The two-component system ChvGI maintains cell envelope homeostasis in *Caulobacter crescentus*

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Abstract

Two-component signal transduction systems (TCS) are often used by bacteria to rapidly assess and respond to environmental changes. ChvG/ChvI is a TCS conserved in α -proteobacteria and known for regulating expression of genes related to exopolysaccharide production, virulence and growth. The sensor kinase ChvG autophosphorylates upon yet unknown signals and phosphorylates the response regulator ChvI to activate transcription. Recent studies in *Caulobacter crescentus* showed that *chv* mutants are sensitive to vancomycin treatment and fail to grow in synthetic minimal media. In this work, we identified the osmotic imbalance as the main cause of growth impairment in synthetic minimal media. We also determined the ChvI regulon and confirmed that ChvI regulates cell envelope architecture at different levels by controlling outer membrane, peptidoglycan assembly/recycling and inner membrane proteins. Furthermore, we identified genes with osmoregulatory properties and confirmed that osmotic upshift is a signal triggering ChvG-dependent phosphorylation of ChvI. In addition, we challenged *chv* mutants with other cell envelope related stress and found that targeting with antibiotics the transpeptidation of peptidoglycan during cell elongation impairs growth of the mutant. Moreover, these antibiotics activate expression of the *chvIG-hprK* operon in ChvI-dependent and independent ways. Finally, we observed that the sensor kinase ChvG fused to a fluorescent protein relocates from a patchy-spotty distribution to distinctive foci after transition from complex to synthetic minimal media. Interestingly, this pattern of (re)location has been described for proteins involved in cell growth control and peptidoglycan synthesis upon osmotic shock. Overall, our data support that the ChvGI

43 TCS is mainly used to maintain cell envelope homeostasis by monitoring osmotic
44 imbalances and damages in the peptidoglycan layer.
45

Introduction

Two-component systems (TCS) equip cells with a rapid sensing and response mechanism to optimise survival in changing and stressful environments. The first component of canonical TCS, a sensor histidine kinase (HK), autophosphorylates upon input signal detection on a conserved histidine residue using the gamma-phosphoryl group of ATP. Thereafter, the HK phosphorylates a conserved aspartate residue of the second component of TCS, a cognate response regulator (RR). In most cases, the RR harbours an output domain that binds to DNA upon phosphorylation to either activate or repress transcription of target genes (Stock *et al.*, 2000; Goulian. 2010; Capra & Laub. 2012). On the other hand, HKs can also exert specific phosphatase activities on their RRs to turn off the response in absence of the input signal (Gao and Stock. 2009; TuAnh and Stewart. 2011).

The Chv (**ch**romosomal **v**irulence factor) TCS conserved in α -proteobacteria and composed of the HK ChvG and the RR ChvI, was first reported as a pathogenic regulator in *Agrobacterium tumefaciens* responding to acid stress (Mantis & Winans. 1993; Li *et al.*, 2002). A study in *Sinorhizobium meliloti* showed that the ChvG homologue ExoS is negatively regulated by the periplasmic protein ExoR (Chen *et al.*, 2008). Further research in *A. tumefaciens* showed that low pH triggers ExoR proteolysis to derepress ChvG/ChvI activity (Wu *et al.*, 2012). ExoR orthologs are absent in other α -proteobacteria, such as in the animal intracellular pathogen *Brucella abortus* and the aquatic free-living bacterium *Caulobacter crescentus* (Castillo-Zéledon *et al.*, 2021; Stein *et al.*, 2021). Nonetheless, the ChvGI-dependent response to acidic conditions seems to be conserved. For instance, the ChvI homologue BvR

in *B. abortus* is phosphorylated in combination of low pH and nutrient depletion conditions, which mimics post-infection conditions (Altamirano-Silva *et al.*, 2018).

In *C. crescentus*, ChvGI activates the expression of the small non-coding RNA (sRNA) ChvR when exposed to acidic stress or DNA damage with mitomycin C, or when cultured in synthetic minimal media (Frölich *et al.*, 2018). Once produced, ChvR subsequently inhibits translation of the TonB-dependent receptor (TBDR) ChvT (Frölich *et al.*, 2018). Interestingly, inactivating of *chvR* or *chvIG* sensitises *C. crescentus* cells to vancomycin treatment whereas a *chvT* mutant makes them resistant to this antibiotic, which suggests that vancomycin passes through the outer membrane via ChvT to reach the periplasm (Vallet *et al.*, 2020). In addition, a *chvIG* knock-out (Δ *chvIG*) mutant could not propagate in synthetic minimal media when cells are inoculated at low density, while inactivating *chvT* in a Δ *chvIG* background partially restored growth (Stein *et al.*, 2021). Although *chvIG* mutants are sensitive to acid stress when grown in minimal media, the primary cause for the growth defect has not been determined.

ChvGI is also known in *C. crescentus* to coordinate its regulation with another TCS, NtrYX, which is under control of the periplasmic protein NtrZ (Stein *et al.*, 2021). NtrZ has been only described in *C. crescentus*, while the HK NtrY and RR NtrX are known for regulating multiple processes in α -proteobacteria such as nitrogen metabolism, motility, virulence and cell envelope integrity (Pawloski *et al.*, 1991; Carrica *et al.*, 2012; Wang, D *et al.*, 2013; Lemmer *et al.*, 2020). Stein *et al.* (2021) showed that both ChvI and NtrX networks significantly overlap and these RRs have opposite regulatory functions in minimal media. For instance, while ChvI acts as a positive regulator of growth, NtrY inhibits growth. Interestingly, repression of growth in synthetic media is

caused by unphosphorylated NtrX, so that inactivating *ntrX* in a Δ *chvI* background also partially restored growth. Actually, NtrY acts as a phosphatase over the phosphorylated NtrX (NtrX~P), while NtrZ inhibits NtrY to presumably maintain high NtrX~P levels.

Here we show that *chvIG* mutants are greatly impacted due to osmotic imbalances in minimal media, but also in complex media supplemented with osmolytes. In agreement with previous data (Stein *et al.*, 2021), deletion of *chvT* and *ntrX* restored growth in hypertonic conditions. We provide a ChvI regulon, using ChIP-seq with polyclonal anti-ChvI antibodies and RNA-seq, which unveiled new targets related to peptidoglycan synthesis and recycling. We also showed that (i) ChvI is phosphorylated upon osmotic shock in a ChvG-dependent way and (ii) expression of the *chvIG-hprK* operon is induced upon treatment with antibiotics. Overall, our results confirm that the ChvGI TCS senses and responds to cell envelope stress by inducing expression of multiple genes related to cell morphology and envelope homeostasis.

Methods

Bacterial strains and growth conditions.

