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Regulation of potassium uptake in *Caulobacter crescentus*

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Abstract

Potassium (K^+) is an essential physiological element determining membrane potential, intracellular pH, osmotic/turgor pressure, and protein synthesis in cells. Here we describe the regulation of potassium uptake systems in the oligotrophic α -proteobacterium *Caulobacter crescentus* known as a model for asymmetric cell division. We show that *C. crescentus* can grow in concentrations from the micromolar to the millimolar range by mainly using two K^+ transporters to maintain potassium homeostasis, the low affinity Kup and the high affinity Kdp uptake systems. When K^+ is not limiting, we found that the *kup* gene is essential while *kdp* inactivation does not impact the growth. In contrast, *kdp* becomes critical but not essential and *kup* dispensable for growth in K^+ -limited environments. However, in the absence of *kdp*, mutations in *kup* were selected to improve growth in K^+ -depleted conditions, likely by increasing the affinity of Kup for K^+ . In addition, mutations in the KdpDE two-component system, which regulates *kdpABCDE* expression, suggest that the inner membrane sensor regulatory component KdpD mainly works as a phosphatase to limit the growth when cells reach late exponential phase. Our data therefore suggest that KdpE is phosphorylated by another non-cognate histidine kinase. On top of this, we determined the KdpE-dependent and independent K^+ transcriptome. Together, our work illustrates how an oligotrophic bacterium responds to fluctuation in K^+ availability.

41 **Importance**

42 Potassium (K^+) is a key metal ion involved in many essential cellular processes. Here
43 we show that the oligotroph *Caulobacter crescentus* can support growth at
44 micromolar concentrations of K^+ by mainly using two K^+ uptake systems, the low-
45 affinity Kup and the high-affinity Kdp. Using genome-wide approaches, we also
46 determined the entire set of genes required for *C. crescentus* to survive at low K^+
47 concentration as well as the full K^+ -dependent regulon. Finally, we found that the
48 transcriptional regulation mediated by the KdpDE two-component system is
49 unconventional since unlike *E. coli*, the inner membrane sensor regulatory
50 component KdpD seems to work rather as a phosphatase on the phosphorylated
51 response regulator KdpE~P.

52

Introduction

Potassium (K^+) is the most abundant monovalent cation within living cells (1, 2) and it is essential to set the membrane potential and intracellular pH (2-5). As K^+ also emerges as an important regulator of bacterial physiology in view of its role in diverse processes (6-17) including biofilm formation, chemotaxis, cell to cell communication or virulence, it is therefore expected that K^+ transport is tightly regulated. In many bacteria, the KdpDE two-component system (TCS) responds to potassium depletion by regulating the expression of the cognate high-affinity K^+ transporter KdpFABC (18). This inner membrane complex consists of four subunits all encoded in the same operon, often together with the *kdpDE* genes (19, 20). In *Escherichia coli*, KdpD is bifunctional inner membrane histidine kinase – harbouring both kinase and phosphatase activities – with 4 transmembrane domains separating two N-terminal cytoplasmic input domains (called KdpD and Usp) and three C-terminal cytoplasmic domains (GAF, HisKA and HATPase_C). While the two last domains are obviously required for the autophosphorylation of KdpD, the GAF domain might be responsible for sensing intracellular K^+ (21) whereas extracellular K^+ might be sensed by the transmembrane domains (22). Based on these data, KdpD was proposed to act as a dual sensor for K^+ , able to sense both extracellular and intracellular K^+ . Whereas the extracellular K^+ was shown to inhibit KdpD kinase activity, its phosphatase activity was stimulated by intracellular K^+ (23). Several proteins have been found to interact with KdpD, such as the universal stress protein UspC identified as a scaffolding protein that interacts with the N-terminal Usp domain. It has been proposed that UspC binding enhances KdpE phosphorylation by KdpD at high K^+ concentration at which KdpE would not be phosphorylated (24). The unphosphorylated form of EIIA^{Ntr}, which belongs to the PTS^{Ntr}, interacts with the catalytic domains KdpD to activate its

autokinase activity (25, 26). K^+ availability ultimately determines the phosphorylation status of the response regulator KdpE and thus provides a tight regulation of *kdpFABC* expression as well as a robust homeostasis in fluctuating environments (23).

Other K^+ transporters have been described in detail in Gram-negative bacteria, such as the inner membrane low-affinity Trk and Kup systems for uptake, and the KefB efflux pump. The Trk potassium uptake system is highly conserved in bacteria and works in a multi-subunit complex comprising the TrkG/H, TrkA and TrkE proteins. TrkH – an extra copy known as TrkG is found in *E. coli* – is the trans-membrane transporter unit, which interacts with the peripheral protein TrkA and the ATP-binding protein TrkE in the cytoplasmic leaflet to control extracellular potassium uptake when high extracellular concentrations of K^+ are available (27-29). Kup is a single protein, constitutively expressed and thought to function as K^+/Na^+ symporter (28, 30, 31).

Recently, it was reported that glutathione controls cell division via negative regulation of KefB in the Gram-negative oligotrophic α -proteobacterium *Caulobacter crescentus* (32). Indeed, mutants unable to synthesize glutathione led to cell filamentation and decreased intracellular K^+ levels. Interestingly, these phenotypes were suppressed by mutations in the KefB efflux pump, suggesting that low intracellular K^+ concentration was responsible for the cell division defect. *C. crescentus* has been extensively used as a model for cell cycle studies since it divides asymmetrically to give birth to a larger sessile stalked cell and a smaller flagellated swarmer cell (33). DNA replication and cell division take place only in sessile stalked cells whereas the swarmer cells remain in a non-replicative but chemotactically active and motile phase able to explore new environments. Once swarmer cells find a suitable – resourceful – habitat for reproduction, they differentiate into stalked cells and concomitantly start DNA

replication. In contrast, upon deprivation of environmental resources, the swarmer cells do not differentiate into replicative stalked cells (34).

In this study, we characterized the response of *Caulobacter* cells grown in environments containing what we defined as limiting, abundant and excessive K^+ concentrations. *In silico* analyses revealed that *C. crescentus* does not have Trk system components but encodes a putative transporter with a cytoplasmic TrkA-like domain. Moreover, in addition to the KefB and KefC efflux pumps, we found genes predicted to code for a Kup and a KdpABC transporters as well as a KdpDE TCS. We generated mutants of these predicted K^+ transporters and assessed their fitness in limiting, abundant and excessive K^+ environments. We also characterized the KdpDE system and determined its regulon by RNA-seq.

Results

Growth of *C. crescentus* at different K^+ concentrations

To primarily assess the impact of K^+ on *C. crescentus* fitness, the wild-type (WT) strain was grown in K^+ -free minimal medium (M2G-K) supplemented with K^+ at different concentrations using either KCl or a combination of K_2HPO_4 and KH_2PO_4 ($K_2HPO_4 + KH_2PO_4$) like the one used in standard M2G minimal medium (**Fig. 1**). First, when phosphate salts were used as K^+ source, we observed that compared to M2G, which contains 7 mM K^+ , growth started to be significantly impaired at concentrations below or equal to 0.025 mM and above or equal to 7 mM (**Fig. 1AB**). Second, by using KCl, we found different K^+ concentrations critical for growth since the WT already failed to grow at 0.05 mM but supports growth up to 25 mM (**Fig. 1CD and Fig. S1A**). Notwithstanding these differences likely due to the PO_4^{3-} and Cl^- counter-anions, the growth of the WT at K^+ concentrations ranging from 0.5 mM to 5

mM was indistinguishable from the one in M2G, that is 7 mM (**Fig. 1**). Therefore, based on these data, we will use in this study potassium phosphate salts at 0.025 mM and 0.5 mM respectively as limiting and abundant concentrations while KCl will be used at 50 mM as excessive concentration.

K⁺ transport and regulation systems in *C. crescentus*

By using *in silico* analyses, we found in the genome of *C. crescentus* NA1000 (NC_011916.1) several genes encoding potential K⁺ uptake [*kup* (CCNA_00130) and *kdpABCDE* (CCNA_01663-1667)] or efflux [*kefB* (CCNA_00204), *kefC* (CCNA_03611) and *kefG* (CCNA_00205)] systems. Although Trk orthologs were not found, a putative transporter (CCNA_01688) containing a cytoplasmic TrkA-like domain was identified. Based on the function of the Kdp, Kef, Kup and Trk systems in other bacteria (2, 12, 35-37), we predicted that K⁺ transport in *C. crescentus* might occur via CCNA_01688, KefBG, KefC and Kup when potassium is abundant in the environment whereas the Kdp system might be inactive (38, 39). In contrast, K⁺-depleted conditions should activate the KdpDE TCS and trigger expression of the high affinity KdpABC transporter (40).

To test these predictions, we first constructed knock-out (KO) mutants for all the K⁺ transport and regulatory systems described above. We successfully inactivated all these genes except *kup*, suggesting it might be essential at least at the K⁺ concentration found in the complex medium PYE used to construct the KO strains. Note that $\Delta kefB$ and $\Delta kdpABCDE$ (hereafter referred to as Δkdp) were respectively used as *kefBG* and *kdp* inactive mutants. Then, we measured the growth for the mutants at different K⁺ concentrations (**Fig. 2**). In limiting (0.025 mM) K⁺ conditions, only Δkdp had a strong growth delay but could still grow (**Fig. 2A**), suggesting that

another transporter is used in K⁺-depletion conditions, at least when *kdp* is inactive. All the KO mutants grew equally well as the WT in minimal medium supplemented with abundant (0.5 mM) K⁺ concentration (**Fig. 2B**) or in complex medium (**Fig. S1B**). At excessive (50 mM) K⁺ concentration, we found that (i) $\Delta CCNA_01688$ had a slight growth delay; (ii) Δkdp had a lower plateau and (iii) $\Delta kefC$ barely grew in comparison to WT (**Fig. 2C**). Interestingly, the growth delay of $\Delta CCNA_01688$ and the lower plateau of Δkdp were not observed in complex PYE medium supplemented with an excess of K⁺ whereas $\Delta kefC$, like in minimal medium, did not grow in these conditions (**Fig. S1C**).

K⁺-dependent essential genome

Transposon mutagenesis coupled to next-generation sequencing (Tn-seq) were used to determine the core set of genes important for adaptation and response to abundant or limiting K⁺ condition (**Fig. 3A**). We used M2G-K with 1 % agar concentration to lower as much as possible K⁺ traces from the agar in our experiment. Indeed, even at 1% agar, the K⁺ traces were sufficient to allow WT cells to grow without exogenous source of K⁺ (on M2G-K, **Fig. 3B**). In contrast, at least 0.025 mM K⁺ had to be supplemented to M2G-K to allow growth of Δkdp while at 0.5 mM K⁺, both the mutant and the WT grew similarly (**Fig. 3B**). Therefore, we decided to use M2G-K without exogenous K⁺ and M2G plates respectively as the limiting and abundant K⁺ conditions for the Tn-seq (**Fig. 3B**).

We analysed the frequency of Tn insertions and observed a bimodal distribution that allowed us visualizing two clusters of genes in each condition, one with low density of Tn insertions (considered as essential genes) and the other one with a higher density

(considered as non-essential genes) (**Figure S2A**). Based on this frequency of Tn insertions, we used Ward's clustering analysis (41) and defined three fitness cost categories, that are (i) essential, (ii) high fitness cost (HFC) and (iii) non-essential. Among the 4186 annotated genes in *C. crescentus*, the percentage of essential genes in both media (M2G and M2G-K) was similar (~12 %), whilst the percentage of HFC genes was lower (~7 %) in M2G-K than in M2G (~13 %). Hence, the percentage of non-essential genes was higher in M2G-K (~ 81 %) than in M2G (~75 %) (**Fig. 3C** and **Table S2**). The shift from the HFC to the non-essential category in *C. crescentus* cells grown in M2G-K could be explained by the reduction of osmotic stress in this medium. Indeed, we showed recently that M2G generates a cell envelope stress for *C. crescentus* due to the high content of monovalent cations (42).

Genes with known or predicted biological functions were grouped according to the classification of cluster of orthologues groups (COG) (**Fig S2B**). This allowed to highlight biological functions that are enriched in abundant and limited K⁺ conditions. Overall, the COG enrichment in the three fitness categories (Essential, High Fitness Cost – HFC and Non-Essential) was substantially similar in both M2G and M2G-K media. The C group “energy production and conversion” genes was the most abundant for all the fitness categories in M2G-K, as well as for essential and non-essential genes in M2G, while for HFC genes it was the second most abundant, after the J group “translation, ribosomal structure and biogenesis”.

