

Proteomic analysis of the *Caulobacter crescentus* stalk indicates competence for nutrient uptake

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Summary

Caulobacter crescentus, a Gram-negative α -purple proteobacterium, is an oligotroph that lives in aquatic environments dilute in nutrients. This bacterium divides asymmetrically. Part of this asymmetric cell division involves the formation of a prosthecum at one pole, referred to as the stalk, which replaces the flagellum of the motile swarmer cell. Little is known about the synthesis or function of the stalk. The stalk is an extension of the cell membranes and peptidoglycan layer, and stalk elongation is stimulated by phosphate starvation. In this study, we have taken advantage of two-dimensional gel (2D gel) electrophoresis as well as the fully sequenced genome of *Caulobacter* to study the proteome of the stalk. We modified a stalk-shedding mutant strain of *Caulobacter crescentus* to increase the yield of stalk material shed and performed 2D gel electrophoresis of purified stalks and cellular fractions. Comparison of the stalk 2D gel with the 2D gels of cell membrane and soluble fractions showed that the stalk is mostly free of cytoplasmic proteins and has a profile very similar to that of the cell membrane. Of the 172 proteins on a stalk 2D gel, we report the identification of 64 spots, corresponding to 39 different proteins present in the stalk of *Caulobacter*. The identifications include several TonB-dependent receptors, two OmpA family proteins, a dipeptidase, GlpQ, two alkaline phosphatases, 3-phytase, a putative TolC protein and 11 proteins of unknown function. These identifications are consistent with the hypothesis that the stalk plays a role in nutrient uptake.

Introduction

Prosthecate bacteria are a diverse group belonging to the

α -Proteobacteria (Stahl *et al.*, 1992; Sly *et al.*, 1999; Weiner *et al.*, 2000). At some stage in their life cycle, all prosthecate bacteria possess one or more extensions of the cell surface called stalks, prosthecae, appendages or hyphae depending on the bacterium and the function of the cellular extension. Prosthecae are cylindrical extensions of the cell surface that are bound by the cell membrane and cell wall of the organism. The life cycle of the dimorphic prosthecate bacteria is conceptually similar (Brun and Janakiraman, 2000). Cell division produces a prosthecate cell and a swarmer cell. These two cell types differ in their morphology, motility and capacity to re-enter the next cell cycle. The swarmer cell is unable to initiate DNA replication and is devoted to dispersal for a portion of the cell cycle. The prosthecate cell, on the other hand, is sessile and is most often found attached to surfaces. The prosthecate cell can initiate DNA replication and is devoted to producing new swarmer cells. Swarmer cells must undergo an ordered series of events leading to differentiation into a prosthecate cell to enter a new cell division cycle.

Dimorphic prosthecate bacteria can be divided into two major groups based on the function of the stalk. In one group, the budding bacteria, the prosthecum is involved in reproduction. Examples of this group include *Rhodomicrobium vannielii*, *Hyphomonas* and *Hyphomicrobium*, which grow and reproduce by budding at the tip of the prosthecum, producing a flagellated swarmer cell. In the other group, which includes *Caulobacter* and *Asticca-caulis*, the prosthecae, called stalks, are not involved in reproduction. In *Caulobacter crescentus*, swarmer cells differentiate into a stalked cell containing a stalk and an adhesive holdfast at the pole that previously contained the flagellum. The *Caulobacter* stalk is an extension of the inner membrane, peptidoglycan and outer membrane of the cell (Poindexter and Bazire, 1964; Pate and Ordal, 1965; Jones and Schmidt, 1973; Schmidt, 1973), with a central core that is devoid of cytoplasmic elements such as ribosomes, DNA or nucleoplasm (Poindexter, 1964).

To synthesize the stalk, the cell has to redirect the biosynthesis of the cell surface in a perpendicular direction at a specific subcellular location at the proper time in the cell cycle. Stalk elongation is controlled in subsequent cell cycles in response to extracellular phosphate concentration. Phosphate starvation can trigger elongation of the stalk to many cell lengths (Schmidt and Stanier, 1966; Schmidt, 1968; Gonin *et al.*, 2000). Because the stalk is

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an extension of the cellular membrane, it provides increased surface area, which is thought to allow cells to take up nutrients including phosphate more efficiently and survive in more nutrient-dilute environments (Poindexter and Bazire, 1964; Porter and Pate, 1975; Larson and Pate, 1976; Poindexter, 1981; 1984). Isolated stalks from *Asticcacaulis biprosthecum* have been found to contain some respiratory and citric acid cycle enzymatic activity, as well as elements of respiratory energy-dependent uptake systems, but they cannot catabolize sugars and amino acids (Jordan *et al.*, 1974; Porter and Pate, 1975; Larson and Pate, 1976). Another function of the stalks, which reduce the buoyant density of stalked cells, may be to help keep the cells at the air–water interface, an obvious advantage for an obligate aerobe such as *C. crescentus* (Poindexter, 1984). In addition, stalked cells are often found attached to surfaces in aquatic environments where inorganic phosphate is the most common limiting nutrient. Increased stalk elongation under these conditions would allow cells to extend away from the surface, thus benefiting from more nutrient flow and avoiding the competition with other bacteria in a nascent biofilm (Poindexter, 1984).