Strains, plasmids and oligonucleotides used in this study are listed in supplementary tables 1, 2 and 3. Plasmid construction details are presented in supplementary methods. *E. coli* strains were grown aerobically in either LB (broth) sigma or LB + 1.5% agar at 37 °C. All *C. crescentus* strains in this study are derived of the NA1000 wild-type strain, and growth was achieved at 30 °C in aerated conditions using either complex medium Peptone Yeast Extract (PYE) or synthetic media supplemented with glucose (M2G or M5GG) as already described in (Ronneau et al., 2016). M2G and M5GG were prepared using M2 (12.25 mM Na₂HPO₄, 7.75 mM KH₂PO₄, 9.35 mM NH₄Cl) and M5 (10 mM PIPES pH 7.2, 1 mM NaCl, 1 mM KCl, 0.37 mM Na₂HPO₄, 0.23 mM KH₂PO₄) salts, respectively, and both supplemented with (0.5 mM MgSO₄, 0.5 mM CaCl₂, 0.01 mM FeSO₄, 0.2% glucose). 1 mM glutamate sodium was added to make M5GG. Modified M2G 0% (Na⁺, K⁺), M2G 25% (Na⁺, K⁺) and M2G 50% (Na⁺, K⁺) were prepared without Na₂HPO₄ and KH₂PO₄ or either 50% or 25% of the Na₂HPO₄ and KH₂PO₄ concentrations in M2G, respectively. *C. crescentus* was grown on plates using either PYE or M2G with 1.5% agar at 30 °C. Growth was monitored measuring OD₆₆₀ in liquid cultures using an automated plate reader (Biotek, Epoch 2) with continuous shaking at 30 °C. Gene expression under the control of inducible promoter (P_{xyIX}) was induced either with 0.1 % D(+)-xylose (Sigma). Expression of enhanced green fluorescent protein (*egfp*) and monomeric cherry *mcherry* protein fusions was induced in fresh exponentially growing cultures (OD₆₆₀ ~ 0.1) for 1 h and 4 h. Generalized transduction was performed with phage ϕ Cr30 according to the procedure described in (Ely, 1991). Antibiotics for *E. coli* were used with the following

final concentrations ($\mu\text{g ml}^{-1}$, in liquid/ solid medium) ampicillin (50/100), kanamycin (30/50), oxytetracycline (12.5/12.5), chloramphenicol (30/20) and for *C. crescentus* kanamycin (5/20), oxytetracycline (1/2.5), mecillinam (100/100), A22 (2.5,5) where appropriate. Plasmid delivery into *C. crescentus* was achieved by either bi- or tri-parental mating using *E. coli* S17-1 and *E. coli* MT607 as helper strains, respectively. In-frame deletions were created by using the pNPTS138-derivative plasmids as follows. Integration of the plasmids in the *C. crescentus* genome after single homologous recombination were selected on PYE plates supplemented with kanamycin. Three independent recombinant clones were inoculated in PYE medium without kanamycin and incubated overnight at 30 °C. Then, dilutions were spread on PYE plates supplemented with 3% sucrose and incubated at 30 °C. Single colonies were picked and transferred onto PYE plates with and without kanamycin. Finally, to discriminate between mutated and wild-type loci, kanamycin-sensitive clones were tested by PCR on colony using locus-specific oligonucleotides.

Spotting assays

Ten-fold serial dilutions (in PYE) were prepared in 96-well plates from 5 ml cultures in standard glass tubes grown overnight at 30 °C in the corresponding media. Cells were then spotted on plates, were incubated at 30 °C for two days and pictures were taken.

β -galactosidase assays

Overnight saturated cultures of *Caulobacter* cells harbouring lacZ reporter plasmids were diluted $\geq 50\times$ in fresh medium and incubated at 30 °C until OD₆₆₀ of 0.3 to 0.5. 100 μl samples were collected in a 96 well plate and kept at -80 °C until measurement.

Then, 50 μ l of aliquots of the previously frozen samples were thaw and immediately treated with 50 μ l Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, pH 7.0) supplemented with 0.1 g polymyxin B and 0,27 % (v/v) β -mercaptoethanol for 30 min at 28 °C. To this, 150 μ l of Z buffer was added, followed by 50 μ l of 4 mg/ml O-nitrophenyl- β -D-galactopyranoside (ONPG). Then, ONPG hydrolysis was measured at 30 °C for 30 min. The activity of the β -galactosidase expressed in miller units (MU) was calculated using the following equation: MU = (OD₄₂₀ $\times$ 1,000) / [OD₆₆₀ $\times$ t $\times$ v] where “t” is the time of the reaction (min), and “v” is the volume of cultures used in the assays (ml). Experimental values were the average of three independent experiments.

Microscopy

Strains grown in PYE were imaged either in exponential or stationary phase using 1.5 % agar pads with the indicated medium. Cells in osmotic shock conditions were pelleted and washed twice with the indicated stress medium and imaged in 1.5 % agar pads maintaining the stress condition. Images were obtained using Axioskop microscope (Zeiss), Orca-Flash 4.0 camera (Hamamatsu) and Zen 2.3 software (Zeiss). Temperature (30 °C) was maintained stable during microscopy analysis using the Tempcontrol 37-analog 1 channel equipment (HemoGenix®) coupled to the Axioskop microscope. Images were processed with ImageJ. Demographs were obtained with MicrobeJ by segmenting each cell, integrating fluorescence, sorting cells by length and plotting fluorescence intensity and cellular widths to indicate the relative position of the protein fusions, in which 0 represents mid-cell and 2 or -2 the cell poles (Ducret *et al.*, 2007).

Protein purification

In order to immunize rabbits for production of ChvI polyclonal antibodies His6-ChvI was purified as follows. A BL21(DE3) strain harboring plasmid pET-28a-*chvI* was grown in LB medium supplemented with kanamycin until an OD₆₀₀ of 0.7 was reached. IPTG (isopropyl-β-D-thiogalactopyranoside) (Thermo Fisher Scientific) was added at a final concentration of 1 mM, and the culture was incubated at 37 °C for 4 h. Then, cells were harvested by centrifugation for 20 min at 5,000 *g* and 4 °C. The pellet was resuspended in 20 ml BD buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10% glycerol, 10 mM MgCl₂, 12.5 mM imidazole) supplemented with complete EDTA-free protease cocktail inhibitor (Roche), 400 mg lysozyme (Sigma), and 10 mg DNase I (Roche) and incubated for 30 min on ice. Cells were then lysed by sonication and the lysate by centrifugation (12,000 rpm for 30 min at 4 °C) was loaded on a Ni-nitrilotriacetic acid (Ni-NTA) column and incubated for 1 h at 4 °C with end-over-end shaking. The column was then washed with 5ml BD buffer, 3ml Wash1 buffer (BD buffer with 25 mM imidazole), 3ml Wash2 buffer (BD buffer with 50 mM imidazole), and 3ml Wash3 buffer (BD buffer with 75 mM imidazole). Proteins bound to the column were eluted with 3ml elution buffer (BD buffer with 100 mM imidazole) and aliquoted in 300 μl fractions. All the fractions containing the protein of interest (checked by Coomassie blue staining) were pooled and dialyzed in dialysis buffer (50 mM Tris [pH 7.4], 12.5 mM MgCl₂).

