in several species of Gammaproteobacteria, there is little knowledge about other possible genes under the control of the CrbS/R system. First, we verified that the behavior of the CrbS/R system in \textit{P. fluorescens} system in \textit{P. fluorescens} \textit{sbw25}, CrbS is encoded by the gene \textit{pflu4471}, CrbR by the gene \textit{pflu1195}, and the acetyl-CoA synthase by the gene \textit{pflu4766}. Although, regulation of \textit{acs} by CrbR has been shown in several species of Gammaproteobacteria, there is little knowledge about other possible genes under the control of the CrbS/R system. First, we verified that the behavior of the CrbS/R system in \textit{P. fluorescens} is as reported in other bacteria. To do so, we constructed strains \textit{ES01 (∆crbS)}, \textit{ES02 (∆crbR)}, and \textit{ES03 (∆acs)} from \textit{P. fluorescens} \textit{SBW25} and tested them for their ability to grow on acetate. As expected, the three deletion mutants were unable to grow on M9 minimal medium supplemented with acetate, unless complemented with the corresponding gene (\textit{Figures 2A–C}). Next, we performed a quantitative proteome analysis of cultures of strains \textit{SBW25, ES01 (∆crbS), ES02 (∆crbR)}), and \textit{ES03 (∆acs)} after incubation for 8 h in M9 minimal media supplemented with acetate. This approach permits the direct comparison of protein abundance between strains, allowing us to distinguish those proteins that changed their abundance as a consequence of acetate utilization from those that were affected by the deletion of \textit{∆crbS}. We identified four proteins, Pflu0110, Pflu0111, Pflu05625, and Pflu05626.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Name} & \textbf{Sequence} & \textbf{Use} \\
\hline
MidMut1 & GGGCTGCGCTATAGAACCTGGCCATCTGAGTTGTAATACCAAGCAGCCCTA & Mapping of the \textit{acs} promoter \\
MidMut2 & TGGGCGCTTGTACCCATCTGCCATGAGTTGTAATACCAAGCAGGGGATC & Mapping of the \textit{acs} promoter \\
S3g-uidAF & CTTATTGGCTGAACAGCTTTGCTGAAA & Deletion of the UidA from pBBR53Gus \\
S3g-uidAR & ACGGTGCTTGTGACGTGAGTTGTAATACCAAGCAGGGGATC & Deletion of the UidA from pBBR53Gus \\
GFPfwrd & ATGAAAGTTAAAGATCTGCGTAAAGGAGAA & Cloning of gfp in pBBR53Gus \\
GFPrev & TTAGATTTCTCGTGCTTTGCTGAGTTGTAATACCAAGCAGGGGATC & Cloning of gfp in pBBR53Gus \\
CbrAgWF & GGGGACAACATCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
CbrAgWR & GGGGACAACATCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
crbSLC5HybR & GGGGACAACATCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
cbraTCSTHybF & CTCCTCCAGATGTCGTTGTCG & Gateway cloning of the cbrA region \\
cbraSLC5HybR & CTCCTCCAGATGTCGTTGTCG & Gateway cloning of the cbrA region \\
crbSdelSTAC1 & AGCGTGGCTGCTTTGCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
crbSdelSTAC2 & AGCGTGGCTGCTTTGCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
crbSdelSTAC1 & AGCGTGGCTGCTTTGCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
crbSdelSTAC2 & AGCGTGGCTGCTTTGCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
cbrAgWF & GGGGACAACATCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
cbrAgWR & GGGGACAACATCTGAGTTGTAATACCAAGCAGGGGATC & Gateway cloning of the cbrA region \\
\hline
\end{tabular}
\caption{Relevant restriction sites are underlined.}
\end{table}
Sepulveda and Lupas

Characterization of CrbS/R in P. fluorescens

FIGURE 2 | Growth curves of deletion mutants of (A) crbS, (B) crbR, (C) acs, and (D) members of the crbS/R regulon in P. fluorescens SBW25. Bacteria were grown in M9 minimal medium with acetate as the sole source of carbon. Results are means for six independent cultures. Relevant phenotypes are indicated in parenthesis.