Unlike the ‘buoyancy’ and ‘extension from a surface’ hypotheses about the function of the *Caulobacter* stalk, the nutrient uptake hypothesis makes specific predictions that are easy to test experimentally. If the stalk is involved in nutrient uptake, certain types of proteins should be contained in the stalk. In this work, we have identified some of the protein components of the stalk of *C. crescentus*. By performing two-dimensional gel (2D gel) analyses on preparations of proteins isolated from *Caulobacter* stalks, we show that the protein composition of purified stalks is similar to that of the cellular outer membrane. We determine the identity of 64 of the 172 protein spots detectable on a stalk 2D gel. These 64 spots correspond to 39 proteins, including TonB-dependent receptors, OmpA-related proteins, a dipeptidase, GlpQ, alkaline phosphatases, 3-phytase, a sensor histidine kinase, a TolC homologue and 11 proteins of unknown function. This indicates that the stalk has the required outer membrane components to increase the nutrient uptake capability of the cell.

Results

Construction of an efficient stalk-shedding strain

In order to purify the large quantities of stalks required for proteomic analysis, we used a mutant that sheds its stalks by dividing frequently at the base of the stalk, NY111d1 (Poindexter, 1978). This mutant has an uncharacterized mutation that causes the stalk-shedding phenotype, but is wild type in other respects; it has holdfasts, forms rosettes and is motile. The stalk shedding of this mutant was much

more efficient when grown under low phosphate conditions (120 μ M). The shed stalks are more buoyant than cell bodies, and the two can be separated relatively efficiently by differential centrifugation. Furthermore, cells with long stalks are more buoyant than cells with short stalks. We thus hypothesized that increasing the length of the stalks might improve the separation of stalks from cell bodies. To increase the yield of stalk material per cell in the stalk-shedding mutant, we introduced a *pstS*::miniTn5 mutation into NY111d1 by transduction. Mutations in *pstS* activate the phosphate starvation regulon and lead to increased stalk elongation (Gonin *et al.*, 2000). The resulting strain, YB2811, had long stalks compared with NY111d1 when grown in PYE medium.

Addition of the *pstS* mutation to strain NY111d1 dramatically improved the yield of stalk material collected and the ability to isolate stalks free from cell bodies. Purification of stalks from YB2811 only required two centrifugation steps: one to remove cell bodies and one to pellet shed stalks. Stalk preparations from YB2811 were essentially free of contaminating cell bodies. On average, the stalk preparations used for this study contained less than one cell body per 1000 stalks. 2D gels of stalk fractions revealed the presence of 172 protein spots. This suggested that the stalk proteome was much less complex than the whole-cell proteome, in agreement with previous microscopy studies indicating that DNA and ribosomes were not present in the stalk (Poindexter and Bazire, 1964; Poindexter, 1981).

We used the DNA stain DAPI to confirm that the stalk and the cellular material attached to some shed stalks did not contain DNA. Out of approximately 1000 cells visualized, all the cells were stained with DAPI (Fig. 1). None of the stalks, those attached to either cells or shed stalks, exhibited any detectable DAPI staining. Furthermore, the small amount of cellular material at the base of the stalk that is sometimes removed with the stalk as a result of aberrant division in the stalk-shedding mutant was devoid of staining. This provides further evidence that cytoplasmic components are not present in the stalk. These results also indicate that the processing of the stalks and the introduction of the *pstS* mutation in NY111d1 had not made the stalks permeable to DNA or other cytoplasmic components, which would result in contamination of the stalk fraction by proteins that are not part of the stalk proteome.