Immunoblot analysis

Proteins crude extracts were prepared by harvesting cells from exponential growth phase (OD₆₆₀ ~0.3). The pellets were then resuspended in SDS-PAGE loading buffer by normalizing to the OD₆₆₀ before lysing cells by incubating them for 10 min at 95 °C. The equivalent of 0.5 ml of cult (OD₆₆₀ = 0.3) was loaded and proteins were subjected to electrophoresis in a 12% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane then blocked overnight in 5% (wt/vol) nonfat dry milk in phosphate buffer saline (PBS) with 0.05% Tween 20. Membrane was immunoblotted for ≥ 3 h with primary monoclonal anti-GFP (1:5,000) antibodies (JL8, Clontech-Takara), then followed by immunoblotting for ≤ 1 h with secondary antibodies: 1:5,000 anti-mouse linked to peroxidase (Dako Agilent), and visualized thanks to Clarity™ Western ECL substrate chemiluminescence reagent (BioRad) and Amersham Imager 600 (GE Healthcare).

Chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) assay.

A ChIP-Seq protocol was followed as described by Coppine *et al.* (2020). Briefly, 80 ml of mid-log-phase cells (OD₆₆₀ of 0.6) were cross-linked in 1% formaldehyde and 10 mM sodium phosphate (pH 7.6) at room temperature (RT) for 10 min and then for 30 min on ice. Cross-linking was stopped by addition of 125 mM glycine and incubated for 5 min on ice. Cells were washed twice in phosphate buffer solution (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) resuspended in 450 µl TES buffer (10 mM Tris-HCl [pH 7.5], 1mM EDTA, and 100 mM NaCl), and lysed with 2 µl of Ready-lyse lysozyme solution for 5 min at RT. Protease inhibitors (Roche) were added, and the mixture was incubated for 10 min. Then, 550 µl of ChIP buffer (1.1%

227 Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], and 167 mM NaCl, plus
 228 protease inhibitors) was added to the lysate and incubated at 37 °C for 10 min before
 229 sonication (2 x 8 bursts of 30 sec on ice using a Diagenode Bioruptor) to shear DNA
 230 fragments to a length of 300 to 500 bp. Lysate was cleared by centrifugation for 10
 231 min at 12,500 rpm at 4 °C, and protein content was assessed by measuring the OD₂₈₀.
 232 Then, 7.5 mg of proteins was diluted in ChIP buffer supplemented with 0.01% SDS
 233 and precleared for 1 h at 4 °C with 50 µl of SureBeads Protein A Magnetic Beads
 234 (BioRad) and 100 µg bovine serum albumin (BSA). One microliter of polyclonal anti-
 235 Chv1 antibodies was added to the supernatant before overnight incubation at 4 °C
 236 under gentle agitation. Next, 80 µl of BSA presaturated protein A-agarose beads was
 237 added to the solution and incubated for 2 h at 4 °C with rotation, washed once with
 238 low-salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl [pH 8.1],
 239 150 mM NaCl), once with high-salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA,
 240 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-
 241 40, 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCl [pH 8.1]), and once with TE buffer
 242 (10 mM Tris-HCl [pH 8.1] 1mM EDTA) at 4 °C, followed by and a second wash with
 243 TE buffer at RT. The DNA-protein complexes were eluted twice in 250 µl freshly
 244 prepared elution buffer (0.1 M NaHCO₃, 1% SDS). NaCl was added at a concentration
 245 of 300 mM to the combined eluates (500 µl) before overnight incubation at 65 °C to
 246 reverse the cross-link. The samples were treated with 20 µg of proteinase K in 40mM
 247 EDTA and 40 mM Tris-HCl (pH 6.5) for 2 h at 45 °C. DNA was extracted using a
 248 Nucleospin PCR cleanup kit (Macherey-Nagel) and resuspended in 50 µl elution buffer
 249 (5 mM Tris-HCl [pH 8.5]). DNA sequencing was performed using an Illumina HiSeq

4000 instrument (Bio.be). NGS data were analysed as described in Coppine et al., 2020.

RNA-seq.

WT and $\Delta chvI$ cells were grown O.N to OD₆₆₀ 0.3 and, then, exposed to 6% sucrose for 4 h. Thereafter, total RNA was extracted with RNeasy® Protect Bacteria Kit from Qiagen and following manufacturers instructions. The quantity and quality (A260/A280 ration) of RNA was determined with a Thermo Scientific™ Nanodrop™ One Microvolume UV-Vis Spectrophotometer. Samples were sent for analysis at Bio.BE company and analysed using Galaxy (Afgan *et al.*, 2018).

In vivo ³²P labelling

Cells were grown overnight in PYE, then washed twice and grown in M5G medium lacking phosphate and was grown overnight in M5G with 0.05 mM phosphate to OD₆₆₀ of 0.3. Then, one milliliter of culture was labelled for 4 min at 30 °C using 30 μCi γ-[³²P]ATP (PerkinElmer). For osmotic shock experiments, samples were incubated with 6% sucrose for 7 minutes at 30 °C. Then, cells were pelleted for 2 minutes at 15000 rpm and the supernatant removed completely without disturbing the pellets. Cell pellets were resuspended in 50 μl Lysis Buffer (50mM Tris pH 7.0, 150mM NaCl, 80 mM EDTA, 2% Triton X100; sterilised with 2 μm Acrodisc syringe filters) by pipetting. Lysed samples were incubated 3 minutes on ice. And 450 μl of cold LBS was added and proteins were collected using centrifugation for 15 minutes at 13000 and 4 °C. Then, 40 μl of SureBeads Protein A Magnetic Beads (BioRad) were washed 4 times in 1 ml PBS + 0.1% Tween (PBS-T) and finally resuspended in 65 μl. Thereafter, 3 μl