By comparing the gene pool in each category for each medium, we identified a set of 537 genes that changed their fitness cost from M2G to M2G-K and vice versa (**Fig. 3D** and **Table S2**), including 153 genes which became exclusively essential (28.5 %), 153 HFC (28.5 %) and 231 non-essential (43 %) in M2G-K. First, we observed that the TCS genes *chvGI*, known to be HFC in M2G due to its response to hyperosmotic

stress (42), became non-essential in M2G-K, thereby supporting that M2G-K is a hypo-osmotic environment compared to M2G. Second, all the *kdp* genes categorised as non-essential in M2G became essential in M2G-K, except *kdpD* which became HFC. In contrast, *kup* moved from the HFC category in M2G to non-essential one in M2G-K. This confirms the importance of the Kdp and Kup systems respectively in limiting and abundant K⁺ conditions. Considering the conservation of the *kdp* system in bacteria, it is intriguing that *kdpD* – encoding the histidine kinase component – did not have the same fitness cost in M2G-K than the genes coding for the transporter (*kdpABC*) and the response regulator (*kdpE*) components. While this supports that KdpE likely regulates, directly, the expression of *kdpABC* genes, it suggests that KdpD and KdpE have different regulatory effects on growth in K⁺-depleted environments. Finally, we did not observe changes in fitness for the other predicted potassium transport systems since all of them were categorised as non-essential genes in both M2G and M2G-K, further supporting that CCNA_01688, KefBG and KefC are not important for growth in abundant and limiting K⁺ conditions, although we cannot rule out some redundancy between these systems.

Kup becomes dispensable in limiting K⁺ conditions

The Tn-seq data suggest that inactivation of *kup* gene could be facilitated at limiting K⁺ concentrations (**Fig. 3D, Table S2**). Kup has been described in different bacteria as the major K⁺ transporter, essential in hyperosmotic conditions using sucrose as osmotic stressor (43, 44). Moreover, in a previous study, we found that the TCS ChvGI in *C. crescentus* positively controls the expression of *kup* in hyperosmotic conditions (42). In agreement with this, we could not obtain a Δkup strain by using a

markerless recombination approach on complex medium (PYE) plates supplemented with 3% sucrose for the SacB-dependent counterselection. Since addition of sucrose considerably increases the osmolality of the media (45), we successfully generated a Δkup mutant on a minimal medium lacking both Na_2HPO_4 and KH_2PO_4 salts and supplemented with 0.5 % sucrose. Interestingly, we observed that the Δkup mutant grew in limiting K^+ concentration, although at a slower growth rate but to a higher plateau compared to the WT (**Fig. 4A**). As expected from the Tn-seq data, we found that Δkup failed to grow when exposed to abundant and excessive K^+ concentrations (**Fig. 4A**). We also confirmed that *kup* is essential in hypertonic conditions since the mutant did not grow in limiting K^+ conditions when 3% sucrose were added. (**Fig. 4A**). Together, these data indicate that Kup allows potassium uptake in abundant and excessive K^+ conditions but can be detrimental for unknown reasons at limiting K^+ concentrations.

While characterizing the growth of Δkdp cells on M2G-K plates (**Fig. 3A**), we inadvertently selected growth suppressors that improved the growth of Δkdp in limiting K^+ conditions (**Fig. S2D**). Whole genome sequencing analyses of several of these suppressors allowed the identification of three mutations in *kup*: *kup*_{A87P}, *kup*_{G253S} and *kup*_{S456R}. Using homologous recombination, we constructed these mutations in WT and Δkdp backgrounds. First, these *kup* mutations did not have any significant fitness cost when K^+ was not limiting (**Fig. 4B**). Second, the single point mutants in an otherwise WT background grew similarly to the WT strain at limiting K^+ concentrations (**Fig. 4B**). Third, each *kup* mutation suppressed the defect in of Δkdp when grown in limiting K^+ condition, although to a lesser extent for *kup*_{S456R} than for the two other suppressor mutations (**Fig. 4B**).

250 To get molecular insight into the mutation-mediated improved K⁺ binding/transport by
251 Kup, molecular dynamics (MD) simulations were carried out on the modeled WT Kup
252 protein and each of the 3 mutants (A87P, G253S, and S456R) in an environment
253 mimicking the lipid composition of *Caulobacter* inner membrane (46, 47) (**Fig. S3A**).
254 Then, Kup coordinates were aligned to one protomer of *Bacillus subtilis* KimA (PDB
255 entry: 6S3K), the only available (Cryo-EM) structure of a KUP family protein (48)
256 (**Fig. 5A**). This Cryo-EM structure corresponds an “inward-occluded” state with two
257 K⁺ cations trapped inside KimA. The 8 residues in KimA described to be involved in
258 the coordination and transport of these two K⁺ cations are conserved in Kup with the
259 following correspondences (KimA/Kup): D36/D54, Y43/Y61, D117/D146, T121/T150,
260 S125/S154, T230/T260, E233/E263 and Y377/Y410.

261 The A87P mutation leads to only a few changes, the WT conformation being mainly
262 retained, but a flipping of E263 towards the orientation described for the
263 corresponding glutamate in KimA (E233) is observed (**Fig. 5B**). The same
264 observation was made with the G253S and S456R mutations (**Fig. 5CD**), suggesting
265 that E263 reversal is structurally relevant for the increase of K⁺ transport by the Kup
266 mutants. Furthermore, although to a lesser extent, the position of another residue is
267 also changed in the 3 mutants. Indeed, D146 points more towards the K⁺ binding
268 cavity, making the carboxylate group more available for coordination (**Fig. 5BCD**).
269 Finally, G253S and S456R mutants are characterized by a reorientation of both Y61
270 and Y410 to a position close to that of Y43 and Y377, respectively, in KimA, with the
271 tyrosyl moiety directed towards the putative K⁺ location (**Fig. 5CD**). Thus, our MD
272 simulations suggest that despite their different locations on Kup, the 3 mutations lead
273 to subtle structural changes in the orientation of key residues described to directly

participate in the binding and/or transport of K^+ . The reorientation of these residues might facilitate binding of K^+ cations at lower concentration.

Altogether, these data indicate that (i) Kup allows potassium uptake in abundant and excessive K^+ conditions, and that (ii) mutations in Kup improve K^+ transport at limiting concentrations, likely by increasing affinity for K^+ .

K^+ - and KdpE-dependent regulons

To further characterise the response of *C. crescentus* to K^+ -depleted conditions, we performed RNA-seq on RNA samples extracted from cells grown in minimal medium in K^+ limiting or abundant conditions. In our experiment, we identified a total of 594 genes – 385 upregulated and 209 downregulated – whose expression was impacted upon K^+ limitation with at least a 2-fold change ($FC \geq 2$) and a false-discovery rate (FDR) lower than 5% ($P_{adj} \leq 0.05$) (**Fig. 6A**). As expected, this experiment confirmed that the expression of *kdp* genes was strongly induced in the K^+ -depleted medium. Among the other upregulated genes, we found genes coding for the phosphate starvation protein PhoH (CCNA_02727), a xylose isomerase family protein (CCNA_01701), a TonB-dependent receptor (TBDR, CCNA_01859), an AraC family transcription regulator (CCNA_01858) and hypothetical proteins (CCNA_03313 and CCNA_03970) (**Fig. 6A** and **Table S3**). Among the top downregulated candidates, we found genes expressing a small RNA (CCNA_R0158), a hemin receptor (CCNA_02277), a putative transporter (CCNA_03022), a protein with a calcium binding EF Hand binding domain (CCNA_02274) and the chaperones GroES (CCNA_00722) and GroEL (CCNA_00721) (**Fig. 6A** and **Table S3**).

As our results suggest that the Kdp system is important for growth upon K^+ depletion, we also performed RNA-seq experiments with a $\Delta kdpE$ mutant grown in limiting K^+

concentrations. Then, we compared the RNA enrichment in $\Delta kdpE$ vs WT to determine which genes are affected by KdpE in a K^+ -depleted condition using the same parameters, $FC \geq 2$ and $P_{adj} \leq 0.05$. Compared to WT, we found 20 upregulated and 19 downregulated genes in $\Delta kdpE$ grown in the K^+ -depleted medium (**Fig. 6B** and **Table S4**). Among the 19 downregulated genes in $\Delta kdpE$ (**Table S4**), 95% (18/19) were also found in the regulon of the WT starved for K^+ , 11 upregulated (including *kdp* genes; **Table S3**) and 7 downregulated (**Table S3**). This suggests that KdpE is strictly required to activate the expression of the 11 genes in a K^+ -depleted environment. On the contrary, the 7 other genes that are repressed in WT cells upon K^+ depletion are even further downregulated in the absence of KdpE, suggesting that KdpE limits the repression of these genes. The expression of the last gene (*CCNA_00930*) was insensitive to K^+ concentration while downregulated in $\Delta kdpE$ (**Table S4**), suggesting that this gene is positively regulated by KdpE but independently of the K^+ concentration. Among the 20 upregulated genes in $\Delta kdpE$ (**Table S4**), only 35% (7/20) were also found in the regulon of the WT starved for K^+ , 2 downregulated (*groEL* and *groES*; **Table S3**) and 5 upregulated (**Table S3**). This indicates KdpE is required to repress *groEL* and *groES* upon K^+ depletion while it limits the activation of the 5 other genes in a K^+ -depleted environment. The remaining 13 upregulated genes in $\Delta kdpE$ (**Table S4**) were insensitive to K^+ availability, suggesting that they are likely repressed by KdpE but in a K^+ -independent way.

Surprisingly, the vast majority (555 genes; 92.8 %) of the 594 genes whose expression changed in the WT grown in K^+ limiting conditions seems to be KdpE-independent, suggesting that other regulators, yet to be discovered, are activated upon K^+ depletion. An alternative explanation might be that $\Delta kdpE$ cells exposed to limiting K^+ concentration suffer from a general stress response, which ultimately

324 leads to global transcriptional changes. This could also explain why more than a third
325 of the 39 genes (14/39) found in the $\Delta kdpE$ regulon were not identified in the K^+
326 regulon.

327 In order to unveil genes whose expression is directly regulated by KdpE, we identified
328 the DNA regions bound by KdpE using ChIP-seq in WT and $\Delta kdpE$ cells grown either
329 at abundant or limiting K^+ concentrations. The highest number of reads were found in
330 the *kdp* promoter (P_{kdp}) only in the WT grown at low K^+ concentrations. Interestingly,
331 this peak was absent in WT grown in abundant K^+ conditions as well as in $\Delta kdpE$
332 both at high and low K^+ concentrations, suggesting KdpE binds to the P_{kdp} only when
333 *C. crescentus* is starved for K^+ , that is likely when KdpE is phosphorylated and
334 abundant. While several other peaks were identified in this condition, these peaks
335 were also present in the $\Delta kdpE$, suggesting that they correspond to unspecific peaks
336 recognized by the polyclonal antibodies. Indeed, while the KdpE protein was only
337 detected in WT cells, at a higher level in limiting than in abundant K^+ conditions,
338 several proteins other than KdpE were detected in $\Delta kdpE$ cells (**Fig. S4A**). Although
339 we do not rule out the possibility that KdpE binds directly several promoter regions in
340 *C. crescentus*, the only confident target identified in our ChIP-seq is P_{kdp} . We also
341 conducted ChIP-Seq in strains in which *kdpE* was expressed under the xylose
342 inducible promoter ($P_{xyIX}::kdpE$) in a $\Delta xyIX \Delta kdpE$ or $\Delta xyIX \Delta kdpDE$ background.
343 Expression of *kdpE* from P_{xyIX} efficiently complemented the growth of both strains in
344 the presence of xylose since $\Delta xyIX \Delta kdpE P_{xyIX}::kdpE$ cells grew similarly to $\Delta xyIX$ or
345 WT cells whereas $\Delta xyIX \Delta kdpDE P_{xyIX}::kdpE$ cells grew at a higher plateau (**Fig.**
346 **S4B**) like $\Delta kdpD$ cells (see below). Moreover, KdpE was easily detected by western
347 blot in both strains although KdpE protein levels were lower in $\Delta kdpDE$ than in $\Delta kdpE$
348 (**Fig. S4C**). Surprisingly, the peak at P_{kdp} was higher in the $\Delta kdpDE$ background than

in the $\Delta kdpE$ one (**Fig. S4D**), suggesting that KdpE could be more active (hyperphosphorylated) in the absence of KdpD and therefore that KdpD mainly acts as a negative regulator of KdpE~P.

