Study of crescentin and its interaction partners in *Caulobacter crescentus*

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Summary

Crescentin is an intermediate filament-like protein which leads to the curvature of Caulobacter crescentus (C. crescentus) cells. A previous study had identified several candidate proteins that might interact with crescentin. In the present study these interactions were further studied.

Seven candidate genes screened from the library were tested by a more stringent bacterial two-hybrid system (BTH). The result showed that PopZ, CC_2998, CC_0548, CC_3145 may interact with crescentin, suggesting that these proteins might be involved in the curvature formation of C. crescentus.

The interaction between PopZ and crescentin was tested by co-immunoprecipitation and time lapse. Co-immunoprecipitation result showed that there might be direct interaction between PopZ and crescentin. However, the time course, which was designed to study the biological relationship between two proteins, did not show significant difference in the distribution of PopZ in either wild type or crescentin mutant strains.

cc_0548 was knocked out in both wild type and crescentin mutant to study the function of this gene, but no morphological abnormality was observed in these strains. In addition, this gene was also fused with mCherry to investigate the distribution of CC_0548, but these strains required further identification.

cc_2998 knockout strains were subjected to stress conditions. Although no significant difference was observed in salinity and osmotic stresses, the expression level of CC_2998 was induced by heat shock.
Introduction

*Caulobacter crescentus*

*Caulobacter crescentus* is a Gram-negative oligotrophic α-proteobacterium which is widely spread in dilute aquatic environments (8). It divides asymmetrically, forming two progeny cells called swarmer cell and stalked cell respectively, which are different in both shape and function. The swarmer cell has a flagellum at one pole and is smaller than the stalked cell. In addition, the swarmer cell is unable to initiate chromosome replication until it differentiates into a stalked cell, shedding its flagellum and building a stalk (a thin cylindrical extension of the cell surface) in the same place. As for the stalked cell, it can continually give rise to a new swarmer cell after each division, acting like a stem cell (11). This unique life style ensures that progeny cells will move to a new location, leading to less competition during growth in an oligotrophic environment. (11)

Unlike *Escherichia coli* (*E. coli*), which initiates multiple rounds of DNA replication during fast growth (19), *C. crescentus* replicates DNA exactly once during each cell cycle (3). During the swarmer-to-stalked cell transition, the cell initiates DNA replication, which starts at a single origin and proceeds bidirectionally (6). The stalked cell elongates as DNA replication and segregation continue. When it comes to the predivisional cell stage, a flagellum is assembled at the pole opposite to the stalk. After the cell division, a swarmer and a stalked cell are generated. The stalked cell immediately initiates a new round of DNA replication, whereas the swarmer cell undergoes an obligatory period of growth and differentiation before starting a new cell cycle (fig 1) (11).

*C. crescentus* has some characteristic features. First, DNA replication occurs only once over each cell division and cells in each developmental stage have distinct polar structures (3, 11). Second, swarmer cells can be separated from stalked cells by density centrifugation so that the cells can be synchronized (3). Because of its easy manipulation in lab and unique development (coordination in cell cycle and cell differentiation), *C. crescentus* has become a model organism for cell cycle studies. One of the important newly characterized cell cycle-dependent proteins is PopZ, which is a proline-rich protein anchoring replication origins at cell poles and thus required during chromosome segregation (2, 7). This protein accumulates at the poles of *C. crescentus* cell, and this distribution requires the cytoskeleton (see below) (2).
Fig 1. Schematics of the cell cycle and development of Caulobacter. (Redrawn from (10)). 1) The swarmer cell sheds its flagellum. 2) The swarmer cell differentiates into a stalked cell and DNA replication begins. 3) The stalked cell elongates as DNA replication and segregation proceed. 4) The replicated DNA segregates into different part of the cell. 5) A new flagellum appears at the pole opposite to the stalk and predivisional cells are formed. 6) After cell division, two structurally different progeny cells are formed. 7) The motile swarmer cell can move to a new place and start differentiation. 8) The stalked cell starts a new cycle of DNA replication once the division is finished.

