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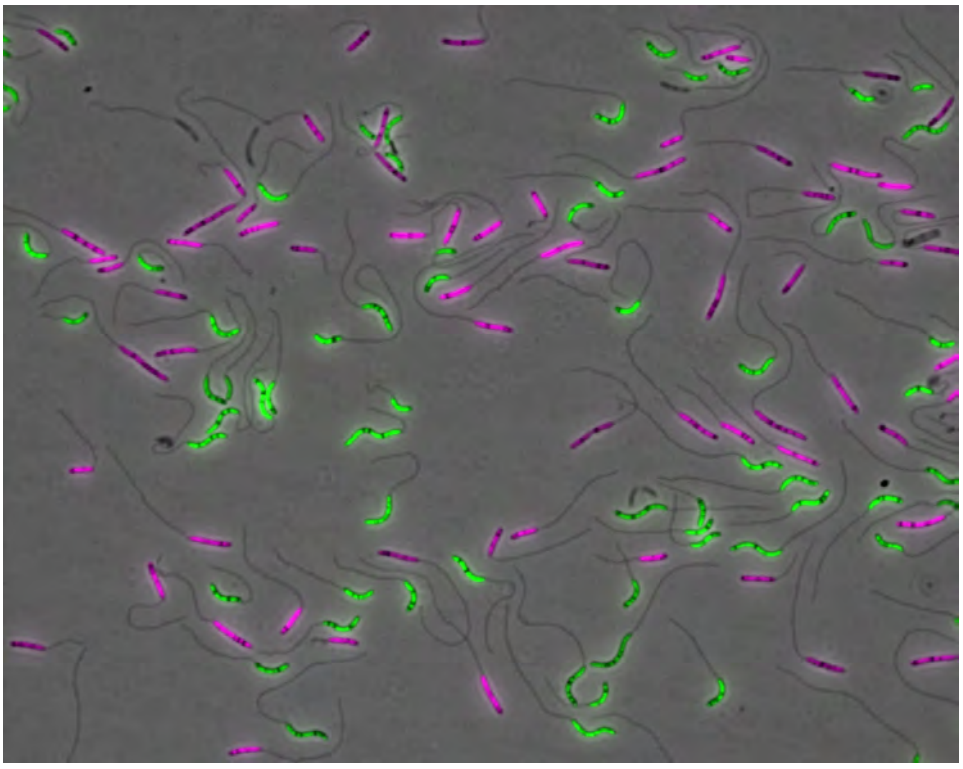
Bacterial Two-hybrid Screening to Study the Role of Crescentin Generating Cell Curvature of *Caulobacter crescentus*



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Summary

Caulobacter crescentus is a ubiquitous freshwater bacterium with a characteristic curved cell shape. This shape is conferred by a protein called crescentin, which localizes to one side of the cell close to the membrane. Crescentin does not have any transmembrane segments and thus no connection to the periplasm to affect the form of the peptidoglycan sacculus. The group I was working in hypothesized that crescentin interacts with other proteins in order to determine the curved cell shape.

To find potential interaction partners I carried out a two-hybrid screening using the BACTH system. It is a bacterial adenylate-cyclase two-hybrid system using the fact that the two catalytic domains of the adenylate-cyclase of *Bordetella pertussis* are separated by a linker region. These domains are not active when physically separated. Fused to interacting proteins, their enzymatic activity can be restored and detected by a *lac*-reporter system. Previously, crescentin has been fused to the gene encoding one of the adenylate-cyclase domains and a genomic library of *C. crescentus* has been fused to the other. The gene *CC3277*, encoding a member of the penicillin binding protein (PBP) family, was picked up in my screening. Crescentin, located on the lateral side of the cell, could affect the function of this specific PBP somehow and thus determine cell shape by affecting the peptidoglycan. In parallel, I worked with candidates found in previous screenings. These were the chemoreceptor McpJ (CC3145) and the hypothetical proteins CC1883 and CC2998. I constructed knockouts of the genes encoding these proteins and fusions to the Red Fluorescent Protein (mCherry). I functionally characterized the proteins encoded by these genes in the same manner as it will be done for the genes I picked up in my screening. Knockouts of these genes did not change the original phenotypes of the wildtype or crescentin mutant. The mCherry fusion to CC1883 in wildtype cells led to an elongated cell shape and possibly multiple constrictions. It seemed to have a great impact on the phenotype. CC2998-mCherry showed uneven distribution in both wildtype and the mutant cells, but a distinct pattern could not be seen.

The chemoreceptor McpJ was located at one or both poles in most wildtype cells and surprisingly in addition I often observed lateral positioning in the vicinity of the cell middle. In the mutant lacking crescentin only polar positioning of McpJ was evident. This indicates that crescentin may have another function apart from determining cell shape, helping other proteins to localize in the cell.

Introduction

Caulobacter crescentus. *Caulobacter crescentus* is a ubiquitous, Gram-negative and non-pathogenic freshwater bacterium. Generally it lives in dilute aquatic systems with phosphorous as limiting nutrient. Phosphorous limitation induces the production of a 30 times longer stalks compared to cells in phosphate-rich medium (Gonin *et al*. 2000). The genome of *Caulobacter* is circular and about 4 Mbp large. It contains 3,767 genes encoding more two-component signal transduction proteins than found in any other bacterial genome sequenced so far (Nierman *et al*. 2001). *Caulobacter* is also a model organism for studies of the bacterial cell cycle and differentiation. Asymmetric cell division in *Caulobacter* results in a stalked cell and a motile swarmer cell. In the latter, replication of the chromosome is repressed but as it differentiates into a stalked cell (S-phase in figure 1) replication and cell division can take place again (Collier *et al.* 2007, Quardokus *et al.* 1996).