FIGURE 3 | Dispersion graph showing the relative abundances (mutant/WT) of proteins in the Δacs and ΔcrbS P. fluorescens strains compared to the wild-type strain (SBW25) when growing on acetate. Colored dots indicate proteins in which direction of change rate differed between the Δacs and ΔcrbS strains. Only proteins with significant changes in abundances ($P \leq 0.01$) are represented.

actP has been shown previously to be transcriptionally activated by CrbR (Jacob et al., 2017) and pflu1813 is found in the same operon as actP (pflu1814) throughout Gammaproteobacteria. This supports the notion that CrbR also regulates the other genes identified through our approach. To confirm this hypothesis we built β-glucuronidase transcriptional fusions of the putative promoter region of each of the genes that code for the identified proteins, plus acs, and tested their expression in different genetic backgrounds. Our results show that all five transcriptional fusions are activated in a wt strain when incubated in M9 medium supplemented with acetate, but not with glucose (Figure 4 and Figure S2). This activation did not happen in the ES01 (ΔcrbS) nor the ES02 (ΔcrbR) strains (Figure 4 and Figure S2), demonstrating that the CrbS/R system controls these genes. Interestingly, in strain ES03 (Δacs), all transcriptional fusions showed high levels of transcription when incubated with glucose. This observation is consistent with the results from the proteome analysis. It could be explained by the continuous triggering of the CrbS/R system, present in this strain, because of the accumulation of acetate from the growth medium and the cell metabolism, due to lack of acetyl-CoA synthase activity (Figure 4). Whether acetate or an unknown related molecule is the signal responsible for triggering the CrbS/R system is still pending to be experimentally determined.

Pflu1813, ActP, and Pflu5625 that increased their abundance in the SBW25 and ES03 strains but decreased it in strain ES01. Since both mutants are unable to grow on acetate, differences in protein abundance between the two strains can be directly linked to the deletion of CrbS (Figure 3).

Pflu0110 Is Necessary for Optimal Growth in Acetate

So far, we have demonstrated that Pflu0110, Pflu1813, ActP, and Pflu5625 are part of the CrbS/R regulon. Pflu0110 is predicted
to be an Acyl-CoA Hydrolase, an enzyme that could hydrolyze acetyl-CoA into CoA and acetate. Pflu1813 is annotated as a putative membrane protein of unknown function that belongs to the DUF485 family. ActP is a cation/acetate symporter, which as mentioned previously, has been shown to be part of the CrbS/R regulon. Seeking to understand the role that these proteins have in acetate utilization we constructed deletion mutants of each of the genes and challenged them to grow on M9 minimal media supplemented with acetate. While strains ES07, ES08, and ES09 (Δpflu1813, ΔactP, and Δpflu5625, respectively) showed a negligible effect, the strain ES10 (Δpflu0110) presented a 5-h delay in reaching stationary phase, which also occurred at a lower optical density (Figure 2D). The minimal impact of pflu1813, actP, and pflu5625 deletions on acetate utilization could be explained by a suppression effect between them. To discard this possibility, we constructed different combinations of double and triple mutants and tested their ability to grow on acetate. Only those in which pflu0110 was deleted showed an impairment of their capacity to utilize acetate on the same scale as strain ES10 (Data not shown).

Characterization of the Crbs/R Activated Promoters

In the next step, we studied the sequence properties of crbR-responsive promoters that mediate specific activation under acetate utilizing conditions. We used 5’ RACE to determine the transcription initiation site (TIS) from the acs and actP promoters. These are located at 34 and 36 base pairs from the first codon of the ORF, respectively. In both regions, immediately after the transcription initiation site, we found a Crc motif (AAnAAnAA) that suggests catabolite repression (Moreno et al., 2015). Additionally, in the first 36 base pairs upstream of the initiation sites we located putative RpoD promoters (Potvin et al., 2008), an observation congruent with an earlier bioinformatic analysis in *P. aeruginosa* (Schulz et al., 2015). Although, we did not succeed to identify the transcription initiation site in the case of genes pflu0110, and pflu5625, we also found putative Crc motifs around 30 nucleotides upstream of the beginning of the ORFs.

To determine the minimal region necessary for CrbR-dependent regulation, we built transcriptional fusions from several derivatives of the acs promoter and measured their activity in M9 medium supplemented with acetate in a wt and a ∆crbR background (Figure 5A). Our results outlined a minimal inducible promoter encompassing the region between the −62 and +1 nucleotides of our original construct (Figure 5 fusion Pracs-35). Most notably, the dramatic loss of induction of the Pracs-35 derivative that ends just after the predicted RpoD promoter, compared to Pracs-62, indicated that an element essential for CrbS/R-mediated induction could be located in this region (Figure 5 fusion Pracs-62 and Pracs-35).