Caulobacter cytoskeletal proteins

C. crescentus is also a good model for studying the prokaryotic cytoskeleton. Cytoskeletal elements are key factors for cell stability/morphology and cell dynamics (12). Although three types of cytoskeletal elements in eukaryotic cells (tubulins, actins, and intermediate filaments) have been described and characterized, it took longer for people to realize that similar structure also exist in prokaryotes (9, 12). One of them is crescentin, whose predicted structure and in vitro polymerization show its similarity to intermediate filaments (IFs) (1). Later, it was demonstrated that the in vivo assembly and dynamics properties are also similar between crescentin and IFs (5, 16). Crescentin forms a filamentous structure which localizes in the inner curvature of the cell (Fig 2). When crescentin is absent in C. crescentus, the bacterium loses its curvature and shows straight morphology (1). One suggested model is that the physical stress provided by crescentin influences the cell wall insertion, leading to cell curvature (4).
**Bacterial two-hybrid system**

Two-hybrid systems are commonly used to find protein-protein interactions (13). The bacterial two-hybrid system is based on reconstitution of adenylate cyclase in *E. coli* (fig 3, 13). The catalytic domain of the adenylate cyclase protein (CyaA) of *Bordetella pertussis* (*B. pertussis*) is composed of two parts, T18 and T25, that do not show catalytic activity when they are physically separated (15). Polypeptides to be analyzed are fused to T18 and T25 respectively, resulting in hybrid proteins. When the two polypeptides interact with each other, T18 and T25 are also brought together and the catalytic function is regained, leading to cAMP synthesis. cAMP, together with the catabolite activator protein, CAP, activates the expression of several genes, including genes of the *lac* and *mal* operons. Thus, medium with lactose (or maltose) as carbon source can be used for selecting those polypeptides that might interact with each other.

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**Fig 3.** The principle of a bacterial two hybrid system (BTH) (Redrawn from (13)). A) The catalytic domain of adenylate cyclase from *Bordetella pertussis* consists of two fragments, T25 and T18. B) When T25 and T18 are physically separated, catalytic activity is lost and no cAMP is synthesized. C) Two interacting proteins bring T25 and T18 together and catalytic activity is regained. D) In the presence of CAP, cAMP binds to the promoter of several catabolic genes and turn on the downstream transcriptions.

pKT25 is a derivative of the plasmid pSU40 and encodes the T25 peptide (the first 224 amino acids of CyaA) of *B. pertussis*. pUT18 and pUT18c are both derivatives of the vector pUC19 (fig 4). They encode the T18 peptide (amino acids 225 to 399 of CyaA) and possess ampicillin resistance. Since the multi-cloning sites are located differently in these two plasmids, both proteins with the tested peptide at the N-terminus or the C-terminus can be obtained. After the genes of interest are cloned in frame into pKT25 and pUT18 (or pUT18c) and co-transformed to *E. coli* BTH101 (a *cya*’ strain), clones that express both chimeric proteins can be selected.
by medium containing ampicillin and kanamycin and the *lac* expression is used to determine if interaction exists between two proteins.

**Fig 4.** Plasmids used in the BTH system. Genes encoding antibiotic resistances are shown in orange, the genes encoding T18 and T25 in green and the multi-cloning sites as MCS.

**Co-immunoprecipitation**

Co-immunoprecipitation is a common technique to test the interactions between two proteins (18). It is based on the assumption that if two proteins interact with each other, they may form a stable complex. For example, there are two interacting fusion proteins, protein 1-mCherry (protein1 fused with a fluorescent tag) and protein 2-Flag (protein 2 fused with a tag which can bind to the resin bound with anti-Flag antibody). If protein 2 is pulled down by the resin with anti-Flag antibody, protein 1 is also very likely to be precipitated so that its presence can be detected by Western-blot with anti-mCherry antibody (fig 5).

**Fig 5.** The principle of co-immunoprecipitation.; Green triangles refer to protein 1-mCherry fusion proteins; pink rectangles refer to protein 2-Flag fusion proteins; blue ovals refer to resin. A) After breaking the cell, all kinds of proteins exist in the lysate. B) Beads with anti-Flag antibodies can specifically bind to protein 2-Flag fusion proteins. C) After wash, if protein 2 interacts with protein 1, the whole complex may remain and the presence of protein 1 can be detected by anti-mCherry antibody.

Prior to this project, a library of genomic fragments cloned in plasmids had been screened with the bacterial two-hybrid system and several candidates that might interact with crescentin had been selected. Since these candidates were from a library that only contained
random fragments of the respective gene, full length genes had been cloned into pUT18 and pUT18c and a BTH assay had been conducted to test these interactions (Henrik Tomenius, unpublished).

Aim
The question I was addressing was the biological function of crescentin. The approach was to find proteins that interact with crescentin and try to describe the protein interaction pathways that crescentin interconnects to achieve its functions. To test the interactions between proteins, two strategies were used: 1) a stringent bacterial two-hybrid assay was used for testing all the candidate genes; 2) direct interactions were tested by co-immunoprecipitation (PopZ and crescentin). To characterize the biological functions of these interactive candidates, different strategies were used: recombinant strains were constructed for studying cc_0548, stress tests were designed for studying the function of cc_2998, a time course was conducted to investigate the relationship between PopZ and crescentin.
Results

Stringent bacterial two-hybrid assay for seven candidate genes

Seven candidate genes that gave positive results in a BTH assay had been selected from the library screening by Henrik Tomenius. He also had cloned these full length genes into pUT18 and pUT18c plasmids for BTH assay on rich medium. However, whether these results could be repeated on minimal medium was unknown, so a stringent test was conducted for these candidates.