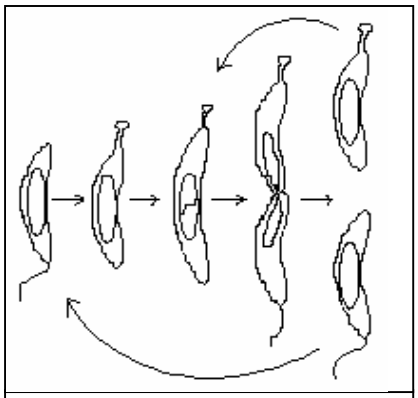


Figure 1. Schematics of the asymmetric cell cycle of *Caulobacter*. Replication of the chromosome and cell division only take place in the stalked cells. After cell division the chromosome is directly replicated again in stalked cells whereas swarmer cells are motile for a certain period of time. A swarmer cell differentiates into a stalked cell before it can go on with its life cycle (modified from Ausmees & Jacobs-Wagner 2003a).

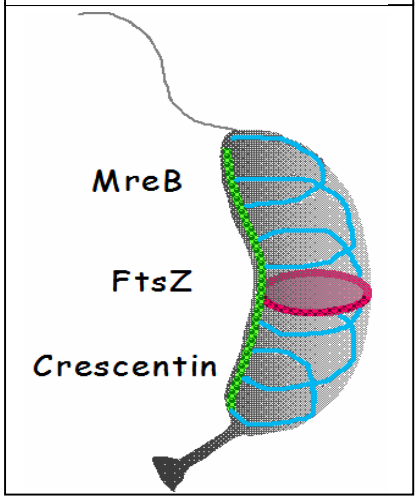


Figure 2. Schematics of filamentous proteins needed to determine cell shape in *Caulobacter crescentus*. FtsZ is shown as a red circle in the cell middle. MreB is shown as grey helical structure. Crescentin, in green is located close to the cell wall on one side of the cell.

Furthermore, *Caulobacter crescentus* has the cell shape of a curved or helical rod (figure 2). To establish coccoid cells like *Staphylococcus* only the protein FtsZ is needed. It is homologous to animal tubulin and forms a ring in the cell middle which is also the site of future cell division (Ma *et al.* 1996.). To establish rod shaped cells, like *Escherichia coli* an additional filament to FtsZ is needed. It is called MreB and forms helical structures, helping the cell to elongate into rods (Vats & Rothfield. 2007). The curved or crescent cell shape of *C. crescentus* needs FtsZ, MreB and the recently found protein crescentin (Ausmees *et al.* 2003b) which is encoded by the gene *creS*. It has the ability to assemble spontaneously into filaments *in vitro* and shows strong similarities to the protein structure of animal intermediate

filaments (Ausmees *et al.* 2003b). In the cell, crescentin is laterally localized close to the membrane, although it does not have transmembrane domains (Ausmees *et al.* 2003b). The molecular mechanisms of how crescentin determines the cell shape are still unknown. The group I was working in assumes that interactions with other proteins are important for crescentin function. Until today it is not exactly known which advantages the crescent cell shape gives to *Caulobacter*. I can only speculate that it might be advantageous for its swimming in freshwater.

The bacterial adenylate cyclase-based two-hybrid system. To find interaction partners so-called two-hybrid systems are commonly used. The first one was the yeast two-hybrid system based on the transcription factor GAL4 (Fields & Song. 1989). A bacterial adenylate cyclase-based two-hybrid system (BACTH) enables screening using only *Escherichia coli* (figure3). The BACTH system (Ladant & Ullmann 1999) takes advantage of the fact that the catalytic domain of the adenylate cyclase from *Bordetella pertussis* consists of two complementary fragments, T25 and T18, that are linked (figure 3A) and not active when physically separated.

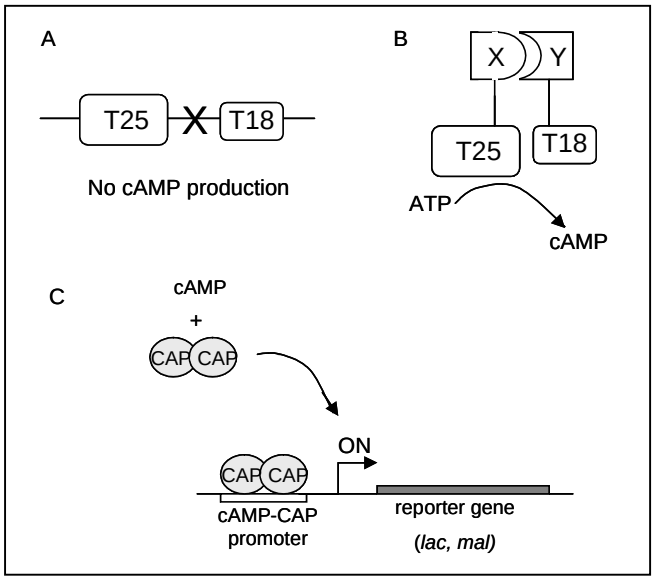


Figure 3. Adenylate cyclase as a reporter of protein–protein interactions. (a) The adenylate cyclase is inactive when the two parts of its catalytic domain, T25 and T18 are not connected. (b) Fusion to interacting proteins (X and Y) can restore the activity and cAMP is produced. (c) cAMP and the catabolic activation protein (CAP) together can turn on expression of several genes, among them the *lac* operon, which can be used for a blue/white screening of interaction partners (modified from Ladant *et al.* 1999).