of anti-ChvI antibody were added to the washed protein A-agarose beads and incubated at RT in a shaker at 1300 rpm. Protein A beads with anti-ChvI antibodies were washed 3 times with PBS-T and 1 time with cold (4 °C) Low Salt Buffer (LSB; 50mM Tris pH 7.0, 100mM NaCl, 50 mM EDTA, 2% Triton X100; sterilised with 2 µm Acrodisc syringe filters), immediately resuspended in 50 µl LSB and kept in ice. ChvI immunoprecipitation (IP) was performed by adding the Protein A beads with anti-ChvI antibodies previously prepared to the cell-free protein samples, and incubating at 4 °C with rotation for 90 minutes. IP samples were washed 1 time in 1ml LSB and 3 times in 1 ml High Salt Buffer (50mM Tris pH 7.0, 500mM NaCl, 50 mM EDTA, 0.1% Triton X100; sterilised with 2 µm Acrodisc syringe filters). Samples were eluted using 25 µl of 2,5X SDS-loading Buffer and incubation at 37 °C for 5 minutes. Samples with SDS loading Buffer were separated from magnetic beads, and 20 µl loaded on a SDS-Page gel and run at 200V-50mA-100W. The gel was dried for 1h at 70 °C under vacuum in a Model 583 Gel Dryer (BioRad). Finally, the dried gel was exposed on phosphoscreen for 5 days and revealed using a Cyclone Plus Phosphor Imager (PerkinElmer).

Results

High osmolyte concentration in M2G impairs growth of *chvIG* mutants

It was shown previously that *C. crescentus chvIG* mutants failed to grow in minimal media with xylose as sole carbon source (M2X), while these mutants grew similarly to WT cells in complex media (PYE) (Stein *et al.*, 2021). Likewise, we observed that mutants inoculated in M2G (with glucose as sole carbon source) in both solid and liquid media were impaired for growth (Fig. 1A-B). To date, the cause for growth impairment in *chvIG* mutants in minimal media has not been determined. Considering that stressful conditions are present in synthetic minimal media (M2G or M2X) but missing in PYE, we tested osmolytes as causative agents. Indeed, the synthetic minimal media M2G contains a higher concentration of osmolytes than the complex media PYE (Hocking *et al.*, 2012). Therefore, we assessed the viability of *chvIG* mutants on plates lacking Na⁺ and K⁺ salts (Fig. 1A; M2G 0% Na⁺, K⁺). In these conditions the *chvI* mutant grew similarly to WT cells. We also reduced these osmolytes in liquid cultures, and observed that growth was restored in the *chvI* mutant when half (and below) of the regular concentration found in M2G was used (Fig. 1B; M2G 50% Na⁺, K⁺). Furthermore, we observed that adding osmolytes (NaCl or sucrose) to complex media impaired growth and decreased viability of a *chvI* mutant (Fig. 1A; PYEX + 40 mM NaCl; PYE + 6% Sucrose). Additionally, ectopic expression of *chvI* from the xylose-inducible promoter ($P_{xyIX}::chvI$) in a *chvI* mutant restored growth in PYEX plates supplemented with NaCl whereas expressing back *chvI* in a *chvIG* mutant did not (Fig. 1A). Finally, while a phospho-mimetic mutant of *chvI* (*chvID52E*) grew similarly to WT, a phospho-ablative mutant of *chvI* (*chvID52A*) failed to propagate

in M2G (Fig. 1C). Together, our data show that a fully functional ChvIG TCS is required to survive and grow in hyperosmotic environments.

Mutations in *chvT* and *ntrX* improve fitness of the *chvI* mutant under osmotic stress

Considering that mutations in *chvT* and *ntrX* partially alleviated the growth impairment of *chvI* mutants in minimal media (Stein *et al.*, 2021), we tested whether *chvT* or *ntrX* inactivation could also partially protect *chvI* mutants from hypertonic conditions. We first confirmed that a $\Delta chvI$ mutant was impaired for growth under osmotic stress by using an excess of KCl in PYE (Fig. 2A). In contrast, neither *ntrX* nor *chvT* inactivation did interfere with growth in PYE + KCl conditions compared to WT (Fig. 2A). Interestingly, we observed that inactivating *ntrX* in a $\Delta chvI$ background restored growth similar to WT, while the $\Delta chvT$ mutation led to cultures with slightly higher OD in liquid cultures. Thus, these data suggest that growth impairment in liquid upon osmotic upshift in a *chvI* mutant is primarily caused by upregulation of ChvT or NtrX.

As expected, $\Delta chvI$ cells poorly grew on PYE plates supplemented with KCl whereas the growth of $\Delta chvT$ cells was similar to the WT on the same plates (Fig. 2B). Surprisingly, in contrast to liquid cultures (Fig. 2A), we observed that $\Delta ntrX$ cells did not grow as the WT in PYE plates but this growth delay was not aggravated in the presence of KCl excess (Fig. 2B). Nevertheless, inactivating either *ntrX* or *chvT* in a $\Delta chvI$ background partially restored growth on KCl plates (Fig. 2B). These data indicate that additional cell envelope defects in *chvI* mutants are responsible for growth impairment on hypertonic plates. Altogether, these data suggest that the homeostasis of the cell envelope is lost in *chvI* mutants, leading to a hypersensitivity to osmolytes.

Fig. 2. Growth and viability of *chvI*, *chvT* and *ntrX* mutants in hypertonic conditions. (A) Percentage of optical density (OD₆₆₀) after 22 hrs of growth in PYE liquid with (dotted bars) or without 50 mM KCl (solid bars). The data represent the average values of biological replicates (n=3, error bars show standard deviation). *** = $p < 0.0001$, single factor ANOVA analysis of OD₆₆₀ after 22 hrs of growth in PYE supplemented with 50 mM KCl compared $\Delta chvI$ cells. (B) Viability of single ($\Delta chvI$, $\Delta chvT$ and $\Delta ntrX$) and double ($\Delta chvI \Delta chvT$ and $\Delta chvI \Delta ntrX$) mutants on PYE agar with or without 50 mM KCl.

ChvI regulon reveals osmotic stress and cell envelope-related target genes

Transcriptomic analyses were previously performed on a $\Delta chvI$ mutant overexpressing the phospho-mimetic mutant *chvI*_{D52E} in PYE (Stein *et al.*, 2021). Nonetheless, cells expressing *chvI*_{D52E} as the only copy, either from its own promoter at the endogenous *chvI* locus ($P_{chvI}::chvI_{D52E}$) or ectopically from the xylose-inducible promoter ($P_{xyIX}::chvI_{D52E}$) led to a slight growth delay and cell filamentation (Fig. 1C and Fig. S1).

This suggests either that ChvI_{D52E} does not perfectly mimic phosphorylated ChvI or that unphosphorylated ChvI is also required for optimal growth in synthetic minimal media. Hence, we determined the ChvI regulon of WT cells grown in M2G by using ChIP-seq with polyclonal antibodies targeting ChvI, which allowed to unveil the DNA regions directly bound by ChvI (Fig. 3A, Supplementary table 4).