***C. crescentus* KdpD regulates KdpE both positively and negatively**

To further assess the role of the *C. crescentus* Kdp system in K⁺-limiting conditions, we first constructed KO mutants of the transport complex ($\Delta kdpABC$) and the TCS ($\Delta kdpDE$) genes. In comparison to the WT, both $\Delta kdpABC$ and $\Delta kdpDE$ mutants had a growth delay at limiting but not at abundant K⁺ concentration (**Fig. 7A** and **Fig. S5A**). Moreover, both mutants grew similarly, suggesting that the *kdpABC* is not expressed in the absence of the TCS, as described in other bacteria.

To study the HK *kdpD* and RR *kdpE* genes independently of each other, we constructed single KO mutants, $\Delta kdpD$ and $\Delta kdpE$. Deletion of *kdpE* resulted in a similar growth delay than the ones displayed by $\Delta kdpABC$, $\Delta kdpDE$ and $\Delta kdpABCDE$ (**Fig. 7A** and **Fig. S5B**). On the contrary, $\Delta kdpD$ behave differently since compared to WT, it had a slight but reproducible growth delay in exponential phase while it later reached a higher plateau in stationary phase (**Fig. 7A**). While the growth defects displayed by both single $\Delta kdpD$ and $\Delta kdpE$ mutants in limiting K⁺ condition could be complemented (**Fig. 7BC**), expressing back *kdpE* in $\Delta kdpDE$ cells led to the same growth behaviour than the single $\Delta kdpD$ mutant, that is a slight growth delay in exponential phase and a higher plateau in stationary phase (**Fig. 7C** and **Fig. S5C**). In addition, the fact that $\Delta kdpE$ is epistatic on $\Delta kdpD$, since $\Delta kdpDE$ grew as poor as $\Delta kdpE$ or $\Delta kdpABC$ (**Fig. 7A**), shows that KdpE is entirely responsible for the $\Delta kdpD$ phenotypes. Together, these data support that both components of the TCS are important for the growth in K⁺-depleted environments and suggest that KdpD and

KdpE have antagonistic roles for growth in such conditions. To better understand the KdpDE regulatory mechanism, we constructed *kdpD* and *kdpE* catalytic point mutants (**Fig. S5D**) and assessed their impact on growth in abundant and limiting K^+ conditions. In agreement with what we observed with the KO mutants, all the catalytic mutants grew similarly to the WT in abundant K^+ conditions (**Fig. S5E**). At limiting K^+ concentrations however, the phospho-ablative mutant of KdpE (*kdpE*_{D56A}) had a strong growth delay, similar to the one displayed by $\Delta kdpE$ (**Fig. 7D** and **Fig. S5F**). In contrast, the phospho-mimetic mutant (*kdpE*_{D56E}) grew similarly to the WT up to the late exponential phase, to finally reach a higher final OD comparable to the one observed with $\Delta kdpD$ grown in same conditions (**Fig. 7D** and **Fig. S5F**). Both the WT KdpE and KdpE_{D56A} proteins were detected at a low but similar level when K^+ concentration is not limiting, such as in complex PYE medium. On the contrary, the level of the KdpE_{D56E} was much higher in these conditions, likely because it constitutively stimulates the expression of the *kdpABCDE* operon (**Fig. S4A**). In contrast, in limiting K^+ conditions, WT KdpE reached high levels, similar to KdpE_{D56E}, whereas KdpE_{D56A} levels did not increase, likely because KdpE_{D56A} cannot be phosphorylated. Together, these data support that *kdpE*_{D56A} and *kdpE*_{D56E} behave respectively as phospho-ablative and phospho-mimetic mutants of KdpE. As expected, a KdpD catalytically dead mutant (*kdpD*_{H670N}) phenocopied $\Delta kdpD$ in limited K^+ (**Fig. 7D** and **Fig. S5F**). Since *kdpE*_{D56A} cells had a much stronger growth delay than $\Delta kdpD$ or *kdpD*_{H670N}, it indicates that KdpE is still phosphorylated despite the absence of KdpD. Thus, KdpE is very likely phosphorylated by another – non-cognate – HK. On the other hand, the fact that *kdpE*_{D56E} cells grew slightly but reproducibly better than $\Delta kdpD$ and *kdpD*_{H670N} suggests that KdpD also phosphorylates KdpE, at least during the early exponential phase of growth. Thus,

KdpD might (i) phosphorylate KdpE in early exponential phase, with at least another HK, and (ii) mainly act as a phosphatase on KdpE~P in late exponential phase, likely to downregulate the expression of *kdp* and restrict growth in K⁺-limiting conditions (Fig. S5G).

To test this hypothesis, we constructed a KdpD kinase-deficient but phosphatase active mutant (K⁻/P⁺) by replacing a conserved phenylalanine residue by a leucine residue (*kdpD*_{F832L}) in the F box involved in ATP binding, in a way reminiscent to what was described for another HK encoding gene, *pleC*_{F778L} (49) (Fig. S5D). Interestingly, we found that unlike the *kdpD*_{H670N}, *kdpD*_{F832L} cells failed to reach a higher plateau in stationary phase (Fig. 7D and Fig. S5F), strongly supporting our hypothesis that KdpD primarily works as a phosphatase in late exponential phase. Moreover, the K⁻/P⁺ mutant *kdpD*_{F832L} grew better than *kdpE*_{D56A} and $\Delta kdpE$ mutants in low K⁺ conditions (Fig. 7D and Fig. S5F), supporting that there is indeed at least another HK that phosphorylates KdpE. Finally, the fact that the *kdpD*_{F832L} mutant had a growth delay compared to WT suggests that KdpD also acts as a kinase for KdpE in early exponential phase (Fig. 7D and Fig. S5F). However, we cannot rule out the possibility that the phosphatase activity of KdpD_{F832L} is impacted by the mutation. For instance, it might be stronger than the WT thereby interfering with the phosphorylation levels of KdpE in early exponential phase. Nevertheless, our results altogether indicate that (i) KdpD works both as a kinase and a phosphatase to control KdpE phosphorylation (KdpE~P) levels depending on the growth phase and K⁺ availability and that (ii) KdpE can be phosphorylated by (an)other non-cognate HK(s) for regulating K⁺ transport in K⁺-depleted environments.

KdpD-dependent regulation of the *kdp* promoter in low K⁺ conditions

424 In order to study the Kdp system at the transcriptional level, a plasmid carrying a
425 $P_{kdp}::lacZ$ reporter fusion was introduced in the different mutant strains, cells were
426 grown in limiting or abundant K^+ conditions and β -galactosidase activity was
427 measured. Consistent with the idea that the Kdp system is a high-affinity potassium
428 transporter, P_{kdp} basal activity in WT cells was barely detectable at abundant K^+
429 concentration while it was strongly induced in WT (normalized to 100%) or even more
430 in $\Delta kdpABC$ cells upon K^+ depletion (**Fig. 8A**). In contrast, P_{kdp} activity was
431 completely annihilated in $\Delta kdpE$ and $kdpE_{D56A}$ cells (**Fig. 8A**), indicating that
432 phosphorylated KdpE (KdpE~P) is strictly required for *kdp* expression both in limiting
433 and abundant K^+ conditions. In support of that, the basal activity of P_{kdp} was even
434 higher in $kdpE_{D56E}$ cells grown at abundant K^+ concentration than in WT cells grown
435 in limiting K^+ conditions (**Fig. 8A**). In contrast, the histidine kinase KdpD seems not to
436 be required for *kdp* expression at low K^+ concentration since P_{kdp} was as active in
437 $\Delta kdpD$ as in WT cells starved for K^+ and still active in $kdpD_{H670N}$ or $kdpD_{F832L}$ cells
438 (**Fig. 8A**). However, when the hydrolase activity of KdpD was inactivated, that is in
439 $\Delta kdpD$ or $kdpD_{H670N}$ cells, the basal P_{kdp} activity at abundant K^+ concentration was on
440 average higher than in WT or $kdpD_{F832L}$, although these differences were not
441 statistically different (**Fig. 8A**). Together, these results suggest that KdpD mainly
442 works as a phosphatase on KdpE~P independently of K^+ availability.

443 To further characterize the role of KdpD in the regulation of *kdp* expression, we
444 measured the activity of the $P_{kdp}::lacZ$ fusion in WT and *kdpD* mutant cells along the
445 growth at limiting K^+ concentration. First, the activity of P_{kdp} in $\Delta kdpD$ cells was
446 slightly lower than the WT during early exponential phase but higher in stationary
447 phase (**Fig. 8B**, compare the yellow to the orange bars at 20-24 hrs and 48 hrs). On
448 the contrary, *kdp* expression was significantly lower both in $kdpD_{H670N}$ (K^-/P^-) and in

kdpD_{F832L} (K^+/P^+) during early exponential phase, but increased when reaching the late exponential only in *kdpD_{H670N}* cells (**Fig. 8B**, compare the blue to the brown bars at 20-24 hrs and 48 hrs).

Altogether, these results support our hypothesis that (i) KdpD mainly works as a phosphatase and that (ii) KdpE is mainly phosphorylated by another non-cognate kinase.

Discussion

As the most abundant monovalent cation used by living cells to drive many cellular processes, regulating potassium (K^+) homeostasis is critical for survival. The oligotrophic α -proteobacterium *Caulobacter crescentus* is cyclically subjected to nutrients deprivation in its natural environments. Here, we characterized the response of *C. crescentus* depending on K^+ availability. First, we determined the range of K^+ concentrations at which *C. crescentus* supports growth. Then, we identified all the genes predicted to code for proteins involved in transport, sensing and regulation of K^+ homeostasis. In addition, we defined the set of genes (i) becoming essential and/or (ii) being induced upon K^+ depletion. This allowed us to find that the low affinity and the high affinity K^+ transporters, respectively Kup and KdpABC, are critical components that maintain K^+ homeostasis in *C. crescentus*. Finally, we dissected the role played by the two-component (TCS) regulatory system KdpDE in the response to K^+ depletion.

By analysing the Tn-seq data, we found that the biological functions for which the highest number of genes that became essential or high fitness cost (HFC) upon K^+ limitation (M2G vs M2G-K) were (i) C “energy production and conversion” (~1%

473 increase of essential and ~6 % increase of HFC) and (ii) J “Translation, ribosomal
474 structure and biogenesis” (~2% increase of essential and ~3 % increase of HFC). In
475 fact, it is not so surprising these two categories are sensitive to K^+ availability since
476 ATP concentration and respiration state are two well-known parameters that drive K^+
477 transport across the membrane. For instance, the electron transport chain
478 determines the membrane potential, which in turn will influence the distribution of
479 other ions like K^+ that accumulate on the cytoplasmic face of the membrane. This
480 might therefore explain why Tn insertions in genes coding for subunits of the
481 respiratory chain or the ATP synthase are counter-selected when K^+ become limiting.
482 On top of this, active transport of K^+ across the membrane by Kdp consumes a lot of
483 ATP, so that decreasing ATP concentration might likely have a negative impact on K^+
484 import. On the other hand, K^+ cations are critical for maintaining the structure and
485 sustaining the ribosomal functions. Not only K^+ is required to stabilize the transfer
486 RNAs (tRNAs), the ribosomal RNAs (rRNAs) and proteins within the ribosomes, but
487 also to increase proofreading by accommodating the right aminoacyl-tRNA in the
488 ribosomal A-site (50, 51). Additionally, many GTPases involved in translation display
489 a K^+ -dependent activation of GTP hydrolysis (1). Thus, the impact K^+ has on
490 translation could explain the lower density of Tn insertion observed in tRNAs and
491 ribosomal genes in cells grown in limiting K^+ conditions.

492 KdpDE is the major bacterial system described in the literature to respond to limiting
493 K^+ availability. The K^+ -dependent regulon of *C. crescentus* allowed us to identify
494 almost 600 genes whose expression changes upon K^+ limitation. Surprisingly, only a
495 small proportion of this regulon depends directly or indirectly on the response
496 regulator KdpE, suggesting that there are unknown transcriptional regulators
497 sensitive to K^+ availability. Based on our Tn-Seq data, genes coding for

transcriptional regulators that became essential in K^+ limiting conditions might constitute good candidates for such a function (**Table S2**).