Proteins of interest can be fused to these fragments (figure 3B). The resulting fusion protein containing the T25 fragment is referred to as "bait" whereas the T18 fusion is called "prey". Interacting polypeptides fused to these fragments can re-induce the enzymatic activity by bringing T25 and the T18 fragments close to each other and lead to cAMP production. In strains lacking endogenous adenylate cyclase activity this can be used to detect protein interactions, since the produced cAMP binds to the catabolic activation protein (CAP) and enables expression of the *lac* operon (see figure 3C). The *lac* operon contains a promoter, an operator, three structural genes (*lacZ*, *lacY* and *lacA*) and a terminator region, where *lacZ* encodes for β-galactosidase. β-galactosidase activity can be detected by adding its substrate, X-Gal (5 -Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) to the growth medium.

Aims. The aim of this project was to find new interaction partners of crescentin which enable it to generate the characteristic curved cell shape of *C. crescentus*. Furthermore I worked with candidate genes that had been picked up in previous screenings, to find out more about the function and localization of their proteins in the cell.

Results

Two-hybrid screening for new potential interaction partners with crescentin.

A library (CC2Hlib) of random fragments of the *Caulobacter* genome fused to the T18 fragment in the plasmid pKT18c ("prey") had been constructed beforehand, as well as an *E. coli* strain containing the "bait" (BTH101 pKT25-creS). To study interactions the "prey" was transformed into an *E. coli* strain already containing the bait. I carried out 27 electroporations in order to transform the prey into the bait-carrying strain. The transformed cells were plated on minimal medium with lactose as the only carbone source and X-Gal as indicator, whereby a selection for lac^+ clones (blue) was obtained. After two and four days I picked 87 lac^+ positive clones. To distinguish between clones that can utilize lactose due to a mutation in the promoter of the lactose operon and not due to the interaction, I retransformed all plasmids into BTH101 pKT25-creS without mutations to achieve a clean genetic background. Out of these 87 clones only 19 were able to cause the lac^+ phenotype in a clean genetic background. Their plasmids were sequenced. Sequence analysis revealed that nine candidates contained a gene fragment in the 5'-3' direction of T18 and three of these were definitely in frame with the T18 reading frame. The genes encoding these proteins, which thus may interact with crescentin are listed in table 1.

Table 1. Candidate genes picked up by the two-hybrid screening

gene name	in frame with T18	function
CC2598	ND*	Gid A family protein
CC2173	ND	lysozyme family protein
CC3238	ND	Holliday junction resolvase
CC2389	ND	cation efflux system protein
CC1028	ND	hypothetical protein
CC1029	ND	hypothetical protein
CC2388	yes	metal ion efflux outer memb. factor prot. fam.
CC3277	yes	penicillin binding protein
CC1026	yes	hypothetical protein

*ND, not determined

Blue colonies can also arise due to false interactions. The library fragment could interact directly with the T25 fragment independent of crescentin. To rule out this kind of false-positives the plasmids of the colonies containing *CC3277*, *CC2388* and *CC1026* were transformed into BTH101 containing pKT25 without crescentin. The resulting colonies were spread on plates containing X-Gal and the colonies containing the penicillin binding protein CC3277 showed no blue color, thus clearly did not interact directly with T25. The result was not clear for CC2388 and CC1026 (data not shown). The remaining candidates were not tester yet for this kind of interaction.

Functional characterization of previously found candidates.

Previous two-hybrid screenings have been carried out (unpublished) in the laboratory as described above, and several putative interaction partners had been found. The genes I went on working with were CC3145, encoding the chemoreceptor McpJ and the hypothetical proteins CC1319, CC2998 and CC1883.

To this point only interactions between crescentin and an often truncated protein encoded by the random genome fragment were studied. To confirm the interaction between the putative protein and crescentin, full-length genes were fused to T18 and tested for interaction (table 2). The genes were amplified from the *C. crescentus* chromosome using PCR. Each protein was tested for interaction with the "bait" as an N-terminal or as a C-terminal fusion to the T18 fragment. Both constructs of CC2998 showed interaction, whereas interaction of McpJ was only detectable when the T18 fragment was fused to its C-terminus (figure 4A, table 2).

Table 2. Full-length fusion to T18

gene	T18 fused to N-terminus of the protein	T18 fused to C-terminus of the protein	function
CC3145	-	+	chemoreceptor McpJ
CC1319	+	-	hypothetical protein
CC2998	+	+	hypothetical protein
CC1883	+	-	biotin-requiring enzyme

In this table + (Lac^+) and - (Lac^-) indicate the phenotype of the BTH101 strain containing pKT25-creS and pKT18 with the corresponding T18 fusion. Only Lac^+ colonies (blue) showed positive interaction between T18 and T25, meaning a possible interaction of the tested protein and crescentin.

This fact, together with its predicted transmembrane domains suggest, that McpJ is membrane-bound. It is not clear which part of the protein is located in the periplasm or the cytoplasm, which means that the T18 fragment might end up in the periplasm. T25 fused to crescentin remains in the cytoplasm, since crescentin is a cytoplasmic protein. The T18 and T25 fragments would not be close enough to be enzymatically active (figure 4B). CC1319 showed interaction when fused to the C-terminus of T18, probably also because of its predicted localization in the membrane. It is not known why the interaction of CC1883 was only detectable when it was fused to the C-terminus of T18. This gene has no predicted transmembrane domains, but the interaction could be disturbed due to the folding of the protein.

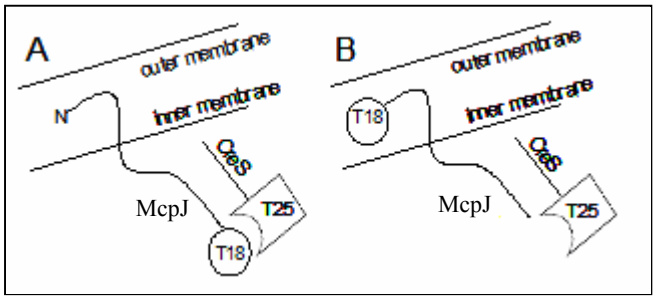


Figure 4. Illustration of the putative localization and interaction of "bait" and "prey" for CC3145. The N-terminus of CC3145 is located in the periplasm. An interaction is only detectable when T18 and T25 are localised close enough to be active (A).