The stalk proteome is similar to the outer membrane proteome

The 172 spots of the stalk gel represent a small set of proteins compared with whole-cell lysates, which produce \approx 850 spots on a 2D gel with the same amount of protein loaded (data not shown). The *Caulobacter* proteome is

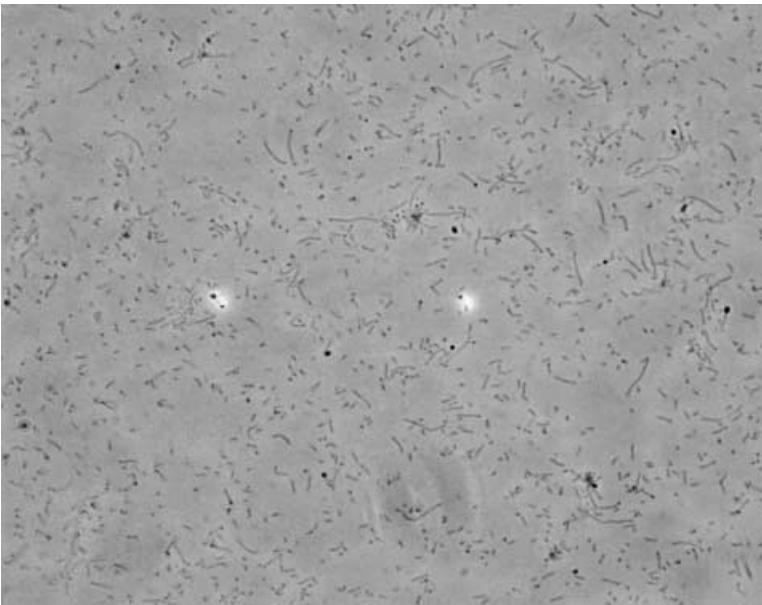


Fig. 1. DAPI staining of stalks and cell bodies. The micrograph is of phase and fluorescence taken simultaneously. YB2811 was grown in HIGG with 120 μM phosphate for 48 h. Stalks were separated from cell bodies by differential centrifugation. The stalk and cell body fractions were stained with 5 $\mu\text{g ml}^{-1}$ DAPI for 10 min followed by five washes in M2 salts.

predicted to be composed of \approx 1800 cytoplasmic proteins, 1430 inner membrane proteins, 325 periplasmic proteins and 140 outer membrane proteins (Nierman *et al.*, 2001; Phadke *et al.*, 2001). The number of stalk proteins detected on 2D gels is similar to the number of predicted outer membrane proteins in the whole proteome, suggesting that stalks are free from cytoplasmic proteins as predicted (Poindexter, 1964). Inner membrane proteins, which may be present in the stalk, are not expected to be found on 2D gels because they are not easily amenable to isoelectric focusing (Santoni *et al.*, 1999; 2000). To confirm that stalks are essentially devoid of cytoplasmic proteins, we used a French press to lyse the cell bodies removed during preparation of the stalks and separated whole-cell soluble proteins from whole-cell membrane proteins by ultracentrifugation. Both soluble and membrane fractions were separated by 2D gel electrophoresis. A comparison of the 2D patterns of whole stalk proteins and whole-cell soluble proteins demonstrates that these fractions are substantially different from one another (Fig. 2A and B). As the cell soluble fraction contains mostly cytoplasmic proteins, this result confirms the prediction that the content of cytoplasmic proteins in the stalk is very low.

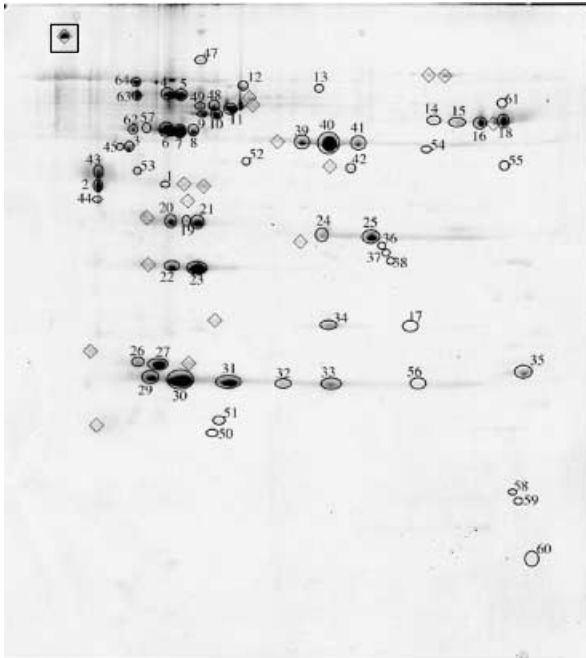
The whole-cell soluble fraction and the whole stalk fraction contained at least 22 proteins in common, as seen by comparison of the two patterns using IMAGEMASTER (Fig. 2A and B). The proteins common between the whole-cell soluble fraction and the whole stalk fraction are also found in the membrane proteome (Fig. 2D) and are thus unlikely to be cytoplasmic proteins. These same proteins can also be extracted from a stalk preparation by washing lysed stalk material with 0.1 M Tris-HCl, pH 8, 2 mM EDTA,