From our experiment, 169 DNA binding sites were identified for ChvI (Fig. 3A, Table S4). About 30% of them (52) correspond to sequences in the vicinity of 101 genes previously identified in the regulon of ChvI_{D52E} in PYE. The 117 remaining peaks correspond to promoter regions of potentially new candidates. Interestingly, the top target is the promoter region of *chvT* itself, suggesting that ChvI regulates ChvT both (i) directly at the transcriptional level and (ii) indirectly at the post-transcriptional level via transcriptional activation of the sRNA *chvR* (Fig. 3A). Beside *chvT* and *chvR*, we identified potential new targeted genes involved in (i) cell division, morphology and peptidoglycan (PG) synthesis, such as *mreB*, *ftsZ*, *ftsN* and *dipM*; and (ii) general stress response (GSR), such as *sigT* (sigma factor T), *nepR* (*sigT* antagonist) and *phyKR* (TCS regulating NepR activity negatively).

By performing RNA-seq on WT and $\Delta chvI$ cells grown in PYE and incubated a few hours in PYE supplemented with 6% sucrose, we found 169 genes with ≥ 1.5 -fold change (Supplementary Table 4). From these binding sites, ~69% correspond to genes identified in our ChIP-seq experiment and ~31% to genes identified in the previous regulon generated by Stein *et al.* (2021) (Table S4). To further confirm some of the new targeted genes, we tested their expression by measuring their promoter activity upon osmotic shock. As shown in Fig 3B, the activity of *dipM* and *ftsN* promoters was significantly lower in the *chvI* mutant compared to WT, while *phyR*

promoter activity was significantly higher in $\Delta chvI$ than in WT. In contrast, the *nepR* promoter had similar activity in both WT and $\Delta chvI$ strains. These results suggest that ChvI can work as an activator (e.g. *dipM* and *ftsN*) or a repressor (e.g. *phyR*) of gene expression.

Interestingly, *hprK* is part of the SigT regulon determined upon osmotic shock (Tien *et al.*, 2018). Given that *hprK* is part of the same operon as *chvIG*, we tested whether the entire operon *chvIG-hprK* could be under the control of SigT. Consistently, we found that the *chvI* promoter (P_{chvI}) was significantly higher in the $\Delta sigT$ mutant compared to WT whereas the activity of P_{chvI} was strongly reduced in the $\Delta chvI$ mutant (Fig. S2A). Thus, SigT is a negative regulator of ChvI while ChvI is subjected to a positive feedback loop. Considering that *phyR* promoter activity was higher in a $\Delta chvI$ background (Fig. 3C), this indicates that ChvI regulates negatively SigT by inhibiting PhyR. Together, these data suggest that both SigT and ChvI antagonise each other.

Overall, our data show that ChvI controls the expression of genes involved in cell envelope homeostasis, in response to osmotic stress.

Figure S1. Growth and morphology of *chvI* mutant strains in synthetic minimal media. (A) Growth of WT, *chvI*_{D52E} and *chvI*_{D52A} mutant strains in M2G. The data represent the average values of biological replicates (n=3, error bars show standard deviation). (B) Morphology of WT, $\Delta chvI$, *chvI*_{D52E} and *chvI*_{D52A} cells after 48 hrs of incubation in M2G. Scale bars in microscopy images correspond to 1 μ m.

Fig. 3. ChvI target genes determined by ChIP-seq and validated by β -galactosidase assays. (A) Genome-wide occupancy of ChvI on the chromosome of *C. crescentus* determined by ChIP-seq on WT strain grown in M2G. The x-axis represents the coordinates on the genome (Mb), the y-axis shows the normalized ChIP-Seq read abundance in reads. Some top hits, corresponding to the promoter regions of *chvT*, *CCNA_R0088*, *ftsN*, *CCNA_02715*, *chvR* and *dipM* operon, are highlighted. (B) Activity of the *dipM*, *ftsN*, *nepR* and *phyR* promoters (Miller Units) in WT (black bars) and $\Delta chvI$ (white bars) cells grown in PYE. The data represent the average values of biological replicates (n=3, error bars show standard deviation). * = $p < 0.05$, ** = $p < 0.01$, single factor ANOVA analysis of β -galactosidase activity.

A

Figure S2. Activity of the *chvI* promoter is regulated by the sigma factor T. (A) Activity of the *chvI* promoter (Miller Units) in WT (black bars), $\Delta chvI$ (light grey bars) $\Delta sigT$ (dark grey bars) grown in PYE. The data represent the average values of biological replicates (n=3, error bars show standard deviation). ** = p < 0.01 Single factor ANOVA analysis of β -galactosidase activity.

ChvI is phosphorylated under osmotic upshift

Since both single $\Delta chvG$ and $\Delta chvI$ mutants are sensitive to osmotic upshift (Fig 1A), this suggests that ChvI might be phosphorylated and activated by ChvG in such stressful conditions. We tested this hypothesis by determining the *in vivo* phosphorylated levels of ChvI (ChvI~P) with or without osmotic shock. In order to facilitate uptake of [γ - ^{32} P] ATP, *Caulobacter* cells are grown in a medium depleted for the phosphate salts Na_2HPO_4 and KH_2HPO_4 (M5GG). Hence, K^+ and Na^+ concentrations in M5GG are respectively 1.01 mM and 1.02 mM, which is much less than in M2G (7.75 mM of K^+ and 12.25 mM of Na^+). At such low concentrations of osmolytes, the $\Delta chvI$ strain grew, as expected, similarly to WT in M5GG (Fig. 4A). In contrast and in agreement with our previous findings, the growth of the $\Delta chvI$ mutant

was severely impaired in M5GG supplemented with 6% sucrose compared to WT (Fig. 4A). Consistent with these data, we observed that ChvI was barely phosphorylated in WT grown in M5GG while no ChvI~P was detected in $\Delta chvG$ (Fig. 4B). More importantly, ChvI was hyperphosphorylated in cells grown under osmotic shock with sucrose (Fig. 4C-D).

Fig. 4. ChvG-dependent phosphorylation of ChvI is stimulated upon osmotic shock. (A) Growth of WT (black) and $\Delta chvI$ mutant (orange) in M5GG with (dashed lines) or without (solid lines) 6% sucrose. The data represent the average value of biological replicates (n=3, error bars show standard deviation). (B) *in vivo* phosphorylation levels of ChvI in WT and $\Delta chvG$ mutant grown in M5GG. (C) *in vivo* phosphorylation levels of ChvI in WT grown in M5GG without (-) or with (+) 6% sucrose. (D) Quantified *in vivo* phosphorylation levels of ChvI in the same growth conditions than in (C). The data represent the average values of biological replicates (n=3, error bars show standard deviation). * = $p < 0.05$, single factor ANOVA analysis of ChvI phosphorylation.