In order to obtain more information about the function and the localization of the candidate genes I constructed knockouts (KO) of these genes and in frame-fusions to mCherry, both in the wildtype and in the Δ*creS Caulobacter* background (table 3).

Table 3. Knock-outs and mCherry fusions in *C. crescentus*.

Gene	mCherry fusion in		KO in	
	wt	Δ*creS*	wt	Δ*creS*
CC3145	+	+	+	+
CC2998	+	+	+	+
CC1319	ND	ND	+	+
CC1883	+	ND	-	-

\+ functional construct

ND construct not yet completed

\- construction failed

The cultures were grown to logarithmic or stationary phase and observed by phase contrast microscopy. The knockouts were observed first. The gene KOs in the wildtype strain resulted no difference in appearance compared to the wildtype (figure 5). Δ*2998* had the same crescent cell shape and size as the wildtype (figure 5 A and C). Similar results were observed for gene KOs in the Δ*creS* strain. The additional KO of the genes I was working with did not change the phenotype compared to the Δ*creS* strain (figure 5 B and D). It was not expected to see a difference in cell shape for the ΔMcpJ mutant, because the function of this protein most likely is not related to cell shape. McpJ is a chemoreceptor and until today not much is known about it. It shows similarities to the well studied chemoreceptor McpA in *Caulobacter*, which has no influence on cell shape (Alley 2001). It was not possible to construct a Δ*1883* mutant, indicating that the gene might be essential for viability.

I succeeded in constructing an mCherry fusion to CC1883 in the wildtype background. 1883-mCherry seemed to be located all over the cell (figure 6A). However, the cells did not show wildtype shape. The cells were elongated, sometimes seemed to have several constrictions and were slow growing. The fusion to mCherry appeared to affect the function of CC1883 dramatically, changing both the phenotype and viability. Thus, I assume that CC1883 might be essential to *Caulobacter*.

The localization of 2998-mCherry was uneven in the cells, but no distinct pattern was detectable comparing swarmer and stalked cells of the wildtype and Δ*creS* mutant (figure 6B and C).

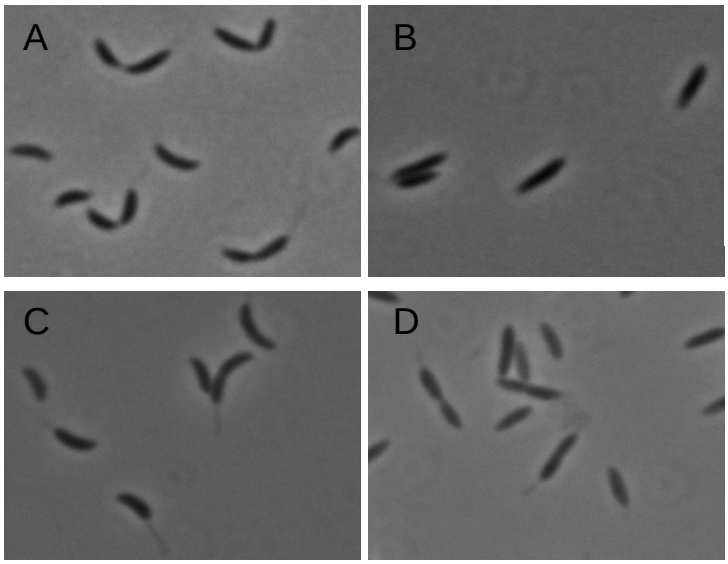
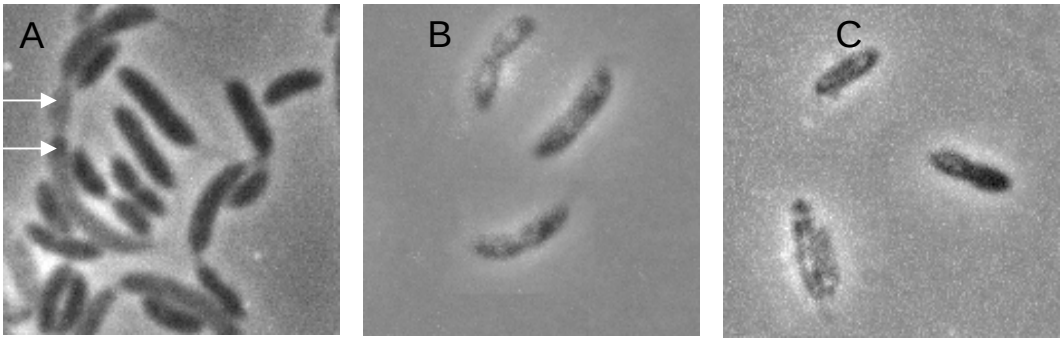


Figure 5. Phenotypes of CB15N (wildtype), the Δ*creS* strain and Δ*2998* strains.
(A) wildtype cells.
(B) Δ*creS.*
(C) Δ*2998*.
(D) Δ*creS Δ2998*.

Figure 6. Localization of 1883-mCherry and 2998-mCherry. (A) 1883-mCherry cells from an overnight culture. Arrows indicate putative multiple constrictions. (B) 2998-mCherry cells from an overnight culture. (C) Cells of *ΔcreS 2998-mCherry* after overnight cultivation.



Chemoreceptors in *E. coli* are predominantly localized at the cell pole (Maddock & Shapiro, 1993) and it could to be similar for *Caulobacter.* As expected, McpJ-mCherry was located at the pole. It was often located at both cell poles and this localisation was maintained even in the Δ*creS* mutant. Furthermore, I often observed lateral positioning of McpJ in the vicinity of the cell middle. This lateral position of McpJ-mCherry could not be seen in the Δ*creS* mutant regardless of the growth phase suggesting that crescentin plays a role in positioning of McpJ (figure 7).