For each gene, the plasmids generating N- and C-terminal fusion proteins (table 1) were transformed to *E. coli* BTH101 containing pKT25-creS or pKT25-popZ. Lac expression was used for scoring on minimal medium plates and all the blue signals were seen as positive results.

Table 1. Bacterial two-hybrid results of candidate genes

<table>
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<tr>
<th>clone</th>
<th>Gene ID</th>
<th>Fused to N- or C-terminal of T18</th>
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<th>Interaction with PopZ (from pKT25-popZ)</th>
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</table>

1 insert in the N- or C-terminal part of T18, see fig 4

2 determined by two-hybrid interaction on minimal medium, see fig 5. +, positive interaction; -, no interaction seen
Fig 6. Results from bacterial two-hybrid assay. Representative samples from ten replicates on M63 minimal plates are shown. Genes for the test (cloned in either pUT18 or pUT18c) are listed above the figure and the genes in pKT25 are listed on the left side. The result is based on lac expression, so the samples showing blue were seen as positive.

As fig 6 shows, crescentin only interacted with PopZ fused to the C-terminus of T18, indicating that the N-terminal fusion may destroy the interaction domain or influence the folding of protein, leading to the loss of interaction. A strong signal was obtained from PopZ with itself, demonstrating that PopZ is a self-associating protein (2, 7). cc_2998 and cc_3145 both gave blue signals in either way of fusion, showing that these genes interact with crescentin in the BTH system. cc_0548 showed positive results on the plate, but it took 7 days for the colonies to grow and only single clones instead of the whole streak grew on the plate. This could be caused by slow growth of the cells, so this result was scored as positive. The rest of full length genes did not show positive results in this stringent test, showing low possibility of interactions between crescentin and those candidates. In conclusion, from the stringent test, I narrowed down the interactive candidates to 4 genes: popZ, cc_2998, cc_0548 and cc_3145, and these interactions in *C. crescentus* should be further studied.

**Co-immunoprecipitation of PopZ and crescentin**

PopZ is a good candidate to be an interaction partner of crescentin’s based on the BTH assay, so a co-immunoprecipitation was conducted to test if a direct interaction exists between two proteins. Three strains were used in this experiment (fig 7). NA928 contains a plasmid with *popZ* fused with *mCherry* under the control of a xylose-inducible promoter (Pxyl), and there is a *flag* tag fused with the crescentin gene on the chromosome. NA926 contains the same plasmid but no tag on the chromosome. This strain was used as a negative control to test whether the resin with anti-Flag antibody could pull down crescentin-Flag fusion protein specifically. NA929 was a second negative control containing *cheB-mCherry* fusion to test whether there was any interaction between mCherry and crescentin (CheB is a methylesterase protein involved in chemotaxis and should not interact with crescentin) (fig 7). Because of the
low protein expression level in *C. crescentus*, all three strains were induced with xylose before co-immunoprecipitation. Fluorescence microscopy was conducted after induction to test whether the mCherry-fused proteins were induced successfully. (fig 8, NA926 induction is not shown).

![Fig 7. Schematics of the genetic setup of the three strains used in the co-IP. mCh refers to mCherry sequence; cat refers to the resistance to chloramphenicol; PxyI refers to xylose promoter. A) Strain NA928 has a crescentin-flag fusion in the genome and a popZ-mcherry fusion in the plasmid. B) Strain NA926 only has a plasmid bearing popZ-mcherry fusion. C) Strain NA929 has a crescentin-flag fusion in the genome and a cheB-mCherry fusion in the plasmid.