followed by the removal of membranes by ultracentrifugation (Fig. 2C). All the proteins in this fraction, which we call the stalk soluble fraction (Fig. 2C), correspond to the proteins found to be in common between the stalk gel (Fig. 2A) and the whole-cell soluble gel (Fig. 2B). The only exceptions are the flagellar proteins (see below). This suggests that the proteins that are similar between the whole stalk gel and whole-cell soluble gel are simply soluble outer membrane proteins or periplasmic proteins, and not cytoplasmic proteins. This was confirmed by the identity of these proteins, as revealed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (see below). As expected, the whole-cell membrane protein 2D gel showed less complexity than the whole-cell soluble protein gel (Fig. 2D and B). Furthermore, comparison of the membrane protein 2D gel (Fig. 2D) with the stalk 2D gel (Fig. 2A) revealed that the vast majority of the 172 stalk protein spots were also present on the membrane protein gel. The proteins present in the stalk fraction, but not those in the membrane fraction, and vice versa, are indicated by boxes. In addition, the pattern of proteins on the stalk 2D gel was similar to the pattern of isolated outer membrane proteins described previously (Phadke *et al.*, 2001).

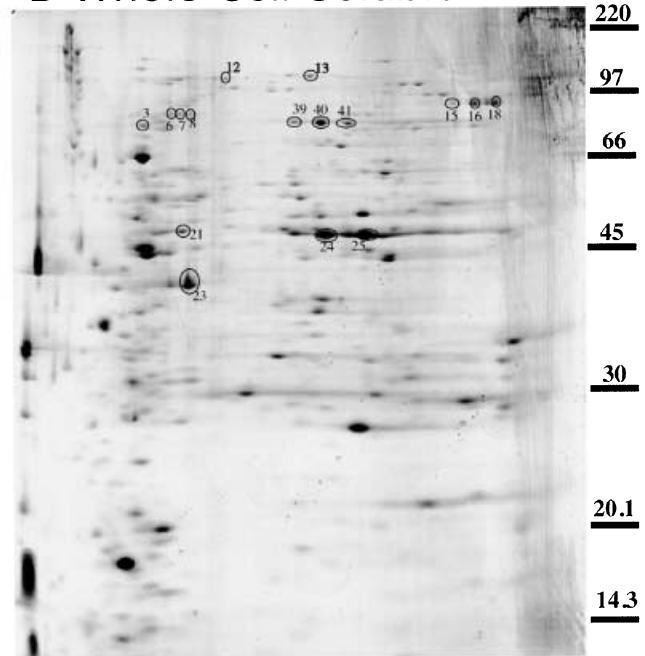
Identification of stalk proteins

We used MALDI-TOF mass spectrometry to identify proteins from the stalk 2D gel (Fig. 2A). We identified 64 individual spots on the 2D gel, which represent 39 different proteins (Table 1). The spots identified are, for the most part, the most abundant proteins on the gel. Many of the

A Stalk



B Whole Cell Soluble



C Stalk Soluble



D Whole Cell Membrane

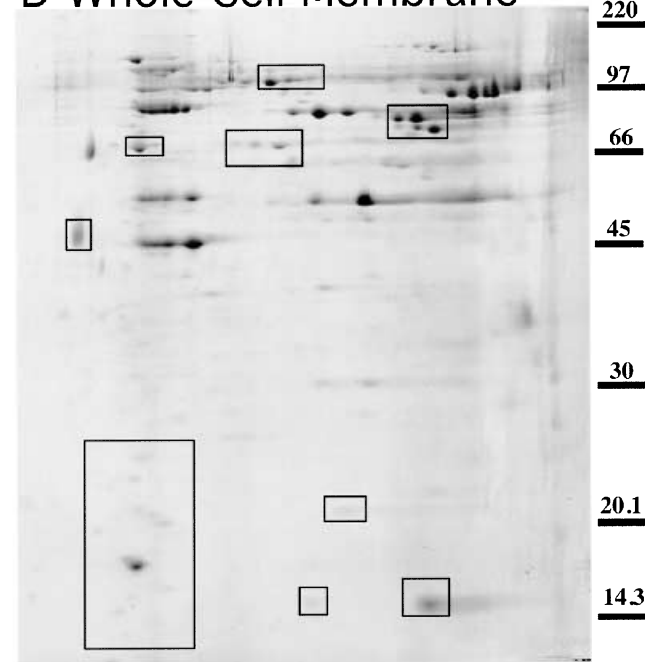


Fig. 2. Two-dimensional gel electrophoresis of various *Caulobacter crescentus* cellular fractions. All gels were loaded with 100 µg of protein and focused using pH 3–10 non-linear strips. The acidic end of the gel lies to the left. Molecular weights are indicated on the right.