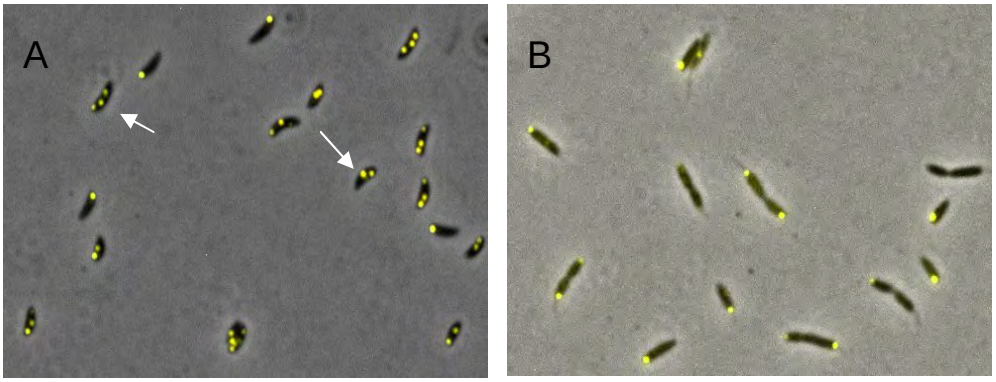


Figure 7. Localization of McpJ-mCherry. The color of the fluorescent dye was digitally changed to yellow for better visualization. (A) 3145-mCherry from an overnight culture. The arrows point to putative lateral spots. (B) *ΔcreS 3145-mCherry* cells after overnight incubation.

Discussion

By using the bacterial two-hybrid system I found a new promising putative interaction partner of crescentin. This gene, *CC3277* encodes a member of the penicillin-binding protein family. Penicillin-binding proteins (PBPs) are transmembrane proteins that are involved in peptidoglycan synthesis and lose their function when binding penicillin or other β-lactam antibiotics. Nathan and Newton (1988) showed that there are at least 14 PBP-like proteins in *Caulobacter*. They synthesize the peptidoglycan shell, the so-called sacculus of the bacterial cell. It is known that the peptidoglycan sacculus is important to maintain the cell shape, because isolated sacculi keep the shape the cell had before. Wildtype *Caulobacter* sacculi are curved, whereas *E. coli* sacculi are rod-shaped. With increasing copy number of crescentin the curvature of the *Caulobacter* sacculi increases as well (Cabeen *et al.* unpublished). Since crescentin is only localized to one side of the cell it could theoretically bind to PBPs on this side and modify their function, leading to cell curvature.

. Fused to mCherry the chemoreceptor McpJ (CC3145) showed polar localization regardless of the growth phase. This implies similarities to McpA, a well studied chemoreceptor in *Caulobacter* (Alley 2001). McpA-GFP is localized to the flagellated pole and the highly conserved domain (HCD) which is present in all chemoreceptors is needed for polar localization. In fact this domain of McpJ was found in the library fragment first picked up to interact with crescentin. Polar or bipolar localization of McpJ could be observed in all growth phases in the wildtype and Δ*creS* strains. This indicates that crescentin is not required for polar localization of McpJ-mCherry and that McpJ-mCherry is localized not only to the flagellated pole. Interestingly, I observed also lateral localization of McpJ-mCherry, closer to midcell, which has never been observed in *Caulobacter* before. Lateral positioning of McpJ was only evident in wildtype cells, but never in the Δ*creS* mutant, suggesting that crescentin is important for localization of lateral clusters of chemoreceptors. Lateral clusters of chemoreceptors have recently been studied in *E. coli* (Thiem *et al.* 2007). They are assumed to localize to future division sites and an additional lateral localization might be important for chemotaxis. This could also be true for *Caulobacter*.

The future perspective of this project is to carry out the functional characterization of the proteins I picked up in my screenings. It will also be necessary to carry out control experiments. The fluorescent dye mCherry could affect the correct localization of the proteins that were tested and influence the results. To construct merodiploid strains would make the results more reliable, because they contain not only the protein fused to mCherry but also a high content of wildtype protein that would locate correctly. Furthermore it would be interesting to test if there are more chemoreceptors in *Caulobacter* that localize similar to McpJ or chemoreceptors in *E. coli*.

Materials and Methods

Bacterial strains, plasmids and media. Strains and plasmids used in this project are listed in tables 4-6. *Escherichia coli* TOP10 and BTH101 were used to construct and maintain plasmids and to amplify library DNA. *E. coli* S17 was used for conjugation of plasmids into *Caulobacter*. The *E. coli* strains were grown in Luria broth medium (10 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone, 1000 ml dest. H_2O, pH 7.2-7.4, when growing on plates 1% agar was added) at 37°C. When colonies were grown on plates LA was used (with 1 % agar and no addition of glucose). BTH101 cells containing pKT25, pUT18c and pUT18 derivatives were grown at 30°C. *Caulobacter* strains were grown at 30°C in PYE (0.2% w/v Bacto peptone, 0.1% yeast extract, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, when growing on plates 1 % agar was added). To select for interaction partners cells were grown on minimal medium containing lactose as the only carbon source and X-Gal as indicator. It was prepared as follows: 2 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 0.5 mg $FeSO_4 \cdot 7H_2O$ and 15 g agar added to 1 liter of water (pH 7.0 adjusted with KOH). After autoclaving 1 ml 1M $MgSO_4 \cdot 7H_2O$, 10 ml 20% lactose, 2 ml 0.5% vitamin B1, X-Gal (final conc. 20 µg/ml), IPTG (final conc. 0.1 M) and appropriate antibiotics (50 µg/ml ampicillin, 25 µg/ml kanamycin) were added. The concentrations of antibiotics used can be seen in table 7.