Lysates of all three strains were prepared and resins coupled to anti-Flag antibody were used to pull down crescentin. The induced and uninduced NA928 strains (fig 8 E, lane 6 and 7) gave a band around 60 kDa, which is what was expected for the PopZ-mCherry fusion. Protein samples from NA927 gave bands of the same size as NA928 (fig 8 E, lane 2 and 4), indicating that the PopZ-mCherry fusion protein was present in both the lysate and the complex that crescentin pulled down. The expected size of CheB-mCherry fusion protein is about 66 kDa, which could be the weak band in the lysate sample (fig 8 E, lane 5). Whether the negative co-IP result of NA929 (fig 8 E, lane 3) was caused by low protein concentration is unknown. The PopZ-mCherry fusion protein from NA926 was not detected (not shown). Based on these results, it is likely that there is direct physical association between PopZ and crescentin.
Fig 8. Xylose induction results and Western-blot analysis of co-immunoprecipitation samples. A – D, fluorescence micrographs of cells containing an mCherry fusion protein (red signal) before and after induction by microscopy. A) N928 before induction; B) N928 after induction; C) NA929 before xylose induction; D) NA929 after xylose induction. E, Western-Blot results of co-immunoprecipitation samples using anti-mCherry antibody. 1) size marker; 2) NA928 after co-IP; 3) NA929 after co-IP; 4) NA928 lysate; 5) NA929 lysate; 6) boiled bacteria (NA 928) without xylose induction; 7) boiled bacteria (NA 928) with xylose induction. The boiled bacterial samples which were induced show the correct size of PopZ-mCherry fusion protein, indicating the protein precipitated from co-IP is the same one.

The biological function of the PopZ-crescentin interaction

Why should PopZ and crescentin interact? As a previous publication showed, PopZ does not cause straight cell shape (7), so PopZ may have nothing to do with crescentin function in generating cell curvature. Yet, it is possible that crescentin is needed for PopZ function. PopZ has been shown to bind to a protein complex binding to the chromosome near the origin of replication (2, 7). This complex is localized to the flagellated cell pole in the swarmer cell (7). As the bacterium differentiates from a swarmer cell to a stalked cell, PopZ binds to the new origin of replication and starts to accumulate at the opposite pole, generating a bipolar localization. To determine whether crescentin gets involved in this movement, NA926 (a wild type) and NA927 (a crescentin mutant) were used to study the distribution of PopZ during one generation.
Swarmer cells were isolated from exponentially growing cultures and inoculated into fresh minimal medium at 30°C, in which they grow and divide synchronously during one cell cycle. At different times, fluorescence microscopy was conducted and the fraction of cells with different PopZ localization (unipolar, bipolar, diffused signal, dots) was calculated.

![Figure 9](image)

The fraction of cells with unipolar localization of PopZ started from close to 1, showing that most cells at the start were swarmers. As the cell cycle proceeded, the fraction of cells with unipolar localization of PopZ decreased, and the bipolar localization increased, indicating the normal growth of the cell. As the bacterium grew older, it started the cell division, generating two daughter cells with PopZ only at one pole, resulting in a slight decrease of bipolar localization. While more cells continued the cell division, the PopZ in the newly formed daughter cells moved to the new pole, compensating the loss of bipolar localization caused by cell division, which lead to the second increase of the fraction of bipolar localization. Although at some times the distribution of PopZ was somewhat different in two strains, the overall trends of PopZ localization appeared to be similar. Thus, crescentin probably does not influence the movement of PopZ.

**Deletion of a lysozyme homologue**

Based on the BTH results, CC_0548 may interact with crescentin. A Blast search with the CC_0548 protein showed that it is a lysozyme family protein, some of which are known to participate in cell wall synthesis (15). Thus, it is possible that this protein might contribute to the cell wall synthesis, resulting in a crescent shape. In order to figure out if this protein is required for the functionality of crescentin, cc_0548 was interrupted with a kanamycin resistant cassette in both NA52 and NA358 as shown in fig 10. Bands of the correct size,
around 800 bp, showed up after amplification, indicating that the insertion was in the right position in these colonies.

Fig 10. The strategy used for knockout (KO) mutagenesis (construction of NA935 and NA936). A part of cc_0548 was first amplified and cloned into pBGS18T plasmid in E. coli DH5α. Then the construct was transformed into E. coli S17 which can transfer the plasmid to C. crescentus recipient strain (NA52 (wt) or NA358 (ΔcreS)). As the plasmid did not replicate in Caulobacter, after mating cells with the amplified fragment recombined in chromosomal DNA were selected by kanamycin and nalidixic acid (to kill E. coli NA931). Several colonies were picked and verified by PCR using primers N127 and N338.

Fig 11. PCR identification of knockout strains with primers N127 and N128. Lane1: size marker; lane 2 to lane 5: CC_0548 knockout in a wild type background (NA52); lane 6 to lane 9: CC_0548 knockout in a Δ creS (crescentin mutant) background (NA358); lane 10: negative control (chromosomal DNA of NA358).

Two cc_0548 knockout strains (NA935, NA936) were then prepared for microscopy to observe the morphology of the cell. However, no significant morphological difference was found between two strains.