Although KdpE is mainly described to regulate the expression of the *kdp* genes, a few studies already suggested it might control other cellular processes. For instance in *E. coli*, 197 differentially expressed genes with a $FC \geq 1.5$ were identified in a *kdpE* knock-out mutant vs WT grown in K^+ limiting conditions (0.1 mM KCl). These genes are involved in transport and metabolism of carbohydrates and nucleotides, energy production and conversion (52). ChIP-seq experiments performed in the plant pathogen *Lonsdalea quercina* subsp. *populi* revealed that KdpE interacts with 44 promoter regions of genes related to cell morphology and division, transcription and translation, nucleotide and carbohydrate metabolism (53).

The analysis of the *C. crescentus* genome allowed us to identify several systems predicted to transport K^+ . By systematically characterizing the growth profile of the corresponding mutants in different K^+ regimes, we found that the high affinity Kdp system primarily serves to import K^+ at limiting concentrations, like in many other bacterial species. Nonetheless, Kdp is unlikely the sole K^+ transport system involved in this context since despite a growth defect, the $\Delta kdpABC$ mutant was still able to grow at low K^+ concentration. Interestingly, we found that the low affinity Kup transporter became dispensable in such limiting K^+ conditions. However, suppressor mutations in Kup (A87P, G253S, and S456R) that improved the growth the Δkdp mutant at low K^+ concentration, without impacting the growth when K^+ was abundant, were selected. A plausible explanation would be that mutations found in Kup increase its affinity for K^+ , thereby increasing K^+ transport to compensate the loss of Kdp. In support of that, MD simulations have revealed structural changes in the putative K^+ binding cavity of Kup. First, all the mutants led to the flipping of E263 to a

position closer to the one described for the corresponding E233 in KimA when bound to K^+ , which likely makes the second binding pocket accessible and would allow the coordination of another K^+ cation. Indeed, the position observed for E263 in the modelled WT Kup configuration appears to interfere with the second K^+ binding site. Second, the carboxylate group of D146 was made more available for K^+ coordination within this second binding site in a way similar to the one described for the corresponding D117 in KimA. Third, compared to the WT Kup, positions of Y61 and Y410 in the G253S and S456R mutants were also found to be shifted towards the positions observed for the corresponding Y43 and Y377 in KimA, thereby exposing to a greater extent their tyrosyl moiety towards the inside of the cavity where K^+ ion is located. Such structural modifications would be induced by local reorganization of H bonds at the mutation sites, resulting in a cascade of subtle conformational changes towards the binding cavity. While the introduction of P87 abrogates two potential H bonds between A87 and the backbone of V83 and V84, S253 can form new H bonds with an adjacent α -helix thanks to its polar side chain (**Fig. S3BC**). Conversely, mutation of S456 into R456 decreases the number of intra-helix H bonds through the loss of the hydroxyl moiety (**Fig. S3D**). The available Cryo-EM structure of KimA likely represents an inward-occluded state in which two K^+ cations are trapped in the intracellular cavity by the ideally positioned Y43, D117, E233 and Y377 residues (48). Thus, our *in silico* results are consistent with a higher K^+ affinity observed experimentally in Kup mutants, likely due to the reorientation of the corresponding Y61, D146, E263 and Y410 residues into a “KimA bound to K^+ ”-like configuration, which consequently enhances K^+ coordination.

Beside Kup, other unknown transporters could participate to K^+ uptake in such limiting conditions. Several putative transporters were identified as essential and high

548 fitness cost in our Tn-seq analysis (CCNA_01159) and up-regulated in the RNA-seq
549 analysis (CCNA_00339, CCNA_01852, CCNA_01587, CCNA_01588, CCNA_02570,
550 CCNA_02571). It is possible that one or several of them second the activity of
551 KdpABC in limiting K⁺ conditions.

552 As in *E. coli*, the expression of *kdp* operon in *C. crescentus* is activated upon K⁺
553 starvation by the KdpDE TCS (54). However, we found surprising differences since in
554 the absence of the histidine kinase (HK) KdpD, (i) *kdp* is still expressed and (ii) the
555 *kdp* promoter (P_{kdp}) is more active in stationary phase. Our genetic dissection of the
556 catalytic activity of KdpDE with point mutants in *kdpE* (phospho-mimetic *kdpE*_{D56E}
557 and phospho-ablative *kdpE*_{D56A}) and KdpD (K⁻/P⁻ *kdpD*_{H670N} and K⁻/P⁺ *kdpD*_{F832L})
558 strongly suggests that (i) KdpE is phosphorylated by another – non-cognate – HK
559 and that (ii) KdpD works mainly as a phosphatase.

560 In *E. coli*, deletion of the *kdpD* results in a prominent growth arrest, of more than 20
561 hours, before restarting growth thanks to KdpE phosphorylation by PhoR. However, it
562 never surpassed the WT plateau as seen with *C. crescentus* Δ *kdpD*, *kdpD*_{H670N} and
563 *kdpE*_{D56E} mutants. Thus, the higher plateau is likely due to the loss of KdpD
564 phosphatase activity. The phosphatase activity of KdpD has been shown in *E. coli* to
565 be stimulated by intracellular K⁺ intracellular levels (23). Four amino acids located in
566 the second periplasmic loop were identified as an extracellular K⁺-specific recognition
567 site. In contrast, the K⁺-recognition site for intracellular sensing remains to be
568 identified. However, although specific residues could not be highlighted, the C-
569 terminal cytoplasmic domain of KdpD was described to display a K⁺-dependent
570 phosphatase activity (20, 55). Authors suggested that the cytoplasmic GAF domain of
571 KdpD may be responsible for intracellular K⁺ sensing. Most GAF domains are
572 involved in regulatory functions through binding to cyclic nucleotides or other small

ligands including ions (55, 56). However, *C. crescentus* KdpD lacks a GAF domain, which suggests that such a connection between intracellular sensing and phosphatase activity does not exist in *C. crescentus* (**Fig. S5D**). Recently, it was reported that *E. coli* KdpD also works as serine kinase in high K⁺ concentrations to control KdpB ATPase activity and ultimately stop Kdp-dependent K⁺ transport (57). The phosphorylated serine residue in KdpB as well as the two Walker (A and B) domains in tandem driving the serine kinase activity of KdpD are conserved in *C. crescentus* (**Fig. S6**), indicating that this post-transcriptional control of K⁺ influx might also work in *C. crescentus*.

As mentioned above, in *E. coli*, the sensor kinase PhoR can crosstalk with KdpE, which allows a $\Delta kdpD$ mutant to grow in a low K⁺ environment after a long lag phase (21). Since PhoR is conserved in *C. crescentus*, it constitutes a good candidate for the non-cognate HK phosphorylating KdpE, although based on our Tn-Seq data the *phoR* gene seems dispensable on low K⁺ plates (**Table S2**).

The nitrogen-related phosphotransferase component EIIA^{Ntr} has been shown in *E. coli* to regulate K⁺ transport, by modulating KdpD kinase activity (26, 58, 59). For instance, in limiting K⁺ conditions (< 5 mM), the unphosphorylated form of EIIA^{Ntr} was shown to directly interact with KdpD and stimulate its kinase activity, thereby promoting phosphorylation of KdpE and the subsequent increase of *kdp* genes expression (25). This EIIA^{Ntr}-based control of KdpD has also been reported in the plant symbionts *Rhizobium leguminosarum* (60) and *Sinorhizobium fredii* (8), two α -proteobacteria closely related to *C. crescentus*. It was later discovered that EIIA^{Ntr} regulates sigma factor selectivity by modulating K⁺ levels. Indeed, the competition between σ^{70} and σ^S for interaction with the RNA polymerase (RNAP) is influenced by K⁺ intracellular levels (61). Depending on the phosphorylation state of EIIA^{Ntr}, the

resulting K^+ intracellular levels preferentially favour the binding of either σ^{70} or σ^S to the core RNAP (61). Interestingly, PTS^{Ntr} components regulate the levels of another signalling molecule – (p)ppGpp – that connects cell division with environmental and metabolic cues in *C. crescentus* (62-64). Knowing that (p)ppGpp also influences gene expression by regulating sigma factor competition (65), it is therefore tempting to speculate that $EIIA^{Ntr}$ and/or (p)ppGpp participate to the K^+ homeostasis in *C. crescentus*, even in other α -proteobacteria.

Methods

Bacterial strains and growth conditions.

All *Escherichia coli* strains used in this thesis were grown aerobically in Luria-Bertani (LB) broth (Sigma). *E. coli* Top10 was used for cloning purpose while *E. coli* MT607 was used for triparental matings. Thermo-competent cells were used for transformation of *E. coli*. All *Caulobacter crescentus* strains used are derived from the synchronizable wild-type strain NA1000. The traditional synthetic medium supplemented with glucose (M2G) was modified by replacing potassium salts by their equivalent sodium salts to obtain a medium without any K^+ (K_0) to which the desired K^+ concentrations could be added. The source of K^+ consists in both KH_2PO_4 and K_2HPO_4 mixed to an equivalent ratio in terms of K^+ concentration. For each experiment, cells were first grown at 30 °C; in Peptone Yeast Extract (PYE), then adapted overnight at 30 °C in K_0 medium supplemented with 0.5 mM K^+ , washed twice in K_0 medium and finally grown at 30 °C in K_0 medium supplemented with the desired K^+ concentration. For growth curve measurements, cultures were diluted to a final OD_{660} of 0.02 in a 96-well plate. Growth was monitored during 48 h with

continuous shaking at 30 °C, in an automated plate reader (Bioscreen C, Lab Systems) measuring OD₆₆₀ every 10 min. For *E. coli*, antibiotics were used at the following concentrations (µg/ml; in liquid/solid medium): chloramphenicol (20/30), kanamycin (30/50), oxytetracycline (12.5/12.5), spectinomycin (100/100), streptomycin (50/100) while for *C. crescentus*, media were supplemented with kanamycin (5/20), oxytetracycline (2.5/2.5), nalidixic acid (20) when appropriate. Genes under the control of the inducible xylose promoter (P_{xyIX}) were induced with 0.1 % xylose. 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 M2G-K) were prepared in 96-well plates from 5 ml cultures in standard universal tubes grown overnight at 30 °C in the corresponding medium. Cells (5µl) were then spotted on plates, incubated at 30 °C for two-to-three days and pictures were taken.

β-galactosidase assays

β -galactosidase assays were performed as already described in (42). Briefly, cultures were either grown to stationary phase directly in a 96-wells plate or grown in flasks from which samples were withdrawn throughout growth and placed at -80 °C in between time points. Permeabilization of cells was performed by incubating cells with lysis buffer (20 mg/ml polymyxin B, β -mercaptoethanol) for 30 min at 28 °C. Then, 50 μ l of 4 mg/ml O-nitrophenyl- β -D-galactopyranoside (ONPG) were added. Lysis and ONPG solutions were prepared using Z buffer as a solvent (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , pH 7.0). Both OD_{420} and OD_{550} were measured every minute for 30 min at 30 °C using a fluorimeter (SpectraMax®, Molecular Devices). Miller Units were calculated using the following formula: $\text{MU} = (\text{OD}_{420} \times 1,000) / [\text{OD}_{660} \times t \times v]$ where t is the reaction time in min v is the volume of culture used in ml. 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: $\text{MU} = (\text{OD}_{420} \times 1,000) / [\text{OD}_{660} \times t \times v]$ 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.