Table 4. *E. coli* strains

strain	relevant features	reference
TOP 10	F⁻ *mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	(Grand *et al.* 1990)
BTH101	F⁻, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1* (Str^r), *hsdR2*, *mcrB1*	(Ladant *et al.* 1999)
S17-1	RP4-2, Tc::Mu Km::Tn7; for plasmid mobilisation	(Simon *et al.* 1983)

Table 5. *Caulobacter crescentus* strains

strain	relevant features	reference
CB15N	Wildtype strain	(Evinger *et al.* 1977)
CB15NΔ*creS*	Wildtype strain with crescentin deleted	(Tomenius, unpubl.)
Δ*3145*	CB15N *3145*::pBGS-Δ*3145,* CC3145 knockout	This study
Δ*creS* Δ *3145*	CB15NΔ*creS 3145*::pBGS-Δ*3145*, CC3145 knockout	This study
3145-mCherry	CB15N *3145*::pBGS-3145-mCherry, CC3145 replaced by 3145-mCherry	This study
Δ*creS* 3145-mCherry	CB15NΔ*creS 3145*::pBGS-3145-mCherry	This study
Δ*1883*	CB15N *1883*::pBGS-Δ*1883,* CC1883 knockout	This study
Δ*creS* Δ*1883*	CB15NΔ*creS 1883*::pBGS-Δ*1883*, CC1883knockout	This study
1883-mCherry	CB15N *1883*::pBGS-1883-mCherry, CC1883 replaced by 1883-mCherry	This study
Δ*creS* 1883-mCherry	CB15NΔ*creS 1883*::pBGS-1883-mCherry	This study
Δ*2998*	CB15N *2998*::pBGS-Δ*2998,* CC2998 knockout	This study
Δ*creS* Δ*2998*	CB15NΔ*creS* Δ*2998*::pBGS-Δ*2998*, CC2998 knockout	This study
2998-mCherry	CB15N *2998*::pBGS-2998-mCherry, CC2998 replaced by 2998-mCherry	This study
Δ*creS* 2998-mCherry	CB15NΔ*creS 2998*::pBGS-2998-mCherry	This study

Table 6. Plasmids

plasmid	relevant features	reference
pKT25	Kan^r, encoding T25 fragment	(Ladant *et al.* 1999)
pKT25-creS	Kan^r, T25 fragment fused to crescentin	(Tomenius, unpubl.)
pUT18C	Amp^r, encoding T18 fragment, multi cloning site upstream of T18	(Ladant *et al.* 1999)
pUT18	Amp^r, encoding T18 fragment, multi cloning site downstream of T18	(Ladant *et al.* 1999)
CC2Hlib	chromosomal library of CB15NΔ*creS* in pUT18c	(Tomenius, unpubl.)
pBGS18T	Kan^r, cloning vector for conjugation into *Caulobacter*	
pBGS18T-mCh	pBGST containing mCherry	This study
pUT18C-3145	full-length fusion of CC3145 with T18	This study
3145-pUT18	full-length fusion of CC3145 with T18	This study
3145-pBGS	pBGS containing full-length fusion of CC3145 with T18	This study
pUT18C-2998	full-length fusion of CC2998 with T18	This study
2998-pUT18	full-length fusion of CC2998 with T18	This study
2998-pBGS	pBGS containing full-length fusion of CC2998 with T18	This study
pUT18C-1319	full-length fusion of CC1319with T18	This study
1319-pUT18	full-length fusion of CC1319 with T18	This study
1319-pBGS	pBGS containing full-length fusion of CC1319 with T18	This study
pUT18C-1883	full-length fusion of CC1883 with T18	This study
1883-pUT18	full-length fusion of CC1883 with T18	This study
1883-pBGS	pBGS containing full-length fusion of CC1883 with T18	This study
pBGS-3145-mCh	pBGS18T-mCh containing an about 400bp fragment of CC3145	This study
pBGS- Δ*3145*	pBGS18T containing an about 400 bp fragment of CC3145	This study
pBGS-1883-mCh	pBGS18T-mCh containing an about 400bp fragment of CC1883	This study
pBGS-Δ*1883*	pBGS18T containing an about 400 bp fragment of CC1883	This study
pBGS-2998-mCh	pBGS18T-mCh containing an about 400bp fragment of CC2998	This study
pBGS-Δ*2998*	pBGS18T containing an about 400 bp fragment of CC2998	This study

Table 7. Antibiotics

antibiotic	final concentration (μg/ml)			
	E. coli liquid LB	*E. coli* LA plates	*Caulobacter* liquid PYE	*Caulobacter* PYE plates
ampicillin	50	100	50	5-10
kanamycin	50	50	20	5
nalidixic acid	-	-	20	-

Preparation and transformation of electrocompetent cells. Cell cultures of BTH101 pKT25-creS and BTH101 pKT25 were grown at 37°C to an OD_{600} of 0.6, chilled on ice, spun down with 6237xg and washed twice with ice-cold sterile water, first with 200 ml and then with 100 ml. The cells were again pelleted as above and resuspended in 5 ml 10% ice-cold glycerol (in water), pelleted as above again and taken up in 0.75 ml 10% ice-cold glycerol. A final cell suspension containing at least 10^{10}cells/ml was aliquoted to 50 µl and shock frozen at -70°C using liquid nitrogen. For electroporation, cells were thawed on ice and 2 µl plasmid DNA was added. Cells and DNA were transferred to a precooled electroporation cuvette and shocked (1.5 mV, 400 Ohms) using the BioRad Gene pulser. Afterwards 1 ml LB was added and the cells were incubated for phenotypic expression for 45 min at 37°C and plated afterwards on selective plates.