ChvGI is sensitive to peptidoglycan synthesis inhibition

Stress response systems surveying cell envelope homeostasis in Gram-negative bacteria are sensitive to different stressful conditions including osmotic shock (Humphreys *et al.*, 1999; Jubelin *et al.*, 2005; Laubacher & Ades. 2008; Cho *et al.*, 2014). Therefore, we first tested growth and viability in other cell envelope stressors,

such as acidic pH, detergent and antibiotics targeting the outer membrane or the PG synthesis machinery. We did not observe significant impacts on fitness between WT and $\Delta chvI$ cells under treatment with acidic stress (Fig. 5A), A22 drug targeting the actin-like protein MreB (Fig. 5B, D), SDS and polymyxin B detergents (Fig. S3A-B). In contrast, exposure to mecillinam, which inhibits the penicillin binding protein 2 (PBP2) transpeptidase activity thereby obstructing PG crosslinking during elongation, significantly impaired growth of $\Delta chvI$ compared to WT (Fig. 5C-D). Considering that a *chvI* mutant was also sensitive to vancomycin (Vallet *et al.*, 2018), which targets the D-Ala-D-Ala moiety of the PG precursors thereby inhibiting PG crosslinking, we assessed the impact of both vancomycin and mecillinam on the *chvI* promoter activity in synthetic media M5GG (Fig. 5.E). We observed that growth was severely affected in the $\Delta chvI$ mutant after six hours of growth with mecillinam and to a lesser extent with vancomycin. In comparison to untreated cells, the activity of the $P_{chvI}::lacZ$ transcriptional reporter significantly increased after six hours of exposure to mecillinam while vancomycin treatment did not influence *chvI* expression. Interestingly, although the activity of $P_{chvI}::lacZ$ decreased in $\Delta chvI$ cells treated with mecillinam, it remained significantly higher than in the untreated $\Delta chvI$ cells. These data suggest that upon mecillinam treatment, the expression of the *chvIG-hprK* operon is induced not only by ChvI itself but also in a ChvI-independent way.

Fig. 5. ChvI expression is activated upon mecillinam treatment (A-C) Growth of WT and $\Delta chvI$ mutant cells in PYE at acidic pH 5.5 (A) or supplemented with either A22 (B) or mecillinam (C). (D) Viability of WT and $\Delta chvI$ mutant upon treatment with mecillinam (Mec) and A22. (E) Activity of the *chvI* promoter (Miller Units) in WT (solid bars) and $\Delta chvI$ (dashed bars) cells grown in minimal media M5GG treated for 6 hrs with vancomycin (Van) or mecillinam (Mec). The data in figures (A-C) and (E) represent the average values of biological replicates (n=3, error bars show standard deviation). The drugs were used at the following concentrations: A22 (2.5 $\mu\text{g ml}^{-1}$), vancomycin (10 $\mu\text{g ml}^{-1}$) and mecillinam (100 $\mu\text{g ml}^{-1}$).

Figure S3. $\Delta chvI$ is not sensitive to SDS and polymyxin B treatment. (A) Viability of $\Delta chvI$ cells on plates supplemented with 0.001 % or 0.0025% sodium dodecyl sulfate (SDS), 10 $\mu\text{g ml}^{-1}$ polymyxin B (Pol B). (B) Growth of WT (black) and $\Delta chvI$ (orange) cells in PYE with (dashed lines) or without (solid lines) 5 $\mu\text{g ml}^{-1}$ polymyxin B (Pol B).

ChvG relocates from patchy-spotty to midcell upon osmotic shock

Intriguingly, the HK ChvG was previously found in an automated large scale analysis for protein localisation as displaying a patchy-spotty distribution typically observed with PG-related proteins (Werner *et al.*, 2009). Therefore, we constructed fluorescent protein fusions to analyse ChvG and ChvI localisation patterns on PYE and M2G pads. First, we confirmed that GFP fusions to the N- or C-terminal extremity of ChvG or ChvI were stable and functional, as attested by western blot analyses and growth assays (Fig. S4AB). Then, fluorescent microscopy images showed that both ChvG N- and C-terminal fusions to GFP displayed patchy-spotty localisation patterns in cells grown in PYE or M2G cultures (Fig. 6A, Fig. S4C). We also confirmed the patchy-spotty localisation pattern for the ChvG C-terminal fusion to GFP in high resolution confocal

microscopy (Fig. S4D). Surprisingly, when cells grown in PYE were washed in M2G and imaged on M2G agar pads, we observed some cells formed foci at mid-cell (Fig. 6A). In contrast, cells grown in M2G, washed with PYE and imaged on PYE pad kept their patchy-spotty localisation pattern (Fig. 6A). This relocation is reminiscent to what was described for the PG-related proteins RodA, PBP2 and PBP1A, whose localization changed from a typical patchy-spotty distribution to mid-cell when cells were shifted from PYE to M2G agarose pads (Hocking *et al.*, 2012).

In contrast to ChvG, none of the ChvI fusions to GFP (ChvI-GFP and GFP-ChvI) displayed patchy-spotty localization. Instead, the signal was diffused all over the cell body, indicating that the protein remains diffuse in the cytoplasm. Neither, we could observe foci formation in these mutants after transition from PYE to M2G pads (Fig. 6B and data not shown). Then, we wanted to assess if the catalytic activity had any impact on the protein localisation. For that, we fused GFP to a full-length catalytic mutant of ChvG to (ChvG_{H309N}), which like $\Delta chvG$ was unable to grow on M2G (Fig. S4E-D). We observed that, similar to the WT ChvG-GFP, the ChvG_{H309N}-GFP showed a patchy-spotty pattern when cells were grown in PYE and relocated at mid-cell when shifted from PYE to M2G (Fig. 6C). However, we noticed that the fluorescent foci were more conspicuous in populations of cells expressing ChvG_{H309N}-GFP fusions than the ones expressing ChvG-GFP. Thus, our data suggest that ChvG co-localizes with PG synthesis machinery independently of its kinase activity.

A

B

C

Fig. 6. ChvG relocates from a patchy-spotty pattern to mid-cell upon osmotic upshift. (A) Localisation of ChvG-eGFP in cells grown in either M2G or PYE and imaged on M2G or PYE agar pads, respectively; or cells grown in PYE, washed in M2G and imaged on M2G agarose pads (PYE → M2G); or cells grown in M2G, washed in PYE and imaged on PYE agarose pads (M2G → PYE). (B) Localisation of ChvI-eGFP in a $\Delta chvI$ background and (C) ChvG_{H309N}-eGFP in a $\Delta chvG$ background grown overnight in PYE and imaged on PYE agarose pads or grown in PYE, washed in M2G and imaged on M2G agarose pads (PYE → M2G). Liquid cultures and pads were supplemented with 0.1 % xylose to allow expression of ChvG, ChvI and ChvG_{H309N} fused to eGFP fusions. Scale bar= 1 μm .