Protein purification and production of polyclonal antibodies

To immunize rabbits for production of polyclonal antibodies, an *E. coli* BL21 (DE3) pLysS strain carrying plasmid pET-28a-*kdpE* was grown in LB medium supplemented with kanamycin and chloramphenicol until an OD_{600} of 0.5. After addition of IPTG to a final concentration of 1 mM, the culture was incubated at 18 °C for 18 hrs. Cells were then harvested by centrifugation for 30 min at 5,000 x g, 4 °C. The pellet was resuspended in 20 ml of binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM MgCl_2 , 12.5 mM Imidazole) supplemented with complete EDTA-free protease cocktail inhibitor (Roche), 400 mg lysozyme (Sigma) and 10 mg

671 DNaseI (Roche) and incubated for 30 min on ice. Cells were then lysed by
672 sonication. After centrifugation at 12,000 rpm for 30 min at 4°C, the lysate was
673 loaded on a Ni-NTA column and incubated 1 hr at 4 °C with end-over-end agitation.
674 The column was then washed with 5 ml binding buffer, 3 ml Wash1 buffer (binding
675 buffer with 25 mM imidazole), 3 ml Wash2 buffer (binding buffer with 50 mM
676 imidazole), 3 ml Wash3 buffer (binding buffer with 75 mM imidazole). Proteins bound
677 to the column were eluted with 3 ml Elution buffer (binding buffer with 100 mM
678 imidazole) and aliquoted in 300 µl fractions. All the fractions containing the protein of
679 interest (checked by Coomassie blue staining) were pooled and dialyzed in Dialysis
680 buffer (20 mM Tris pH 8, 500 mM NaCl, 10% glycerol). Purified KdpE was used to
681 immunize rabbits (CER Groupe, Marloie, Belgium).

682 **Tn-seq analysis.**

683 A mini-Tn5 was introduced in *C. crescentus* NA1000 WT strain by bi-parental mating.
684 Briefly, overnight cultures of recipient and donor strains (grown in LB supplemented
685 with kanamycin) were mixed in 95:5 ratio to a final volume of 1 ml. Cells were
686 harvested by centrifugation at 9,000 rpm (7,600 x g) at room temperature for 2 min.
687 The supernatant was removed, and 1 ml of fresh PYE medium was added to wash
688 the pellet. A second centrifugation step was done to remove again the supernatant.
689 Thereafter, the pellet was resuspended in 50 µl and spotted on PYE agar and
690 incubated at 30 ° C for 4h. Cells were collected, resuspended in 10 ml M2G or M2G-
691 K and washed twice with the same volume. Dilutions (10^{-6}) were plated on M2G or
692 M2G-K plates supplemented with aztreonam ($5\mu\text{g ml}^{-1}$) and kanamycin ($5\mu\text{g ml}^{-1}$) and
693 incubated at 30 ° C for 5 days. Then, at least 3×10^5 colonies were collected in M2G
694 or M2G-K supplemented with 10% glycerol and stored at -80 °C. Genomic DNA was
695 then extracted following the *Nucleospin Tissue Kit* protocol (Macherey-Nagel) and

resuspended in 50 μ l elution buffer (5 mM Tris-HCl [pH 8.5]). DNA sequencing was performed using an Illumina NextSeq (paired-end 2x75) instrument (Fasteris, Geneva, Switzerland). Reads corresponding to the mini-Tn5 insertion sites were first filtered with the 5'-GGTTGAGATGTGTATAAGAGACAG sequence before being processed as described in (66). Briefly, filtered reads were mapped on the genome of *C. crescentus* NA1000 (GenBank accession number NC_011916.1) and converted to Sequence Alignment/Map (SAM) format using the Burrows-Wheeler Aligner (BWA) and SAMtools, respectively, from the Sourceforge server (<https://sourceforge.net/>). Next, the number of reads overlapping each genomic position was computed using custom Python scripts. The total number of reads for internal 80% of each ORF in each condition were then computed using custom Python scripts. For comparing strains, the total number of reads was normalized by the ratio of the number of reads between the two strains. Ward's clustering analysis was performed to define the fitness categories as previously reported (41), that are (i) essential, (ii) high fitness cost (HFC) and (iii) non-essential. The number of clusters was arbitrarily set to 10 with ORFs from cluster 1 classified as essential, from cluster 2 as high fitness cost, and those from clusters 3-10 as nonessential.

For the M2G library, genes with values of Tn insertions / bp ≤ 0.01333333 were considered as essential, $0.01333779 \leq$ and ≤ 0.04063018 as HFC, and ≥ 0.04081632 as non-essential. For the M2G-K library, genes values of ≤ 0.0047112 were considered as essential, $0.0047909 \leq$ and ≤ 0.0211178 as HFC, and ≥ 0.0215792 as non-essential.

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

ChIP-Seq was performed as already described in (66). Briefly, 80 ml of mid-log-phase cells (OD_{660} 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 Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4) resuspended in 450 μ l TES buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 100 mM NaCl), and lysed with 2 μ l 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 μ l of ChIP buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, protease inhibitors) were added to the lysate and incubated at 37 °C for 10 min before sonication (2 x 8 bursts of 30 sec on ice using a Diagenode Bioruptor) to shear DNA fragments to a length of 300 to 500 bp. Lysate was cleared by centrifugation for 10 min at 12,500 rpm at 4 °C, and protein content was assessed by measuring the OD_{280} . Then, 7.5 mg of proteins was diluted in ChIP buffer supplemented with 0.01% SDS and precleared for 1 hr at 4 °C with 50 μ l of SureBeads Protein A Magnetic Beads (BioRad) and 100 μ g bovine serum albumin (BSA). One microliter of polyclonal anti-KdpE antibodies was added to the supernatant before overnight incubation at 4 °C under gentle agitation. Next, 80 μ l of BSA presaturated protein A-agarose beads were added to the solution and incubated for 2 hrs at 4 °C with rotation, washed once with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), once with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]), and once with TE buffer (10 mM Tris-HCl [pH 8.1] 1 mM EDTA) at 4 °C,

745 followed by a second wash with TE buffer at RT. The DNA-protein complexes were
746 eluted twice in 250 µl freshly prepared elution buffer (0.1 M NaHCO₃, 1% SDS).
747 NaCl was added at a concentration of 300 mM to the combined eluates (500 µl)
748 before overnight incubation at 65 °C to reverse the cross-link. The samples were
749 treated with 20 µg of proteinase K in 40mM EDTA and 40 mM Tris-HCl (pH 6.5) for 2
750 h at 45 °C. DNA was extracted using a Nucleospin PCR cleanup kit (Macherey-
751 Nagel) and resuspended in 50 µl elution buffer (5 mM Tris-HCl [pH 8.5]). DNA
752 sequencing was performed using an Illumina NextSeq 550 (paired-end 2x75 or
753 2x100) instrument (Seqalis). NGS data were analysed as described in (66).

754 **RNA-Seq**

755 WT and *ΔkdpE* cells were grown overnight to OD₆₆₀ ~ 0.3 in M2G-K 0,025 mM K⁺
756 and WT in M2G-K 0,5 mM K⁺. Thereafter, total RNA was extracted with RNeasy®
757 Protect Bacteria Kit from Qiagen and following manufacturer's instructions. The
758 quantity and quality (A260/A280 ration) of RNA was determined with a Thermo
759 Scientific™ Nanodrop™ One Microvolume UV-Vis Spectrophotometer. RNA-Seq
760 TTRNA libraries were prepared from total RNA after ribodepletion with the Illumina
761 Ribo-Zero Plus rRNA Depletion kit and sequenced with Illumina NovaSeq 6000
762 (paired-end 2x100) instrument (Seqalis). NGS data were analysed using Galaxy
763 (<https://usegalaxy.org>) (42). Briefly, FastQC was used to evaluate the quality of the
764 reads; HISAT2 was used to map the reads onto the NA1000 reference genome
765 (NC_011916.1) and generate bam files; featureCounts was used to generate counts
766 tables using bam files and DESeq2 was used to determine differentially expressed
767 genes. The Volcano plot was generated using GraphPad Prism 9 software.

768 **Statistical analyses**

All the statistical analyses were performed using GraphPad Prism 9 software. A *P* value of <0.05 was considered as significant.

Molecular dynamics simulations

Protein systems were prepared for simulations from the three-dimensional model of Kup from *Caulobacter crescentus* (UniProt ID: Q9ABT9 or B8GXM1) generated with AlphaFold2 (67). A87P, G253S, and S456R mutants were manually edited from the WT structure at the corresponding position via the PyMOL software (version 2.5.2). As the putative potassium binding site of Kup is located within its transmembrane helices, the N- (residues 1 to 38) and C-terminal (residues 499 to 665) domains were truncated to reduce computational costs. The resulting structural models (WT, A87P, G253S, and S456R) were properly protonated at pH 7.5 via the ProteinPrepare tool implemented in PlayMolecule (68). The CHARMMGUI suite was used for building the lipid bilayer, the insertion of Kup, and for the generation of input files using the Membrane Builder interface (69, 70). Based on the reported composition of *Caulobacter* inner membrane, a mix of 90% 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) and 10% tetraoleoyl cardiolipin (TOCL2) was selected to better reflect the native environment of Kup (46, 47). Each protein structure was oriented along the z axis, perpendicular to the lipid bilayer, with the transmembrane helix constituted from residue R77 to M104. The upper leaflet contained 65 DOPG and 7 TOCL2 molecules, whereas the lower leaflet was composed of 63 and 7 DOPG and TOCL2 molecules, respectively. KUP-embedded lipidic systems were solvated in TIP3P water, neutralized with K⁺ cations, and supplemented with 150 mM KCl. All-atom molecular dynamics (MD) simulations were carried out with the GROMACS 2023.1 suite package (71) on Kup WT, A87P, G253S, and S456R systems, using CHARMM36m force field ((72). Overall calculation efficiency was

improved by hydrogen mass repartitioning (HMR), allowing to increase the time step to 4 fs. The energy of each system was first minimized within 5000 steps using the steepest descent method. Systems were further equilibrated successively at a temperature of 300 K and a pressure of 1 bar by the use of Berendsen thermostat and barostat, respectively, for a total of 1875 ps. Production MD were performed at 300 K and 1 bar with the Nosé-Hoover thermostat and the Parinello-Rahman barostat for 1 μ s each. Long-range electrostatic interactions were taken into account by means of the Particle Mesh Ewald (PME) summation. Periodic boundary conditions (PBC) were set in all three dimensions, and atom trajectories were recorded every 4 ps. The final frame of each run was extracted and visualized with the PyMOL software (version 2.5.2). The latter was also used to create the presented snapshots and figures.

Data availability

ChIP-Seq, RNA-Seq and Tn-Seq data have been deposited to the Gene Expression Omnibus (GEO) repository with the accession numbers [GSE253227, GSE253228, GSE253229].

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Competing financial interests

The authors declare no competing financial interests.

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Figure legends

Figure 1. Impact of K⁺ on *C. crescentus* growth. Growth of WT in M2G-K supplemented with different K⁺ concentrations using K₂HPO₄ + KH₂PO₄ (A-B) and KCl (C-D) as K⁺ source. Data represent average, n=3, and error bars= ±SD. Other concentrations tested are presented in Fig. S1.

Figure 2. Deletion of K⁺-related genes impact *C. crescentus* growth. Growth of WT, $\Delta kefB$, $\Delta kefC$, $\Delta CCNA_01688$ and $\Delta kdpABCDE$ mutants in minimal medium M2G-K supplemented with (A) limiting (0.025 mM), (B) abundant (0.5 mM) and (C) excessive (50 mM) K⁺ concentrations. Data represent average, n=3, and error bars= ±SD.

Figure 3. Tn-seq profile of *C. crescentus* cells in limiting K⁺ conditions. (A) Diagram of the Tn-seq experiment. As explained in detail in Material and Methods section, *E. coli* (*E. coli*) cells were used to deliver a mini Tn5 vector into *C. crescentus* (*C. crescentus*) by conjugation. Mating spots were washed twice in either M2G or M2G-K. Cells were plated in either M2G or M2G-K square plates supplemented with kanamycin (Km) and aztreonam (Az) to counter select *C. crescentus* cells with Tn5 insertions (green cells) over *C. crescentus* without insertions and *E. coli* cells. Cells were collected to complete > 3 x 10⁵ clones. Thereafter gDNA extraction and Tn5 directed sequencing was performed to determine the frequency of insertions for each gene in *C. crescentus* genome and determine genes that are essential, high-fitness cost or non-essential in M2G and M2G-K plates. (B) Growth of WT and Δkdp cells in M2G-K

plates without or supplemented with K^+ . (C) Number of Transposon insertions (#Tn insertions) against the length (#bp) of 4186 genes in M2G and M2G-K media. Non-essential genes are highlighted in grey, high-fitness cost genes in light blue, and essential in dark blue. (D) Representation of genes that changed in their fitness-cost categories according to analysis done in M2G and M2G-K (C). (C-D) The percentage of genes in each category is indicated following the colour code.