To test the transformation efficiency of these electrocompetent cells 2 µl CC2Hlib were transformed into BTH101 pKT25CreS, which yielded $1x10^8$ individual transformants per electroporation. The transformation efficiency was determined with a dilution series.

Preparation and transformation of $CaCl_2$-competent cells. Cells were grown to an OD_{600} of 0.6, chilled on ice and centrifuged (2900xg for 10 min at 4°C). The pellet was taken up in ice-cold sterile 0.1 M $CaCl_2$ and left on ice for 30 minutes. It was centrifuged again (2900xg for 10 min at 4°C) and taken up in a smaller volume of $CaCl_2$ and the cells were kept on ice for 3 hours and shock frozen in liquid nitrogen afterwards. For transformation 2 µl plasmid DNA was added and the sample incubated on ice for 30 min. A heat shock was performed for 2 min at 42°C and afterwards the cells were chilled on ice for 5 min. 5 ml LB was added and phenotypic expression was carried out for 45 min at 37°C.

Bacterial two-hybrid screen. Crescentin had been cloned previously in frame with the T25 fragment into pKT25 (figure 1) and a genomic library of *C. crescentus* strain CB15NΔ*creS* had been constructed in pUT18c (Ladant & Ullmann, 1999).

To construct the library, now referred to as CC2Hlib (*Caulobacter crescentus* library for two-hybrid screening) the *C. crescentus* genome had been fragmented by sonication and blunt end fragments ligated into pUT18c, yielding $5x10^6$ individual clones (Tomenius *et al.* unpublished). For the later two-hybrid screening 27 electroporations were carried out (CC2Hlib into BTH101 pKT25CreS) which means that according to the transformation efficiency $2.7x10^9$ clones were screened. The transformants were selected on minimal medium containing X-gal, IPTG and antibiotics. To rule out false positives due to promoter mutations the pUT18c plasmids of the lac^+-colonies were retransformed into BTH101 pKT25-creS. Plasmids of clones that were positive again were isolated and sequenced and a BLAST-search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was done to obtain further information about the interacting fragments and their full-length sequence.

Sequence analysis and database searches. BLAST was used to find the full-length sequence of positive library fragments. The DAS (http://www.sbc.su.se/~ miklos/DAS/) algorithm was used to predict transmembrane domains. To construct primers, gene knockouts, mCherry fusions and further sequence analysis the Jellyfish software was used.

Polymerase chain reaction. Primers were designed according to the information obtained by the BLAST-search and used to construct knockouts and mCherry-fusions of the particular genes (table 8). For PCR a mastermix without template DNA was prepared beforehand (table 9). Template DNA for colony PCR was added using toothpicks. The PCR conditions are stated in table 10.

Table 8. Oligonucleotides

gene name		restriction enzyme	nucleotide sequence	use
CC1883	F	BamHI	attta**GGATCC**cATGTCGAACCCCAAGGCCCCCGCCGA	cloning [1]
CC1883	R	KpnI	attta**GGTACC**gCCTCGATGACGACGAGCGGCT	cloning [1]
CC1883KO	F	BamHI	attta**GGATCC**cCCAAGGCCCCCGCCGATCCGGT	KO[2]
CC1883KO	R	KpnI	attta**GGTACC**tGCCGACCATCGGCGACTTCA	KO[2]
CC1883mChr	F	BamHI	atttta**GGATCC**tGAACTGACCGCCGCGCCCGTCAA	mCherry fusion[2]
CC1883mChr	R	KpnI	atttta**GGTACC**cTCGATGACGACGAGCGGCTCGCCGAA	mCherry fusion[2]
CCMcpJ	F	BamHI	attta**GGATCC**cATGACGACCGCCATGAACATT	cloning [1]
CCMcpJ	R	KpnI	attta**GGTACC**gcGCCCCGACCGCCTGCAGCCCTCA	cloning[1]
CCMcpJKO	F	BamHI	atttta**GGATCC**gCCATGAACATTCCCAAGAAGCT	KO[2]
CCMcpJKO	R	KpnI	attta**GGTACC**tTCTGGGCGATGTTCTTGACCT	KO[2]
CCMcpJmChr	F	BamHI	attta**GGATCC**gACAGCATCACCTTCCAGACCAA	mCherry fusion[2]
CCMcpJmChr	R	KpnI	attta**GGTACC**gCCCCGACCGCCTGCAGCCCTCA	mCherry fusion[2]
CC2998	F	SalI	attta**GTCGAC**cTTGAACGGTGCGGGGCGAAGCGCCA	cloning[1]
CC2998	R	KpnI	attta**GGTACC**gcGTTCCCCGCCACCGTCGTCACCACCTT	cloning[1]
CC2998KO	F	HindIII	attta**AAGCTT**gCGGGGCGAAGCGCCAGCTTCA	KO[2]
CC2998KO	R	KpnI	atttta**GGTACC**tCATGCGGATCGGGACCCAAAT	KO[2]
CC2998mChr	F	BamHI	attta**GGATCC**aTGGCGGTCTGCAGACCTTCGTGA	mCherry fusion[2]
CC2998mChr	R	KpnI	atttta**GGTACC**gTTCCCCGCCACCGTCGTCACCACCTT	mCherry fusion[2]

[1] into vector pUT18
[2] into vector pBGS18T
F forward primer yielding the N-terminal part of the protein encoded by the fragment
R reverse primer yielding the C-terminal part of the protein encoded by the fragment

Table 9. Preparation of mastermix for different PCR volumes

	mastermix for		
	50 µl	100 µl	200 µl
10xPCR Buffer (conc. Tris-HCl and $(NH_4)_2SO_4$ company secret, 15 mM $MgCl_2$)	5 µl	10 µl	20 µl
Q-buffer (company secret)	10 µl	20 µl	40 µl
dNTPs (10 mM)	5 µl	10 µl	20 µl
primer forward / reverse (10 µm)	1.25/1.25 µl	2.5/2.5 µl	5/5 µl
Taq polymerase (Fermentas) (5 units/µl)	0.5 µl	0.5 µl	1 µl
H_2O	27 µl	54.5 µl	109 µl