An Imperfect Inverted Repeat Is Required for CrbR-Mediated Transcriptional Activation

CrbR is a member of the LuxR family, whose members are known to recognize and bind inverted repeats (Evangelista-Martínez et al., 2006; Antunes et al., 2008). To identify any conserved motifs required for CrbR-mediated promoter induction, we obtained the sequence of the putative promoter region of *acs* homologs from 31 Gammaproteobacteria (*Table 3*) and ran a motif search using the MEME, a tool for the discovery of novel, ungapped motifs in sequences (Bailey et al., 2009). We were able to identify an imperfect inverted repeat pattern within the 5’ end of the coding strand, which was present in all the sequences analyzed. Particularly, in the *acs* promoter of *P. fluorescens* SBW25, it is located between the −52 and −36 bases from the transcription initiation site, immediately before...
the start of the RpoD promoter (Figure 5A). Not only is this observation congruent with our results from the mapping of the acs promoter, but the identified inverted repeat is also located in the putative promoter regions of actP, pflu5625, and pflu0110 (Figure 5B). Moreover, we performed a motif search of the putative promoter regions of a total of 112 identified homologs of acs, actP, pflu5625, and pflu0110 in 31 species of Gammaproteobacteria. We found the imperfect inverted repeat in 91 (81%) of the analyzed sequences (Table 3) and determined a consensus sequence using MEME (Figure 5C). Consistent with the absence of the CrbS/R system in members of the family, the identified motif was not found in the vicinity of the homologs of these genes in enterobacteria. All the evidence we gathered pointed to a role of this inverted repeat in the transcriptional activation of the CrbS/R regulon in the presence of acetate, so we named it the ACTU motif (acetate utilization).

To test this hypothesis, we constructed transcriptional fusions with two derivatives of the acs promoter with one (Pracs1M) and four (Pracs4M) point mutations, respectively, which break the symmetry of the repeat. Boxed bases indicate mutations inserted to disrupt the ACTU motif. Activities of transcriptional fusions of derivatives of the acs promoter in a wild-type or a ΔcrbS strain under Acetate-utilizing conditions. Miller units are expressed as means ± Standard deviations of results from at least three independent experiments. (B) Sequence of the ACTU motif of the CrbS/R regulon and the acs promoter derivatives with one (Pracs1M) and four (Pracs4M) point mutations, respectively, which break the symmetry of the repeat. Boxed bases indicate mutations inserted to disrupt the ACTU motif. (C) Sequence Logo of the ACTU motif.

Finally, we used the FIMO module of the MEME suite, that finds individual motif occurrences in a sequence database (Grant et al., 2011), to prospect for additional genes regulated by the CrbR/S system in P. fluorescens SBW25. Besides the genes identified in this work, the program predicted new occurrences of the palindromic sequence in the upstream region of several genes (Table 4). These predictions should be approached cautiously as the q-value suggests they may not be significant. However, this was the case for pflu0110, which we have experimentally proved to be regulated by CrbR. Therefore, experimental verification should be obtained for the most interesting predictions, for example, genes pflu3808 and pflu4952, which code for Isocitrate dehydrogenase and Fumarase, respectively. Both enzymes are part of the TCA cycle, and particularly the Isocitrate dehydrogenase is located at the branching point of the glyoxylate cycle that cells activate when growing on acetate (Wolfe, 2005).
The SLC5 Domain of CrbS Can Replace the SLC5 Domain of CbrA