Figure 4. Impact of deletion and overexpression of the *kup* gene. (A) Growth of WT and Δkup mutant in minimal medium M2G-K supplemented with limiting (0.025 mM), abundant (0.5 mM), excessive (50 mM) K^+ concentrations or limiting (0.025 mM) K^+ concentrations and 3% sucrose. (B) Growth of WT, *kup*_{A87P}, *kup*_{G253S} and *kup*_{S456R} in an otherwise WT or Δkdp background, in abundant and limiting K^+ conditions. Data represent average, n=3, and error bars= $\pm$ SD.

Figure 5. Molecular dynamics-highlighted structural changes of the putative K^+ binding sites of Kup mutants. (A) Snapshot of Kup WT after 1 μ s of simulation. The protein is represented in cyan as cartoon, the considered mutation sites are colored in magenta and indicated by arrows, and the residues composing the KimA-based K^+ binding cavity are highlighted as sticks in dark blue. (B) Snapshot of the KimA-based K^+ binding site of Kup_{A87P}, (C) Kup_{G253S}, and (D) Kup_{S456R} after 1 μ s of simulation. On each of these panels, WT Kup (cyan) and mutated Kup (magenta) aligned to KimA (PDB entry: 6S3K; light grey) are shown as cartoon, conserved residues between Kup and KimA constitutive of the K^+ binding pocket are represented as sticks in the corresponding color, and K^+ cations as orange spheres. The displayed amino acid numbering only corresponds to Kup sequence.

Figure 6. K⁺ and KdpE-dependent regulon. (A-B) Volcano plots representing the relation between the log₂ fold change (FC) and -log False Discovery Rate (FDR) on gene expression between (A) WT in limiting vs abundant K⁺ condition, and (B) $\Delta kdpE$ vs WT in limiting K⁺ condition. Genes identified are presented as dots. Significant down- and up-regulated genes are presented as blue (A) and purple (B) dots, while genes with no significant alterations are presented as black dots. Top 10 down and upregulated genes are highlighted for each analysis.

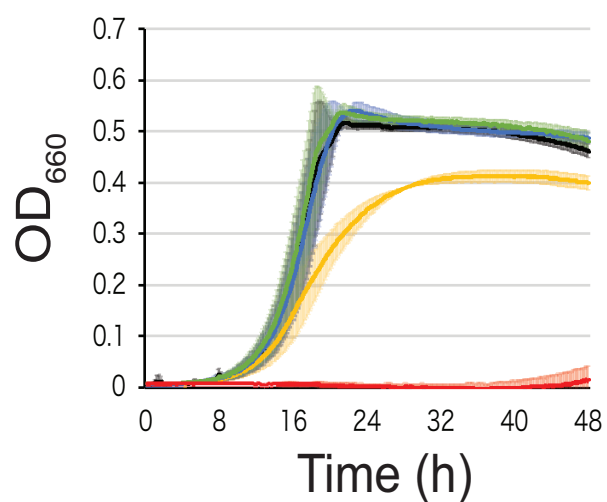
Figure 7. KdpD downregulates KdpE to control growth in low K⁺. (A) Growth of WT, $\Delta kdpABC$, $\Delta kdpD$, $\Delta kdpE$ and $\Delta kdpDE$. (B) Growth of WT and $\Delta kdpD$ carrying either pMR10 empty vector (EV) or pMR10 $P_{kdp}::kdpD$. (C) Growth of WT, $\Delta kdpE$, $\Delta kdpDE$ expressing *kdpE* from the *xylX* locus ($P_{xylX}::kdpE$). (D) Growth of *kdpD* and *kdpE* catalytic point mutants. Growth was done in minimal medium M2G-K supplemented with limiting (0.025 mM) K⁺ concentrations (A-D), and tetracycline for plasmid selection (B) or with 0.01 % xylose for the induction of *kdpE* expression (C). Data represent average, n=3, and error bars= $\pm$ SD.

Figure 8. Regulation of *kdp* expression (A) Relative β -Galactosidase activities of WT and *kdp* mutants carrying a transcriptional $P_{kdp}::lacZ$ fusion grown in M2G-K supplemented with limiting (0.025 mM) or abundant (0.5 mM) K⁺ concentrations. The data were normalized to the WT grown in limiting K⁺ conditions (blue bars). (B) Relative β -Galactosidase activities of WT and *kdp* mutants carrying a transcriptional $P_{kdp}::lacZ$ fusion along the growth in M2G-K supplemented with limiting (0.025 mM). The data were normalized to the WT grown in limiting K⁺ conditions (orange bars) after 20 hrs of growth. Data represent average, n=3, and error bars= $\pm$ SD. Means were statistically compared using a two-way ANOVA, followed by Tukey's multiple

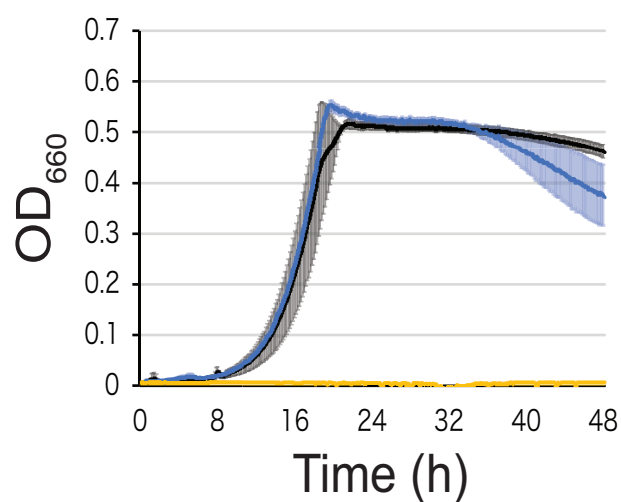
1085 comparisons test; not significant (NS), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P <$
1086 0.0001 (****).