Table 10. Polymerase chain reaction conditions

temperature	duration	
95 °C	2.30 min	
96 °C	0.30 min	29 cycles
42 °C	1.30 min	
72 °C	2.00 min	

Cloning and plasmid DNA purification. Cloning was carried out using standard protocols (Sambrook 2001). In brief the chromosomal DNA was amplified by PCR and cut with appropriate enzymes. The vector backbone was cut with corresponding enzymes. For the two-hybrid screening the pKT18 vector was used, whereas for KOs and mCherry fusions the pBGS18 vector was used. Both insert and vector were purified to get rid of restriction enzymes, mixed and ligated together using T4 ligase (Fermentas). All plasmid DNA was purified using the Macherey-Nagel NucleoSpin® plasmid purification kit which lyses pelleted cells with SDS and alkali. After neutralization through the next buffer, cell debris was pelleted at 11498xg and the supernatant was loaded onto a column. An ethanolic buffer removed contaminations and the pure plasmid DNA was eluted with 50 µl sterile water.

Construction of knockouts and mCherry fusions. The BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) yielded the full-length sequence of the genes. For gene knockouts the part of the corresponding gene encoding a short N-terminal part (~400 bp, obtained by PCR) of the protein without the first three codons was ligated into pBGS18T (figure 8A). This construct results in a non-functional protein, thus a protein KO. For mCherry fusions the part of the corresponding gene encoding a short C-terminal part (~400 bp, obtained by PCR) of the protein without the stop codon was ligated in frame to *mCherry* (Shaner *et al.* 2004) in pBGST-mCherry (figure 8B) resulting in a translational fusion. Homologous recombination by a single cross-over event inserted the plasmid into the chromosome because pBGST is not replicated in *Caulobacter*. The selection was carried out by addition of kanamycin to the selection medium.

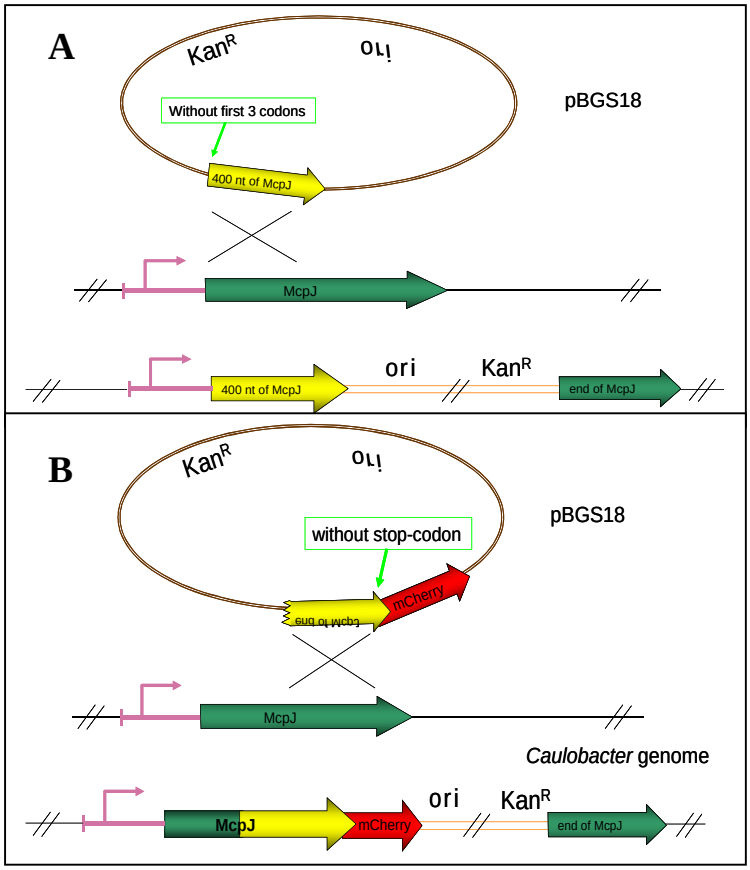


Figure 8. Construction of KOs and mCherry fusions. (A) Construction of KO of McpJ in the *Caulobacter* genome. To construct this KO the CCMcpJKO forward and reverse primers (table 8) were used, yielding respectively the N-terminal and the C-terminal part of the protein encoded by the fragment. (B) Construction of mCherry fusion to McpJ in the *Caulobacter* genome. Here the CCMcpJmChr forward and reverse primers (table 8) were used respectively to construct a translational fusion of McpJ and mCherry.

Mating. Conjugation was used to introduce plasmids into *Caulobacter*. To achieve this 1 ml overnight culture of *Caulobacter* and 0.1 ml of *E. coli* S17 donor strain were spun down separately 3421xg for 2-3 min. The pellets were mixed and resuspended in 1 ml PYE and spun again. Almost all supernatant was removed and the pellet was resuspended in a minimal volume PYE. The thick cell suspension was spotted on PYE plates without antibiotics and not spread out. After 4 h at 30°C or overnight incubation at room temperature the spot was scraped up from the plate and spread on PYE plates containing 20 μg/ml nalidixic acid to kill *E. coli* and suitable antibiotics to select for the conjugated plasmid.

Microscopy. Phase-contrast and fluorescence microscopy were carried out using an Axioplan II imaging fluorescence microscope with appropriate filter sets and an Axiocam charge-coupled device camera (Carl Zeiss Light Microscopy). The Axiovision software and Adobe Photoshop 7.0 were used to process images.

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