In bacteria, there is mounting evidence for the existence of multi-protein complexes that link transport and signaling. For example, several signal transduction systems use permeases, ABC-transporters, or soluble substrate-binding proteins as co-sensors (Tetsch and Jung, 2009; Västermark and Saier, 2014). In this context, CrbS and CbrA represent a unique type of sensor, in which the signaling and transport domains are connected within the same polypeptide. In the case of CbrA, previous data suggest that the protein not only senses histidine, but is also capable of internalizing it, and that this process is dependent on signaling. Physical coupling between the N-terminal SLC5 domain and the C-terminal Histidine kinase of CbrA is required for function (Zhang et al., 2015). These observations raise the possibility that transport of the substrate through the SLC5 domain elicits a signal, which could trigger or modulate the activity of the histidine kinase. To test this hypothesis, we generated strain ES17, a double ΔcbrA ΔcrbS deletion mutant. When tested, this strain was not able to grow on acetate as a carbon source nor in histidine as a carbon and nitrogen source (Figures 7A–D). Next, using overlapping PCR, we built two chimeric constructs, from CbrA and CrbS, in which the promoter and the SLC5 domain regions of each gene were exchanged. A conserved arginine located immediately before the STAC domain was used as the crossover point (Figure 1 and Figure S1 and Supplementary File 1). These constructs were used independently to complement strain ES17, giving rise to strains ES18 (SLC5-CbrS/HK-CbrA) and ES19 (SLC5-CbrA/HK-CrbS; Figure S1). Both strains were then challenged to grow in M9 media supplemented with histidine or acetate or a combination of both. Strain ES18 was able to thrive in any media containing histidine (Figures 7A,B). The presence of acetate in the medium had no effect, as long as histidine was also added (Figures 7B,D). These results show that, in CbrA, substrate transport is not needed for the induction of the signal. The previously observed lack of activity of the histidine kinase when not physically coupled to the SLC5 domain can be explained then by increased instability, misfolding, or failure to dimerize. Strain ES19 was not able to grow in any of the conditions tested (Figures 7A–D). Two factors may explain this. (I) The chimera is expressed from the cbrA promoter, which may not be active under acetate-utilizing conditions; (II) CrbS may have additional structural limitations.

TABLE 3 | Search for the ACTU motif in homologs of genes from the P. fluorescens SBW25 CrbS/R regulon in the +, Gene found with the ACTU motif. −, Gene found without the ACTU motif in the promoter region.

| Strain                                      | acs | actP | pflu0110 | pflu5625 |
|---------------------------------------------|-----|------|----------|----------|
| Pseudomonas aeruginosa PA01                 | +   | +    | +        | actP     |
| Pseudomonas brassicacearum NFM421           | +   | +    | +        | +        |
| Pseudomonas entomophila L48                 | +   | +    | +        | +        |
| Pseudomonas fluorescens SBW25               | +   | +    | +        | +        |
| Pseudomonas fulva 12X                       | +   | +    | NF       | +        |
| Pseudomonas mendocina                        | +   | +    | NF       | −        |
| Pseudomonas montelii SB3078                 | +   | +    | +        | +        |
| Pseudomonas poae RE1114                     | +   | +    | +        | +        |
| Pseudomonas putida BIRD-1                   | +   | +    | +        | +        |
| Pseudomonas resinovarans                    | +   | +    | +        | +        |
| Pseudomonas stutzeri A1501                  | −   | +    | +        | −        |
| Pseudomonas. syringae 144B8                 | +   | +    | +        | +        |
| Vibrio anguillarum 775                      | +   | +    | NF       | +        |
| Vibrio cholerae O395                        | +   | +    | NF       | +        |
| Vibrio fischeri MJ11                       | +   | +    | NF       | +        |
| Vibrio fumissii NCTC 11218                  | +   | +    | NF       | +        |
| Vibrio vulnificus CMCP6                     | +   | +    | NF       | +        |
| Acinetobacter oleivorans DR1                | +   | +    | +        | actP     |
| Photobacterium profundum SS9                | +   | +    | NF       | +        |
| Pseudomonas atlantica T6c                   | +   | +    | +        | NF       |
| Pseudomonas haloplanktis TAC125             | +   | +    | +        | NF       |
| Shewanella baltica BA175                    | +   | +    | NF       | +        |
| Shewanella frigidimarina NCIMB 400          | +   | +    | NF       | actP     |
| Shewanella putrefaciens 200                  | +   | +    | NF       | +        |
| Shewanella woodyi ATCC S190                  | +   | +    | NF       | +        |
| Thalassoliutus oleivorans MILL-1             | +   | +    | NF       | actP     |
| Xanthomonas albiziana GPE PC73              | −   | +    | NF       | NF       |
| Xanthomonas axonopodis pv: citri str. 306    | +   | +    | NF       | NF       |
| Xanthomonas campestris pv: Campestris        | +   | −    | +        | NF       |
| Xanthomonas citri subsp. citri Aww12879      | +   | +    | NF       | NF       |
| Xanthomonas fuscans 4834R                    | +   | +    | NF       | NF       |
| Citrobacter rodentium ICC168                 | −   | −    | NF       | NF       |
| Cronobacter sakazakii BAA-894               | −   | −    | NF       | NF       |
| Dickeya Dadanti 3937                        | −   | −    | NF       | NF       |
| Enterobacter cloacae EcWSU1                  | −   | −    | NF       | NF       |
| Eschenichia coli DH1                        | −   | −    | NF       | NF       |
| Klebsiella pneumoniae                       | −   | −    | NF       | NF       |
| Legionella pneumophila                      | −   | −    | NF       | NF       |
| Salmonella enterica                         | −   | −    | NF       | NF       |