Figure S4. ChvG relocates from a patchy-spotty pattern to mid-cell upon osmotic upshift.

(A) Growth of $\Delta chvI$ and $\Delta chvG$ mutants complemented with *chvI* and *chvG* N- and C-terminal eGFP fusions. (B) Immunodetection of eGFP fusions with GFP antibodies. The expected molecular weights for eGFP, eGFP-ChvI, ChvI-eGFP, eGFP-ChvG and ChvG-eGFP are 26.94, 54.33, 55.75, 85.88, 86.06 kDa, respectively. (C) Localisation of eGFP-ChvG in a $\Delta chvG$ background grown overnight in PYE and M2G and imaged in PYE and M2G agarose pads, respectively. (D) Confocal microscopy images of $\Delta chvG$ cells expressing eGFP-ChvG cells grown in PYE. (E-F) Growth upon endogenous (E) and ectopic expression (F) of *chvG*_{H309N} in M2G. The data represent the average value of biological replicates (n=3, error bars show standard deviation). Expression of eGFP fusions from P_{xyIX} was induced with 0.1 % xylose.

The N-terminal extremity of ChvG determines localisation and relocation

ChvG is a HK anchored in the membrane thanks to 2 transmembrane helices which delimit a periplasmic sensor domain (amino acids 50-221), with a cytoplasmic signal transduction histidine kinase domain (amino acids 242-534) (Fig. 7A). To assess which of these ChvG domains are essential for localisation, we fused to mCherry ChvG versions harbouring either the complete or truncated sensor (ChvG₁₋₂₇₄ and ChvG₁₋₁₁₄, respectively) or the catalytic (ChvG₂₇₃₋₅₃₄) domain. First, we confirmed that full-length ChvG fused to mCherry displayed the same localisation and relocation patterns (Fig. 7B, F) than the ones described for GFP fusions (Fig. 6A). Interestingly, the truncated or complete sensor domain alone (ChvG₁₋₁₁₄, ChvG₁₋₂₇₄) fused to mCherry were both localized as patchy-spotty in PYE (Fig. 7C-D). However, only the ChvG₁₋₂₇₄-mCherry fusion relocated as foci upon transition to M2G (Fig. 7G-H). Unlike the sensor domains, the catalytic domain (ChvG₂₇₃₋₅₃₄) fused to mCherry displayed neither the patchy-spotty nor the foci pattern of localization and was instead diffusely localized in the cytoplasm (Fig. 7E, I). Altogether, our results indicate that the complete periplasmic sensor domain of ChvG edged by transmembrane domains are required for relocation as foci upon osmotic shock whereas the first 114 amino acids are sufficient to determine the patchy-spotty pattern of localization.

Fig. 7. Localisation of truncated ChvG variants. (A) Conserved domains in the HK ChvG and position of the amino acids defining the truncated proteins. (B-I) Localisation of ChvG WT (B, F) and truncated versions (C-E, G-I) in cells grown in PYE and imaged on PYE agarose pads (B-) or in cells grown in PYE, washed in M2G and imaged on M2G agarose pads (PYE → M2G) (F-I). Liquid cultures and pads were supplemented with 0.1 % xylose. Scale bars in microscopy images correspond to 1 μ m.

Discussion

The α -proteobacterial TCS ChvGI has conserved cell envelope regulatory functions (Lamontagne *et al.*, 2007; Heckel *et al.*, 2014; Ratib *et al.*, 2018; Vallet *et al.*, 2020; Stein *et al.*, 2021), but the exact perceived signal that triggers the ChvG-dependent phosphorylation of ChvI remains unknown. We found that hyperosmotic shock triggers ChvI phosphorylation and treatment with antibiotics targeting PG synthesis proteins induces *chvIG* expression. We also showed that ChvI regulates expression of genes related to PG synthesis, such as *ftsN* and *dipM*, and to osmotic and oxidative stress regulation, such as *nepR*, likely explaining why ChvGI system is required for viability in these stressful conditions. Other targets related to cell envelope architecture, identified by ChIP-seq and RNA-seq experiments, include genes coding for the β -barrel assembly machinery (Bam) complex; components of the Tol system; several outer membrane proteins (OMP) such as RasFa, CCNA_03820 and CCNA_01956; and the cytoplasmic FtsZ-binding protein ZapA. Altogether, our results support a role for ChvGI in *C. crescentus* as a safeguard of cell envelope homeostasis by sensing cell wall-related damages and regulating the expression of genes related to cell division and envelope architecture.

We observed that similarly to $\Delta chvI$, the endogenous expression of phospho-mimetic (*chvID52E*) or phospho-ablative (*chvID52A*) failed to grow in hypertonic conditions such as those found in synthetic minimal media (M2G or M2X). However, ectopic expression of *chvID52E* partially alleviated growth defect of $\Delta chvI$ in these conditions, in agreement with a previous report (Stein *et al.* (2021)). These observations indicate that ChvI phosphorylation dynamics is tightly regulated to ensure optimal growth of *C. crescentus* in hyperosmotic environments. Therefore, an important level of

phosphorylated ChvI might be required even in mild cell envelope stress conditions.

In addition, constitutive activation of a cell envelope stress response system can be detrimental, as already shown in *E. coli* with the sigma factor E (σ^E), the inner membrane stress regulator Cpx or the regulator of capsule synthesis Rcs (Missiakas *et al.*, 1997; Pogliano *et al.*, 1998; De las Peñas *et al.*, 2003; Grabowicz & Silhavy. 2017). Indeed, overactivation of σ^E causes overexpression of sRNAs inhibiting expression of integral OMPs, therefore weakening the outer membrane integrity (Nicoloff *et al.*, 2017). Likewise, constitutive Cpx activation disturbs cell division and morphology causing mis-localisation of FtsZ as well as overexpression of L,D-transpeptidase enzyme LdtD (Pogliano *et al.*, 1998; Delhaye *et al.*, 2016). Similarly, overactivation of the RR RcsB results in overexpression of the small outer membrane lipoprotein OsmB, which is toxic through yet unknown mechanisms (Grabowicz & Silhavy. 2017). In any of the previous cases, hyperstimulation can lead to deregulation of cell envelope components.