Figure 1

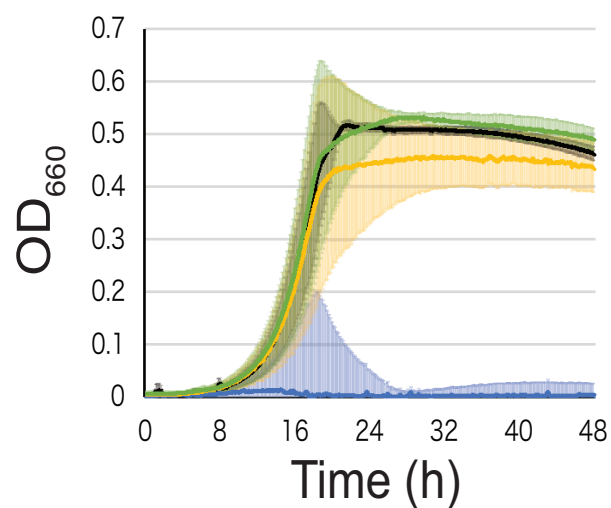
A



B



C



D

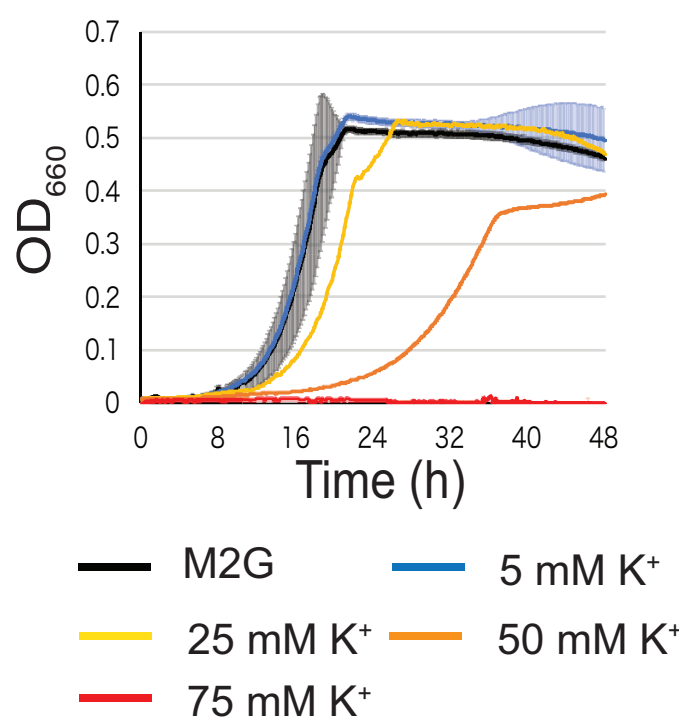


Figure 2

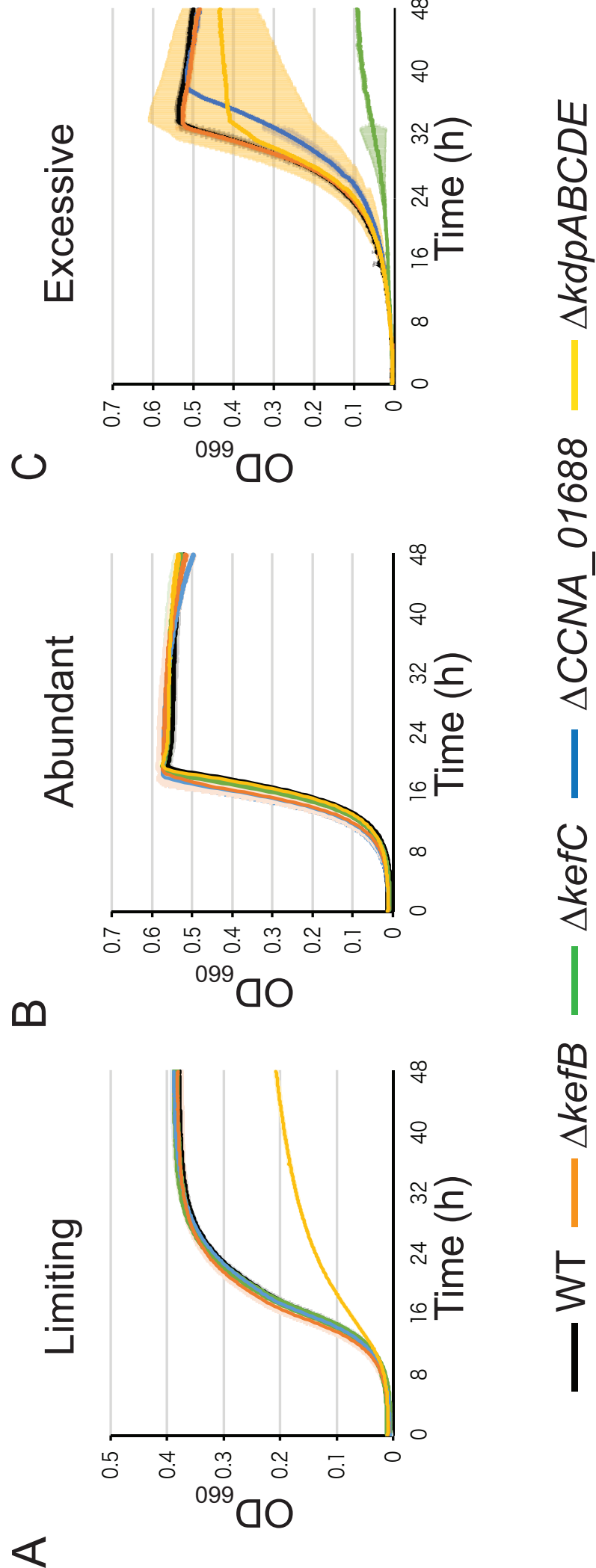


Figure 3

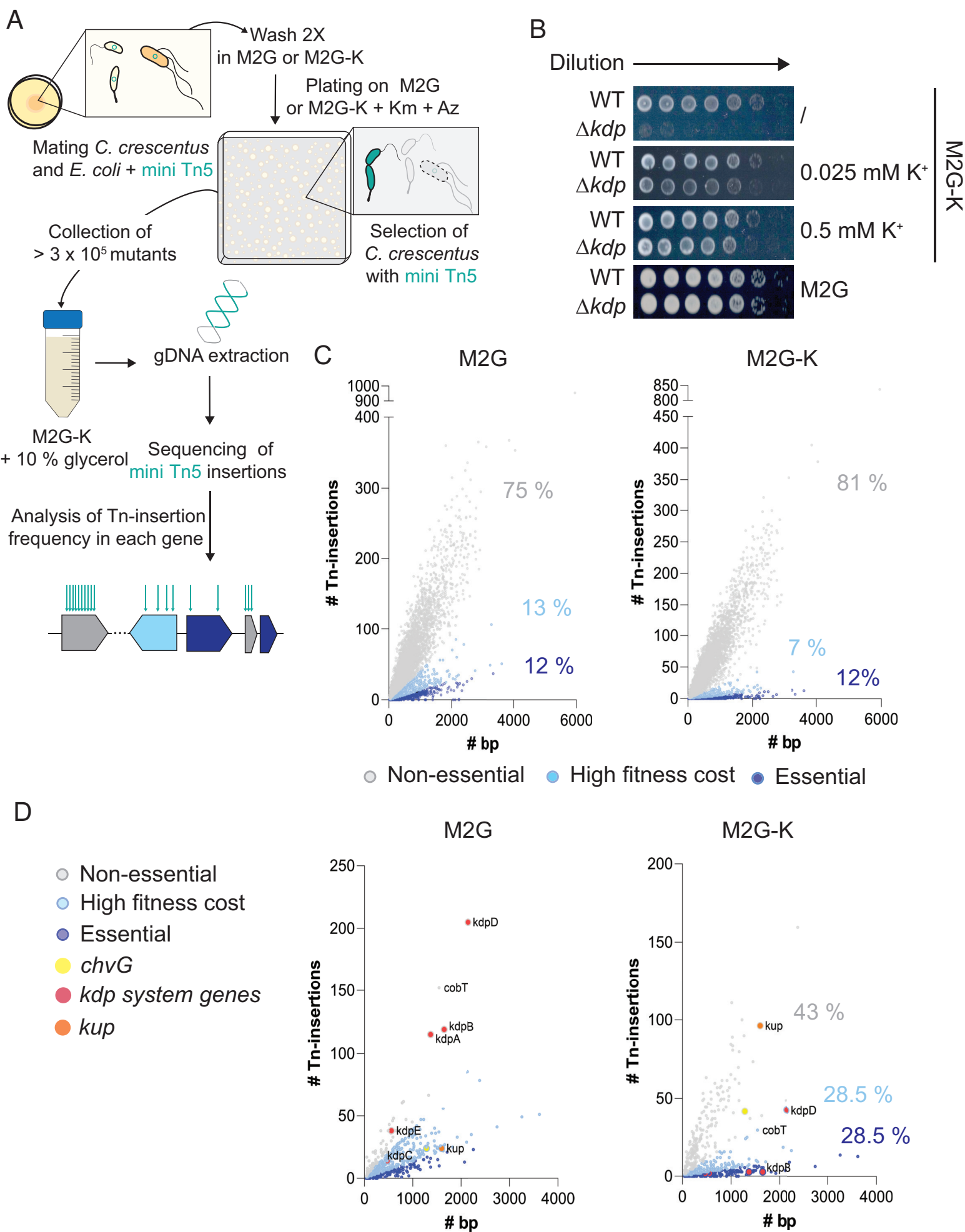
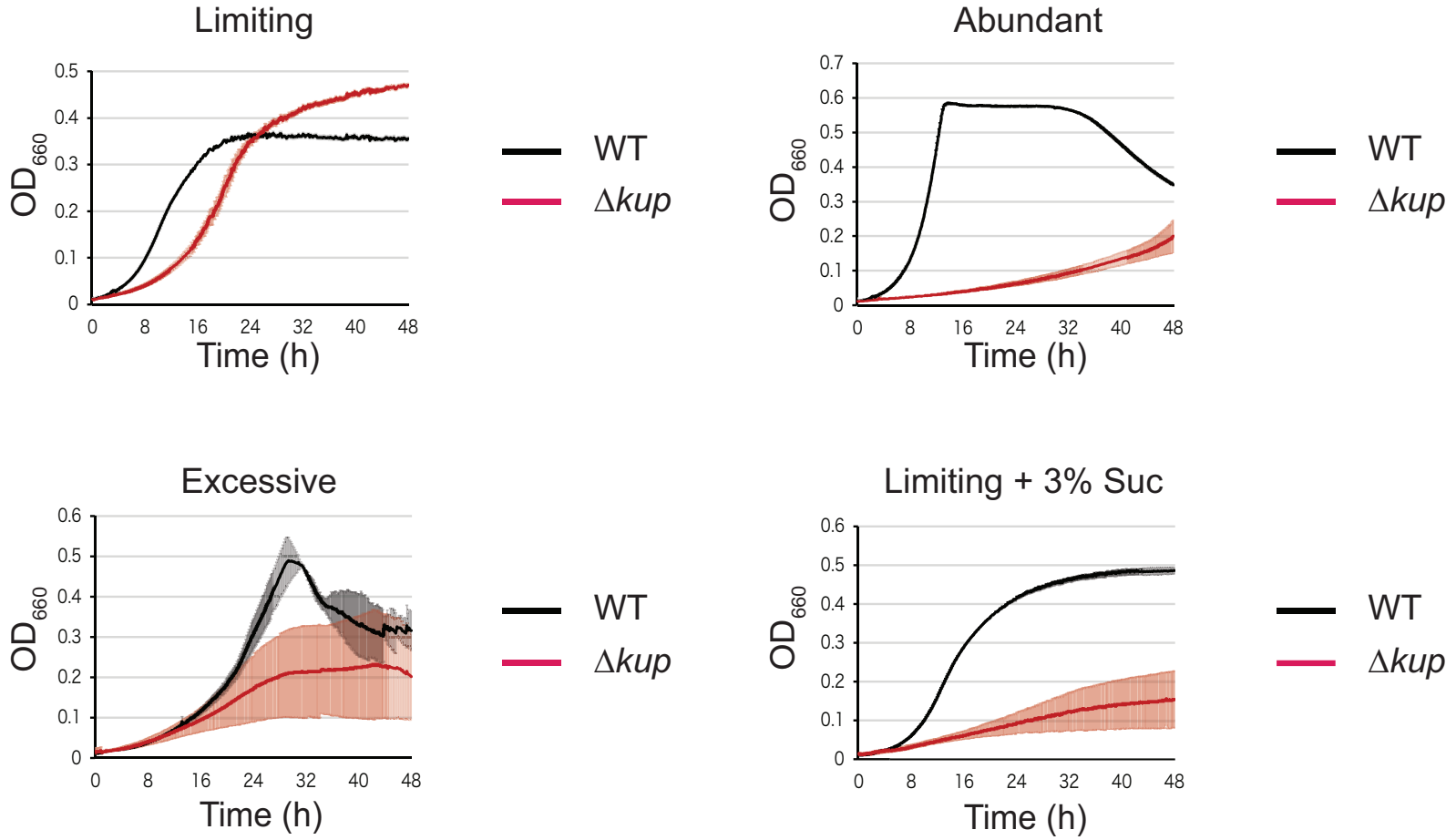