NF, Gene not found in the genome. actP, Gene transcribed in an operon from the actP promoter.
TABLE 4 | New genes with a predicted ACTU motif in *P. fluorescens* SBW25.

| Gene     | Annotation                          | q-value | Matched_ACTU motif sequence                                      |
|----------|-------------------------------------|---------|------------------------------------------------------------------|
| PFLU4766 | Acs Acetyl-coenzyme A synthetase     | 0.00315 | TACTACCATCGTCGAAT                                                  |
| PFLU0447 | Methyl accepting chemotaxis protein | 0.00315 | TACGACTAAGTGCTAAA                                                   |
| PFLU1813 | Hypothetical protein                 | 0.0196  | TAATACTAAGTCGTCT                                                   |
| PFLU5624 | Transcriptional elongation factor g6A | 0.077   | CTAGACTTACGTCCAA                                                  |
| PFLU5625 | Putative Signal-transduction protein| 0.077   | CTAGACTTACGTCCAA                                                  |
| PFLU3314 | CheV chemotaxis protein             | 0.239   | TACTACCATCGTCGAAT                                                  |
| PFLU1406 | Ribosomal protein R50              | 0.345   | TACGACTAAGTGCTAAA                                                   |
| PFLU3808 | NAD(P)+ Isocitrate dehydrogenase    | 0.517   | TACGCTTAAAGTCGCAC                                                  |
| PFLU1460 | Putative phage regulatory protein   | 0.517   | TACGACCCCTGTCATG                                                   |
| dsbA     | Thiol/disulfide interchange protein | 0.552   | TAAACACTAGTGCAAT                                                   |
| PFLU4471 | CrbS                                | 0.6     | GTCAAGCAAGGTCGGGT                                                  |
| PFLU181 | tRNA-Met                           | 0.633   | TAATCCCTTGGTCTAG                                                  |
| PFLU1110 | Acyl-CoA Hydrolyase                 | 0.633   | CGTGACCATGTCGGAT                                                   |
| PFLU1212 | Polymine ABC transporter            | 0.657   | TACGACTAAGTGCTAAA                                                   |
| PFLU4952 | Fumarase                            | 0.717   | AAGGACCTAAGTCGGGT                                                  |
| PFLU4951 | Iron/sulfur-binding oxidoreductase  | 0.737   | TTTGCCCATGTCGGG                                                    |
| wssB     | Cellulose synthase                  | 0.808   | CACGCGCTTGGTCAAG                                                   |
| PFLU4643 | Nitrate reductase                   | 0.828   | GCGACCAAAGTGGCA                                                    |
| PFLU3097 | Aldehyde dehydrogenase             | 0.916   | TACGACCCCTGTCGGG                                                   |
| PFLU2727 | SAM dependent methyltransferase     | 0.916   | TACGCCCCAAAGTGGC                                                   |
| PFLU4120 | Multi-drug transporter              | 0.93    | CACGCCCAAGTTCGG                                                   |

Shaded lines indicate genes experimentally identified in this work. The q-value is the minimal false discovery rate at which a motif occurrence is assumed significant. We used a value of q < 0.05 as a threshold for significance.