In *C. crescentus*, the general stress response (GSR) sigma factor SigT is also activated upon osmotic imbalance thanks to a complex network comprising the sRNA (GsrN), the anti-SigT regulator (NepR), the histidine phosphotransferases (LovK and PhyK) and the response regulators (MrrA, LovR and PhyR) (Alvarez-Martinez *et al.*, 2007; Lourenço *et al.*, 2011; Foreman *et al.*, 2012; Lori *et al.*, 2018; Tien *et al.*, 2018).

In steady-state conditions, NepR impedes SigT-dependent transcription. Upon either oxidative stress or osmotic imbalance, the histidine phosphotransferase MrrA activates PhyK to further phosphorylate PhyR. Once phosphorylated, PhyR~P interacts with NepR to release SigT from inhibition and allow the expression the SigT-dependent regulon. Interestingly, we found that ChvIG and GSR are interconnected

but this connection is counterintuitive. Indeed, we observed (i) that deletion of *sigT* led to higher *chvI* promoter activity and (ii) that ChvI directly represses *phyR* expression, suggesting that ChvI and SigT antagonise each other despite being sensitive to hyperosmotic conditions. However, we cannot exclude the possibility that the cell envelope homeostasis is sufficiently disrupted in both single mutants to correspondingly activate the other functional system, *i.e.* ChvGI in $\Delta sigT$ through P_{chvIG} and SigT in $\Delta chvI$ through P_{phyR} . Alternatively, the antagonistic regulation might allow these two systems to respond to different levels of osmotic imbalances. For instance, activation of ChvGI at low hypertonic conditions could down-regulate GSR whereas SigT would be sensitive to higher salts concentrations at which ChvGI would be tuned down.

In the absence of a functional ChvGI system, *C. crescentus* cannot propagate in minimal media except if the TonB-dependent outer membrane protein ChvT or if NtrX is concomitantly inactivated (Stein *et al.*, 2021). ChvGI is known to indirectly down-regulate ChvT by directly activating the expression of the sRNA ChvR (Fröhlich *et al.*, 2018). Our ChIP-seq data showed that ChvI also likely regulates *chvT* expression directly by binding to its promoter region. This dual – transcriptional and post-transcriptional – control indicates the ChvT outer membrane protein is an important player in cell envelope homeostasis. In support of that, a $\Delta chvI \Delta chvT$ double mutant grew better than a $\Delta chvI$ single one in hyperosmotic regimes. Interestingly, NtrXY has been also described as a cell envelope regulator in α -proteobacteria. NtrYX controls succinoglycan and exopolysaccharide (EPS) production, and salt stress response in *S. meliloti* (Wang *et al.*, 2013; Calatrava *et al.*, 2017). In *Rhodobacter sphaeroides*, NtrXY confers resistance to membrane disruptive agents and regulates the expression

of genes coding for PG and EPS synthesis enzymes, lipoproteins and cell division proteins (Lemmer *et al.*, 2017; Lemmer *et al.*, 2020). Thus, confirming that *ntrX* inactivation in a $\Delta chvI$ background partially restored growth in the presence of high concentration of osmolytes further supports the importance of the NtrZXY system, together with ChvGI, in the cell envelope stress response in α -proteobacteria.

We showed that ChvGI also regulates sensitivity to the antibiotic mecillinam that inhibits PG transpeptidation. Previously, Vallet *et al.* (2020) showed that *chvI* mutants failed to grow when exposed to vancomycin, which also impedes PG crosslinking by directly interacting with the D-Ala-D-Ala moiety of the PG precursors. Here, we showed that $\Delta chvI$ cells were sensitized to mecillinam and to a lesser extent to vancomycin. However, although both antibiotics inhibits the transpeptidation step of PG synthesis, only mecillinam treatment induced *chvIG* expression. This is likely due to the fact that intracellular concentrations of vancomycin remain low in WT cells since the TonB-dependent receptor ChvT is poorly expressed and incorporated in outer membrane. However, treatment with cefixime, cefotaxime and sodium deoxycholate did not show differences in cell viability between WT and $\Delta chvI$ cells (Vallet *et al.* 2020). Furthermore, the activation of *chvR* expression upon cefotaxime or sodium deoxycholate exposure was shown to be ChvI-independent. In addition, we showed here that $\Delta chvI$ cells were not more sensitive to treatment with SDS, polymyxin B or the MreB inhibitor A22. Considering that MreB is a key cytoplasmic component of the elongasome complex, of which PBP2 and other proteins are part, it is therefore likely that ChvGI rather senses specific targets in the periplasm to assess cell envelope stress. Notwithstanding that, other stress response systems, yet to be discovered or

characterised in the context of the aforementioned stressors, might be involved in responding to the damages that do not activate ChvGI.

We observed that ChvG has a patchy-spotty pattern localisation when grown in complex and synthetic minimal media, while it relocates as foci at mid-cell upon transition from complex to minimal media. The patchy-spotty localisation pattern has been described for multiple PG-related proteins in *C. crescentus*, including MreB, MreC, Pal, PBP1A, PBP2, RodA, TolB (Figge *et al.*, 2004; Divakaruni *et al.*, 2005; Werner *et al.*, 2009; Hocking *et al.*, 2012; Billini *et al.*, 2019). Moreover, RodA, PBP2, PBP1A, which are proteins involved in PG polymerization and crosslinking, have been reported to relocate at mid-cell upon osmotic upshift in a FtsZ-dependent but MreB-independent manner (Hocking *et al.*, 2012). It would be interesting to check whether this ChvG localisation patterns are conserved among α -proteobacteria, in particular in Rhizobiales since they do not encode MreB orthologs. In *E. coli*, it has been reported that the PBP2 periplasmic portion physically interacts with those of PBP1A and RodA to mediate PG assembly and to ensure proper cell elongation (Banzhaf *et al.*, 2012; van der Ploeg *et al.*, 2015). It is yet to determine whether ChvG interacts with PBP2, PBP1a, RodA and/or others with similar localisation patterns. Nonetheless, it is tempting to speculate that ChvG interacts and co-localises with PG-related enzymes as a cell envelope safeguard system in α -proteobacteria. A recent study in the Gram-positive bacterium *Bacillus thuringiensis* showed that upon treatment with the antibiotic cefoxitin, the putative PBP protein PbpP derepresses the extracytoplasmic function sigma factor P (σ^P) which increases resistance to β -lactams (Nauta *et al.*, 2021). Future experiments will aim to determine the ChvG interactome under cell envelope undisturbed and threatening conditions, and dissect its activation

mechanism as well as its connection with the general stress response, not only in *C. crescentus* but also in other α -proteobacteria.

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Author Contributions

A.Q-Y. and R.H. conceived and designed the experiments. A.Q-Y. performed all the experiments except otherwise stated. A.M. purified the ChvI protein and helped with some plasmids constructs. A.Q-Y., J.C. and R.H. analyzed the data. A.Q-Y. and R.H. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

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