Study of subcellular localization of CC_0548
In order to compare the intracellular localization of CC_0548 in wild type and crescentin
mutant *Caulobacter*, a fluorescence tagged CC_0548 strain was needed. To construct this, NA697 was mated with NA52 (wt) and NA358 (ΔcreS) as shown in figure 12. When the selected clones were prepared for microscopy, very weak fluorescent signals were observed. However, when primers N335 and N336 were used to identify these clones, no band showed up. Since no positive control was available for PCR identification, it was hard to tell whether the negative results were caused by PCR failure or incorrect insertion of the fragment in the chromosome and these two strains required further investigation.

**Stress tests of CC_2998**

CC_2998 is a protein homologous to OsmC, which is thought to be sensitive to environmental changes (17). Thus, NA833 and NA834 were used for testing (fig 13). Both strains have cc_2998 fused with mCherry in the genome, which was used to quantify the protein expression level, but they originate from different strains: NA833 has a wild type background while NA834 has a crescentin mutant background. Two strains were subjected to different stress conditions to test whether CC_2998 is involved in stress responses. Three conditions were tested: salinity stress, osmotic stress, and heat shock. Before stress treatment, each strain was grown to logarithmic phase and diluted to OD600 0.1. After one hour of growth, the culture was split into two flasks, with one subjected to stress and the other serving as a negative control. In the salinity stress test, strains were exposed to 85 mM NaCl and samples were collected after 3 and 7 hours for fluorescence microscopy and viable counting. A decrease of the viable bacteria (data not shown) showed that both strains were sensitive to high salt concentration, but no significant difference of CC_2998-mCherry levels were observed between the stressed cells and the controls. In the osmotic stress, both strains were...
treated with 150 mM sucrose for 3 hours. From viable counting and microscopy results (data not shown), almost all cells were broken and dead after 3 hour-exposure.

Fig 13. Schematics of the genetic setup of the strains used in stress tests. The lines and squares in the figure show parts of the genome. *mCh* refers to mCherry sequence. A) NA833 has an *mCherry* fused with *cc_2998* in a wild type genomic background. B) NA834 has an *mCherry* fused with *cc_2998* in a crescentin mutant genomic background.

Fig 14. Fluorescence intensity of NA833 and NA834 with and without heat shock. After 15-minute heat shock the expression levels of CC_2998 in two strains were measured by fluorescence intensity. The mean RGB value shows the intensity of fluorescence. Blue bars refer to strains without heat shock and red bars refer to strains with 15 minute-heat shock. The error bars show the standard deviation (n=20). According to the t-test, there was a significant difference of fluorescence intensity between samples with and without induction. *p= 4.4 \times 10^{-12}; **p= 7.1 \times 10^{-9}

In the heat shock test, both strains were exposed to 48°C for 15 min before they were collected for measuring fluorescence intensity. The expression level of CC_2998 was twice as high in the heat-shocked cells. Because the heat shock only lasted for 15 minutes, a time
period that was much shorter than the generation time, it is likely that this increase of protein expression was due to new synthesis and induced by the heat shock. Thus, CC_2998 appeared to be a heat inducible protein. Interestingly, the expression of CC_2998 in NA834 without heat treatment appeared to be slightly higher than that in NA833, but this still remained a question whether the phenomenon was caused by the loss of crescentin.
**Discussion**

In this study, I confirmed the possible interactions of crescentin with several previously identified candidate proteins by stringent tests in BTH system, and I obtained several good candidates showing strong interactions in the test. One is PopZ, a cell cycle-dependent protein, whose physical association with crescentin was now also demonstrated in vitro by co-immunoprecipitation. But when, how and why the PopZ-crescentin interaction occurs in vivo remains a problem. One interesting cell cycle stage that needs to be further investigated is the predivisional stage, in which crescentin filaments need to be broken at the cell division site and distributed into two daughter cells (1). Crescentin filaments are quite stable in vivo, so this “break up” might require other molecules for assistance. Besides, since this phenomenon should be precisely regulated in the cell cycle, and PopZ is a cycle-dependent protein, this crescentin-PopZ interaction might happen at this time point.

CC_0548, a lysozyme family protein, is another promising candidate, but no straight cell shape was observed in the knockout strain, indicating this interaction may not be needed for cell shape. However, the possibility that other genes compensate for the loss of CC_0548 cannot be ruled out, so the overexpression of this gene will be done.

According to the results I obtained so far, it is interesting that none of the interactive candidates are transmembrane proteins, since the localization of crescentin suggests that it somehow attaches to the cell membrane. But instead, a cell cycle-dependent protein, a lysozyme-like protein and a chemoreceptor were identified as its interaction partners. Thus, there is an intriguing possibility that crescentin is not only needed for cell shape, but has other functions. The results that crescentin interacts with CC_2998 and chemoreceptors which are involved in sensing the environment suggest that crescentin, and perhaps the curved cell shape are important for fitness in the natural environment.
Acknowledgements

First of all, I would like to express my sincere gratitude to my supervisor, Dr. Nora Ausmees, for giving me a chance to complete the degree project, for her patience, encouragement and continuous support from the initial to the final level. I also would like to thank Professor Karin Carlson, whose course introduced me into an amazing research field.