DISCUSSION

Despite the characterization of the CrbS/R system and its effect on pathogenesis in different species of Gammaproteobacteria, the study of its role as a regulator of acetate utilization has been limited to its control of acetyl-CoA synthase. In this work, we identified and characterized new genes that are regulated by this system. *Pflu0110* was the only gene whose deletion impaired the ability of *P. fluorescens* to grow on acetate, while deletion of *pflu1318*, *actP*, and *pflu5625* had no apparent effect. These genes could, however, play a role under natural conditions. *Pflu0110* is predicted to code for an Acyl-CoA Hydrolase. CoA is the major acyl group carrier in cells, required for the metabolism of carbohydrates, amino acids, fatty acids, and ketone bodies; it is used to modulate the activity of enzymes and transcriptional factors. Under conditions in which acetate is present in high concentration and the main energy source, it is possible to predict a scenario where the intracellular pool of CoA becomes depleted, as it is incorporated into the acetate utilization pathway. As a result, it will be unavailable for other processes that require CoA, hindering cell growth. The activity of the Acyl-CoA hydrolase antagonizes that of the acetyl-CoA synthase, so it is feasible to propose that its role in acetate utilization is to maintain the availability of CoA for other cellular processes. *Pflu1813* encodes a membrane protein of unknown function. However, since it is always found in an operon with *actP*, even in organisms that lack the CrbS/R system, it is probably functionally associated with ActP. ActP was characterized in *E. coli* as an acetate transporter with narrow specificity. As in *P. fluorescens*, an *E. coli* ActP-deficient mutant showed no impairment when growing on acetate (Gimenez et al., 2003). Since cells can transport acetate by passive diffusion, it has been proposed that ActP is required for scavenging acetate in micromolar concentrations (Gimenez et al., 2003). The scale at which we monitor growth on acetate requires concentrations that range between 2.5 and 20 mM, which do not resemble the conditions in nature. As a result, even though we can follow the overall process of acetate utilization, the subtle adjustments and processes in a poor and competitive environment may not be triggered, or are overwhelmed, and go undetected. To circumvent these limitations, we propose the use of experimental setups that better resemble natural conditions, like microcosms (Craig et al., 2004) or the use of infection models, as has been recently done with *P. entomophila* and *Drosophila* (Jacob et al., 2017). The same may explain the lack of phenotype of the *pflu5625* mutant. *Pflu5625* is annotated as a signal-transduction protein with CAMP-binding (Berman et al., 2005), CBS (Baykov et al., 2011), and nucleotidyltransferase domains, predicted to regulate protein activity by nucleotidylation in response to changing levels of CAMP, or other unknown molecules (Aravind and Koonin, 1999). A detailed characterization of this protein is required to identify these signals as well as the pathways in which it is involved.

The mapping of the promoter regions of *actP* and *acs* allowed us to determine the minimal fragment required for expression and induction of genes controlled by the CrbS/R system. Inside this region, we identified an inverted repeat.
FIGURE 7 | Growth curves of *P. fluorescens* SBW25 strains carrying CrbS/CbrA chimeras. Bacteria were grown in M9 minimal medium complemented with (A) 20 mM histidine, (B) 20 mM histidine and 5 mM acetate, (C) 20 mM acetate, and (D) 20 mM acetate and 5 mM histidine. Results are means for six independent cultures. Relevant phenotypes are indicated in parenthesis. When histidine is at a 20 mM concentration, it is the only carbon and nitrogen source. Otherwise, ammonium chloride is used as the nitrogen source.

needed for CrbR-dependent promoter activation, which we named the ACTU motif. *Acs* and *actP* are the only members of the *P. fluorescens* CrbS/R regulon that are conserved among the Gammaproteobacteria while *pflu0110* and *pflu5625* are restricted mostly to the pseudomonales and the vibriionales. Additionally, the ACTU motif was not found in the upstream region of genes of the Enterobacteriaceae, which lack CrbR and CrbS. Altogether, these observations show that, although a conserved set of acetate-utilization genes is present in the Gammaproteobacteria, diversification in accessory genes and regulation has occurred across the lineage.

Chimeras have been proved to be useful tools for the structural and functional characterization of Two-component systems (Utsumi et al., 1989; Skerker et al., 2008; Capra and Laub, 2012; Mondéjar et al., 2012; Ganesh et al., 2013). Our results with the SLC5-CrbS/HK-CbrA chimera show that the histidine kinase domain from CbrA does not depend on a signal triggered by the transport of histidine through the SLC5 domain to activate CbrB. This finding is consistent with the role of these systems in the detection of compounds that are often found in low levels in nature. If signaling depended on transport, under micromolar concentrations of the substrate the activity of the transporters would compete with that of the sensors, risking a premature interruption of induction. We rather propose that the fusion of the SLC5 domain and the histidine kinase in the STAC containing TCST sensors allows for early detection and fast response of subtle concentrations of substrate in a highly competitive environment.

Our tests with the ΔSTAC mutants may have been limited, as discussed earlier, by the scale at which we can measure the ability to utilize carbon sources. The STAC domain is present in all proteins in which an SLC5 domain is linked to the sensor of a Two-component system. This hints for an important role of this domain under conditions that we have not reproduced. We are currently designing new CrbS/CbrA chimeras in order to answer these questions and to continue to pursue the characterization of the processes modulating sensing and signaling in this particular group of proteins.

**AUTHOR CONTRIBUTIONS**

ES and AL: Conception and design, acquisition of data, analysis and interpretation of data, writing of the article.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02287/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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