Figure 4

A



B

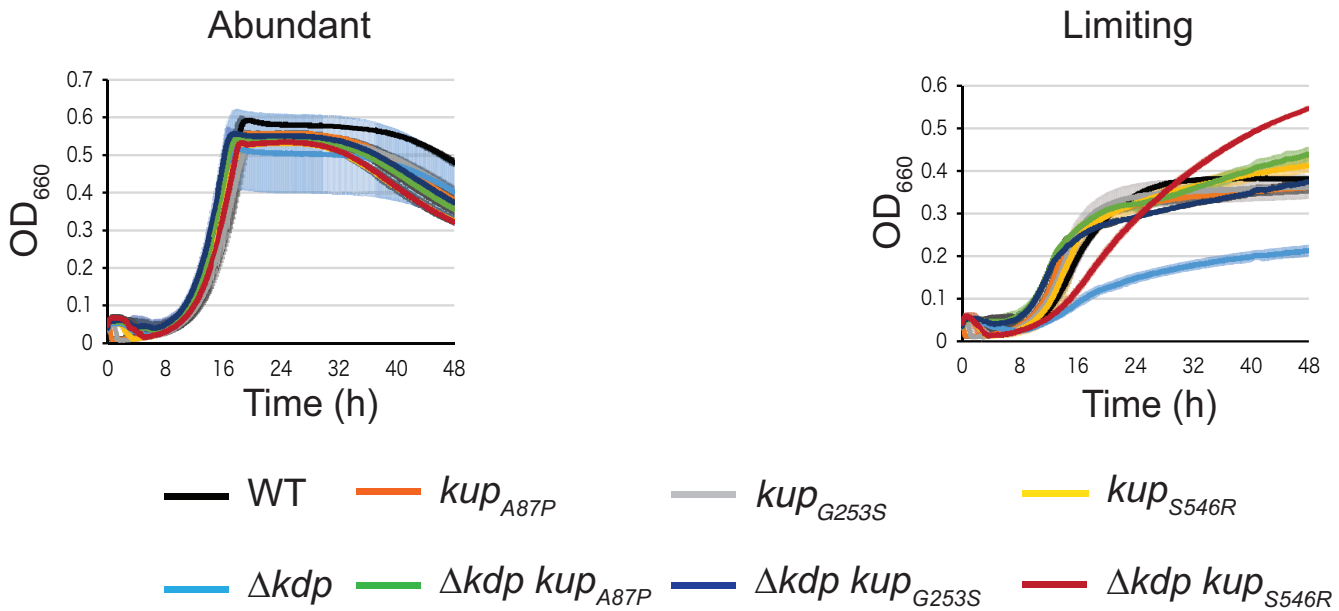


Figure 5

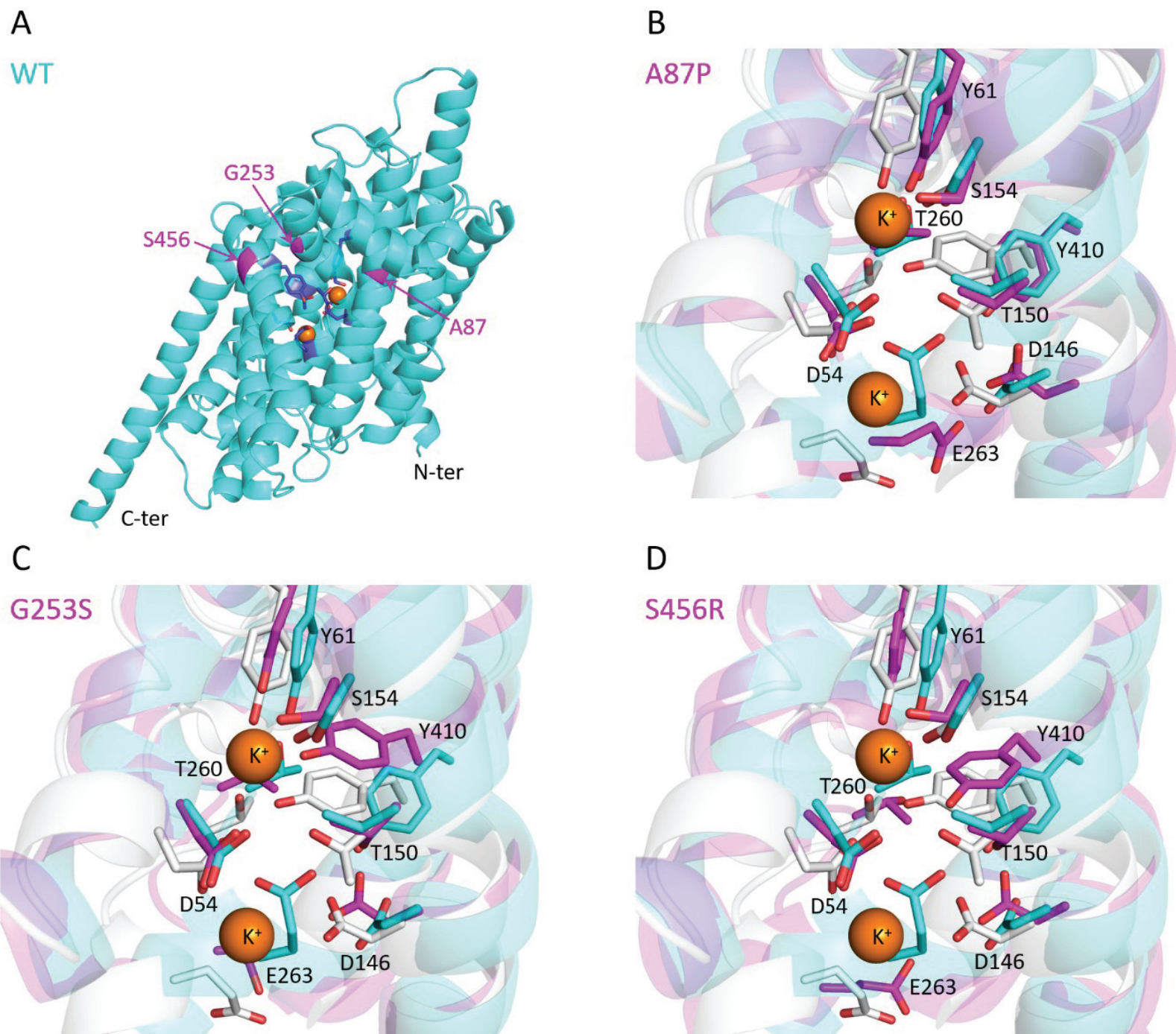
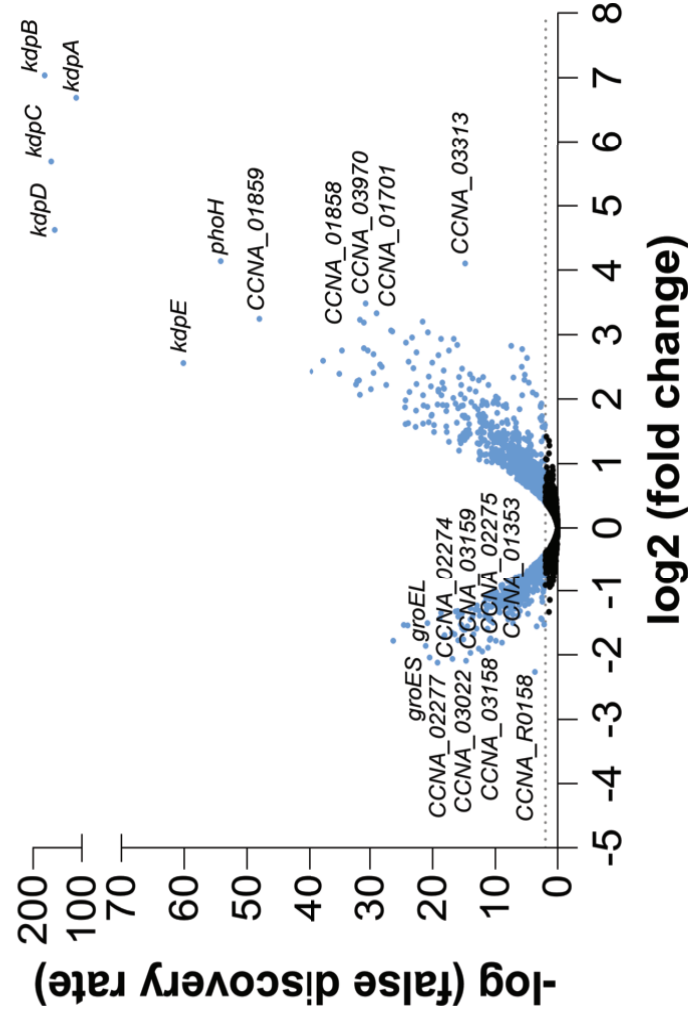


Figure 6

A

WT limiting vs abundant [K+]



B

$\Delta kdpE$ vs WT low [K+]

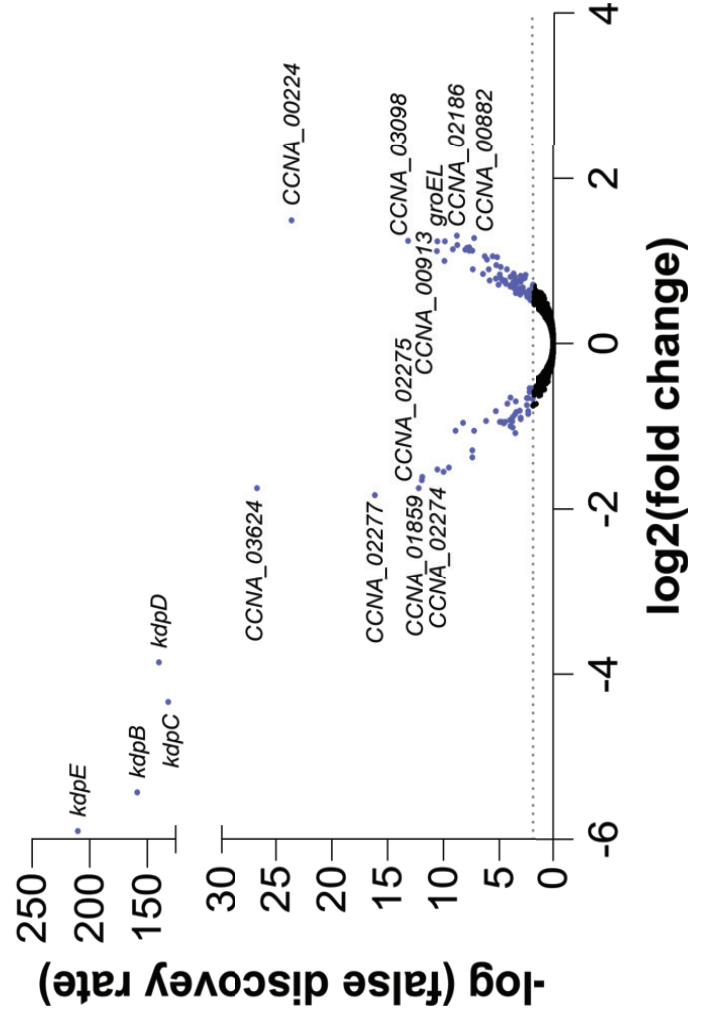
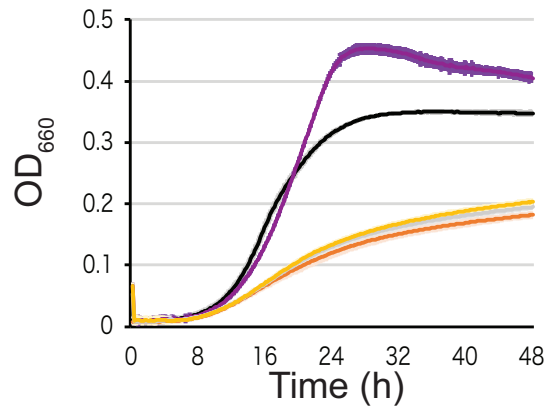


Figure 7

A

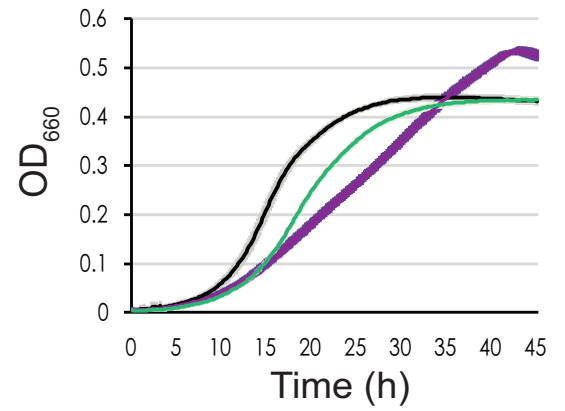
Limiting



— WT — $\Delta kdpABC$ — $\Delta kdpD$
— $\Delta kdpE$ — $\Delta kdpDE$

B

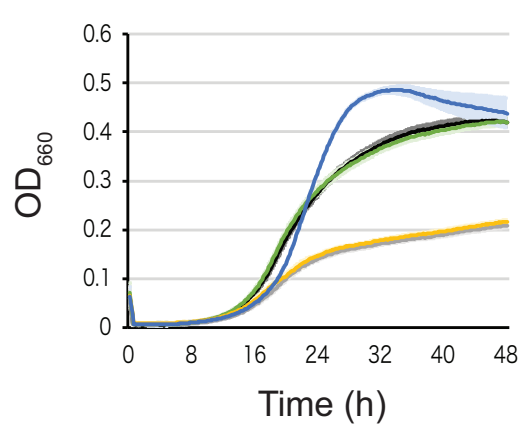
Limiting



— WT EV — $\Delta kdpD$ EV
— $\Delta kdpD$ $kdpD^+$

C

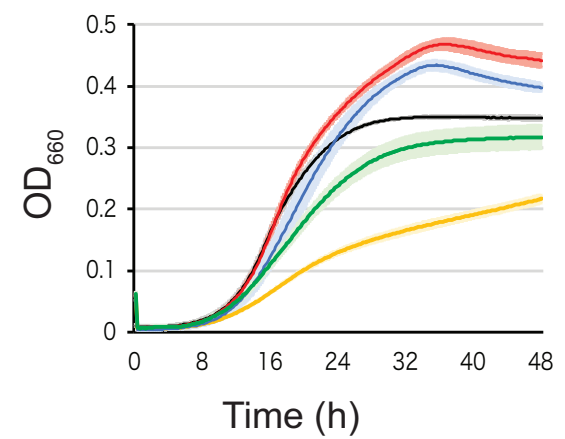
Limiting + 0.01% xyl



— WT — $\Delta kdpE$
— $\Delta kdpDE$
— $\Delta kdpE$ $P_{xyIX}::kdpE$
— $\Delta kdpDE$ $P_{xyIX}::kdpE$

D

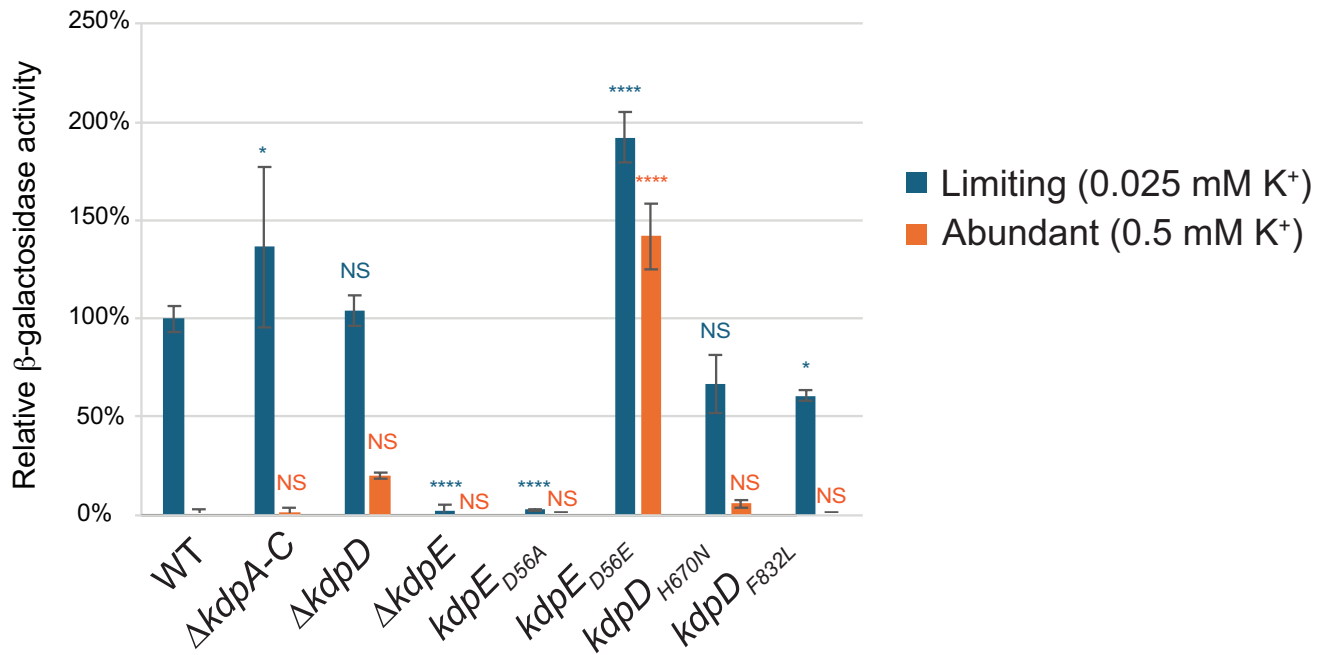
Limiting



— WT — $kdpE_{D56A}$
— $kdpE_{D56E}$
— $kdpD_{H670N}$ — $kdpD_{F832L}$

Figure 8

A



B