My sincere thanks also go to Sonchita Bagchi for her patient teaching of lab skills and data analysis. Thanks to Xiaodong Ma and Yu Chen for enlightening discussion and useful suggestions.

Last but not least, I am forever in debt to my parents for their support and understanding.
**Materials and methods**

**Strains, plasmids and oligonucleotides**

The strains, plasmids and oligonucleotides are listed in Table 2 to Table 4 respectively.

### Table 2. strains

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<tr>
<td>NA834</td>
<td>CB15N ΔcreS cc&lt;sub&gt;2998::cc_2998&lt;/sub&gt;-mCherry</td>
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</tr>
<tr>
<td>NA935</td>
<td>CB15N Δcc&lt;sub&gt;0548&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>NA936</td>
<td>CB15N ΔcreS Δcc&lt;sub&gt;0548&lt;/sub&gt;</td>
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</table>

### Table 3. Plasmids

<table>
<thead>
<tr>
<th>stock name</th>
<th>plasmid description</th>
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<tbody>
<tr>
<td>pNA90</td>
<td>pJS14 (vector for protein expression)</td>
<td>(14)</td>
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<tr>
<td>pKT25</td>
<td>vector for two-hybrid assay</td>
<td>BACTH kit, Euromedex</td>
</tr>
<tr>
<td>pUT18</td>
<td>vector for two-hybrid assay</td>
<td>BACTH kit, Euromedex</td>
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<tr>
<td>pUT18c</td>
<td>vector for two-hybrid assay</td>
<td>BACTH kit, Euromedex</td>
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<tr>
<td>pNA138</td>
<td>pBGS18T (vector for mating)</td>
<td>(20)</td>
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<tr>
<td>pNA917</td>
<td>pJS14-pXyl-mCherry-CheB</td>
<td>lab stock</td>
</tr>
<tr>
<td>pNA579</td>
<td>pKT25-creS</td>
<td>lab stock</td>
</tr>
<tr>
<td>pNA902</td>
<td>pKT25-popZ</td>
<td>lab stock</td>
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<tr>
<td>pNA697</td>
<td>pBGS18T-ce&lt;sub&gt;0548&lt;/sub&gt;-mCherry</td>
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</tr>
<tr>
<td>pNA699</td>
<td>pBGS18T-ce&lt;sub&gt;0548&lt;/sub&gt;</td>
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<tr>
<td>pNA701</td>
<td>pUT18-popZ</td>
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Cultivation

*Caulobacter* strains were grown at 30°C in PYE or M2G minimal medium and washed with M2 as described by Ely (8). *E. coli* S17 and DH5α were grown at 37°C in LB medium as described by Sambrook *et al.* (16). The strains used for BTH assay were grown on LB plates at first and then transferred to M63 medium supplemented with maltose (2 g (NH₄)₂SO₄, 13.6 g KH₂PO₄, 0.5 mg FeSO₄·7H₂O and 15 g agar in 1000 ml H₂O). After autoclaving, 1 ml of 1 M MgSO₄·7H₂O, 15 ml of 20% maltose, 2 ml of 0.05% vitamin B1 (thiamin) were added.

Table 4. Oligonucleotides

<table>
<thead>
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<th>annotation</th>
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<td>N335</td>
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<td>CC0548KOkpnR</td>
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</tr>
<tr>
<td>N338</td>
<td>CC0548mChrkpnR</td>
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<tr>
<td>N127</td>
<td>M13_forw</td>
<td>gtaaaaagcagggcagt</td>
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</tbody>
</table>

¹ upper case: restriction enzyme site; lower case: sequence of the gene; italics: additional bases which improve the efficiency of the restriction enzymes.

The construction of *cc_0548* knockout donor strain

*cc_0548* was amplified from *C. crescentus* genome (lab stock) by primers N335 and N336 (Table 4), and the amplified fragment was purified with a PCR purification kit (Qiagen). The PCR product as well as the plasmid pBGS18T (pNA698), were digested with KpnI and BamHI (Fermentas) according to the guidance provided by the manufacturer. After digestion, the PCR product and linearized pBGS18T were purified again with a PCR purification kit (Qiagen) and the two DNA pieces were ligated overnight with T4 ligase (Fermentas). The construct was then transformed into chemically competent *E. coli* DH5α and kanamycin resistance was used for selection. The plasmid was prepared and transformed to *E. coli* S17. Kanamycin was used to select the correct clone as a donor strain (NA931).

Polymerase chain reaction (PCR)

A 25 µl reaction system was used for PCR amplification, with 0.2 µM primers, 0.25 mM dNTP (for each). A PCR kit (Qiagen) containing buffers and Taq polymerase was used according to the manufacturer’s instruction. For the amplification of *cc_0548* fragment,
primers N335 and N336 (Table 4) were used. The PCR conditions were as follows: 1) initial denaturation at 96 °C for 10 min; 2) 35 cycles of amplification including a 30-sec denaturation step at 95 °C, a 45-sec annealing step at 54 °C, and a 15-sec extension step at 72 °C; 3) a final 10-min extension at 72 °C. As for the identification of NA935 and NA936, primers N127 and N338 (Table 4) were used. The PCR conditions were as follows: 1) initial denaturation at 96 °C for 15 min; 2) 35 cycles of amplification including a 30-sec denaturation step at 95 °C, a 45-sec annealing step at 45 °C, and a 1-min extension step at 72 °C; 3) a final 10-min extension at 72 °C.

**The construction of cc_0548 knockout and mCherry fusion strains by mating**

The *Caulobacter* recipient strains (NA52 and NA358) and *E. coli* S17 donor strains (NA931 for knockout construction and NA697 for mCherry fusion construction) were inoculated to LB and PYE respectively to obtain an overnight culture for the experiment. On the next day, 1 ml *Caulobacter* culture and 0.1 ml *E. coli* S17 were spun down at 4000 × g for 3 min and the supernatants were discarded. Pellets of both strains were resuspended and mixed in a total volume of 1 ml PYE. The mixture of two strains was spun down again and the supernatant was removed. The mixed pellet was then resuspended in a small volume of PYE and spotted onto a PYE plate without antibiotics. The plate was incubated at 30°C overnight. The blob was scraped and spreaded on the PYE plate with nalidixic acid (20 μg/ml, for killing *E. coli*) and antibiotic to select the *Caulobacter* containing the plasmid.

**Synchronization of Caulobacter**

Strains were inoculated into PYE medium 2 days before synchrony. On the second day, bacteria were reinoculated in 30 ml PYE to obtain cultures with OD660 at 0.2-0.4. 3 ml Ludox (colloidal silica, pH 7, Sigma-Aldrich) and cell culture were mixed in pre-chilled Corex tubes and centrifuged at 30000 × g for 30 min at 4°C. After centrifugation, the top band (stalk, predivisional cells) and most supernatant were removed, while the bottom band (swarmer cells) was transferred into a falcon tube. 12 ml M2 solution was mixed with swarmer cells and cells were spun down at 2500 × g for 15 min at 4°C to get rid of the residual Ludox particles. Most supernatant was pipetted off and pellets were resuspended and transferred to Eppendorf tubes. Cells were centrifuged again at 2500 × g and washed twice with M2. The pellets were resuspended in M2G for growth.

**Preparation of chemically competent E. coli cells**

The mid-log phase bacteria were transferred to sterile tubes. After incubation on ice for 5 min, the cells were spun down at 5000 × g for 10 min at 4°C. The supernatant was taken away and the cells were resuspended in a total of 80 ml cold TFBII (30 mM KC2H3O2 [potassium acetate], 100 mM RbCl, 10 mM CaCl2·2H2O, 50 mM MnCl2·4H2O, 15% glycerol, pH 5.8). After incubating on ice for another 5 min, the cells were spun down again and resuspended in a total of 8 ml cold TFBII (10 ml MOPS, 10 mM RbCl, 75 mM CaCl2·2H2O, 15% glycerol). The cells were then incubated on ice for 15 min before being aliquoted into tubes. All the cells were frozen in liquid N2 and stored at -70°C.

**Isolation of plasmids from E. coli**

20
1.5 ml *E. coli* was harvested and resuspended in 100 μl Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, 100 μg/ml RNase, pH 8.0) in an eppendorf tube. 200 μl Solution 2 (0.2 M NaOH, 1% SDS) and 200 μl chloroform were then added to the suspension and mixed gently for lysis. After one-minute cell lysis, 150 μl Solution 3 (24.9 g CH₃COOK and 5 ml HCOOH in 100 ml water) was added and the mixture was vortexed briefly and spun down for two minutes at 14000×g. The aqueous phase was transferred to a new tube and DNA was precipitated with 800 μl ice-cold ethanol before a second centrifugation at 14000×g. The supernatant was discarded and the pellet was air-dried and dissolved in 50 μl water.

**Transformation**
The chemically competent cells were thawed and incubated on ice for 10 min. Less than 100 ng DNA was added to the tubes with cells and incubated for another 30 min on ice before a heat shock at 42°C for 90 sec. After ice incubation for 1-2 min, 800 μl LB was added to the tube and the cells were incubated at 37°C with agitation for 1 hour before plating.

**Bacterial two-hybrid assay**
*E. coli* BTH101 strains containing pNA579 or pNA902 were made chemically competent and transformed with pUT18 (or pUT18c) derivatives with different genes cloned. The transformants were spread on LA with ampicillin (100 μg/ml) and kanamycin (50 μg/ml) for selection. 10 colonies were picked and streaked on M63 minimal medium containing IPTG (1 mM), X-gal (1 mM), ampicillin (100 μg/ml) and kanamycin (50 μg/ml), and incubated at 30°C for up to 7 days.

**Co-immunoprecipitation**
Strains were inoculated into PYE medium 2 days before co-immunoprecipitation. For NA928 and NA926, bacteria were reinoculated into 150 ml PYE on the second day. When the OD660 reached 0.15, xylose was added to the final concentration of 0.2% to the medium for 1.5 h before the sample was centrifuged at 5000 g for 15 min. For NA929, xylose was added to the final concentration of 0.2% to the medium for induction immediately after reinoculation on the second day. The bacteria were collected when OD660 came to 0.4 and centrifuged at 5000 g for 15 min. Cell pellets were washed with Wash Buffer (20 mM HEPES, 100 mM NaCl, 0.05% Triton-X100) and resuspended in 800 μl IP Buffer (20 mM HEPES, 100 mM NaCl, 20% glycerol, 0.3% Triton-X100) containing a protease inhibitor cocktail (Sigma-Aldrich, 10 μl/ml buffer). Samples were treated with lysozyme (0.2 mg/ml) for 2.5 h at 30 °C before sonication. Lysates were then treated with DNaseI (15U, Fermentas) and MgCl₂ (10 mM) and incubated on ice for 10 min (With a higher concentration of MgCl₂ added, DNaseI worked even at a low temperature to make lysates less viscous.) before centrifugation at 13000×g for 15min. Cleared lysates were incubated with ANTI-FLAG M2 Affinity resin which contains the antibody (Sigma) for 3 hours and washed 4 times with Wash Buffer. The resin was then resuspended in 30 μl loading buffer (100 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 15% glycerol, 0.2 mg/ml bromophenol blue) and boiled for 10 min. These samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).
Sodium dodecyl sulfate polyacrylamide gel electrophoresis
A 10% polyacrylamide gel (separating gel: 2.4 ml H2O, 1.25 ml Tris-HCl (1 M, pH 8.8), 0.05 ml SDS (10%), 1.25 ml acrylamide (Biorad 40%, 37.5:1), 0.05 ml ammonium persulfate (10%), 5 μl TEMED; stacking gel: 1.42 ml H2O, 0.25 ml Tris-HCl (1M, pH 6.8), 0.02 ml SDS (10%), 0.26 ml acrylamide (Biorad 40%, 37.5:1), 0.02 ml ammonium persulfate (10%), 2 μl TEMED) was prepared with BIO-RAD Mini-PROTEAN Tetra Electrophoresis System. The gel was run vertically at 80 volts for stacking gel and 120 volts for separating gel in a BIO-RAD cassette with proper amount of running buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% SDS). A prestained protein ladder (PageRuler, Qiagen) was used as size standard.

Western blot
When the SDS-PAGE was terminated, the BIO-RAD cassette was disassembled and the stacking gel was separated from the separating gel and discarded. A piece of polyvinylidene fluoride (PVDF) membrane (Millipore Immobolin–P transfer membrane) which was approximately the same size as the separating gel was placed in methanol for 1 minute for activation and then placed against the gel. The gel and PVDF membrane were put in the transfer cassette with pads and three layers of filter paper covering both sides. The transfer was run with a BIO-RAD transfer system in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) at 4℃ either at 20 mA overnight or at 100 mA for 2 hours.
When transfer was complete, the membrane was incubated in PBST (NaCl 8 g, KCl 0.2 g, Na2HPO4 1.44 g, KH2PO4 0.24 g, 0.1% Tween-20 in 1000 ml H2O) with 5% nonfat milk powder added for 2 hours. After blocking, the membrane was washed with PBST for four times (10 minutes for each), and incubated with rabbit anti-mCherry antibodies(1:1000, Clontech) for 1 hour. The membrane was then washed for another four times with PBST before incubating with horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG polyclonal antibodies (1:3000, Dako) for 1 hour. The wash with PBST was repeated for four times and the membrane was developed by enhanced chemiluminescence (ECL) kit (GE Healthcare) and several films with different exposure time were taken.
Reference


Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 175-176