- A. Stalk proteins.
- B. Soluble cell body proteins.
- C. Soluble stalk proteins.
- D. Cell membrane proteins.

Proteins identified by MALDI-TOF are circled and numbered in (A) for reference in Table 1. Proteins present in the membrane gel and in the soluble gel that are not present in the stalk gel are indicated by a box (B and D), and those spots only present in the stalk gel are also indicated by a box (A). Those proteins that could not be identified are indicated by a diamond (A).

Table 1. Identification of proteins on the stalk 2D gel.

Spot	CC no. ^a	ORF ^b	Size (Da)	Conf ^c	COG GRP	COG ^d	Identification	Solubility (TM domains) ^e	Localization ^f
1	2149	6461	92 459	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
2	170	2635	53 187	99		None	Hypothetical protein	Soluble	OM
3	902	4069	61 201	99	N	1749	FlgE hook protein	Soluble	OM
4	2924	643	102 818	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
5	2924	643	102 818	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
6	2149	6461	92 459	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
7	2149	6461	92 459	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
8	2149	6461	92 459	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
9	454	3196	88 902	99	P	1629	TonB-dependent receptor	Soluble	SigPep ^h
10	210	2709	92 480	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
11	171	2637	97 310	99	P	1629	TonB-dependent receptor	Soluble	OM
12	925	4119	112 906	99		None	OmpA family protein	Soluble	OM
13	288	2871	117 218	98		None	Hypothetical protein	Soluble	OM ^g
14	3146	1077	88 828	99	P	1629	TonB-dependent receptor	Soluble	SigPep ^h
15	3146	1077	88 828	99	P	1629	TonB-dependent receptor	Soluble	SigPep ^h
16	3146	1077	88 828	99	P	1629	TonB-dependent receptor	Soluble	SigPep ^h
17	3147	1079	89 272	99	P	1629	TonB-dependent receptor	Soluble	OM
18	3146	1077	88 828	99	P	1629	TonB-dependent receptor	Soluble	SigPep ^h
19	1375	5003	49 865	99		None	Conserved hypothetical protein	Soluble	SigPep ^h
20	3494	1781	47 605	99	N	1360	OmpA family protein	Soluble	PP ^g
21	3494	1781	47 605	99	N	1360	OmpA family protein	Soluble	PP ^g
22	3494	1781	47 605	99	N	1360	OmpA family protein	Soluble	PP ^g
23	3494	1781	47 605	99	N	1360	OmpA family protein	Soluble	PP ^g
24	163	2621	50 225	99		None	Hypothetical protein	Membrane (1)	OM ^g
25	163	2621	50 225	99		None	Hypothetical protein	Membrane (1)	OM ^g
26	1460	5156	28 238	99	N	1344	FijL	Soluble	Fla ⁱ
27	1460	5156	28 238	99	N	1344	FijL	Soluble	Fla ⁱ
28	1460	5156	28 238	99	N	1344	FijL	Soluble	Fla ⁱ
29	1461	5158	28 000	99	N	1344	FijK	Soluble	Fla ⁱ
30	1461	5158	28 000	99	N	1344	FijK	Soluble	Fla ⁱ
31	792	3852	27 927	99	N	1344	FijM	Soluble	Fla ⁱ
32	600	3480	29 168	98		None	Hypothetical protein	Soluble	OM
33	792	3852	27 927	99	N	1344	FijK Lor M	Soluble	Fla ⁱ
34	3444	1677	29 243	99		None	Hypothetical protein	Soluble	PP ^{g,i}
35	2257	6670	29 567	99	R	500	Hypothetical protein	Soluble	SigPep ^j
36	300	2897	44 051	98	R	1228	Xaa-Pro dipeptidase	Membrane (1)	PP
37	2925	644	39 763	98		None	Hypothetical protein	Membrane (1)	PP
38	3272	1336	37 947	99	C	584	GlpQ	Soluble	PP
39	1750	5691	69 353	99	P	1629	TonB-dependent receptor	Soluble	OM
40	1750	5691	69 353	99	P	1629	TonB-dependent receptor	Soluble	OM
41	1750	5691	69 353	99	P	1629	TonB-dependent receptor	Soluble	OM
42	455	3198	61 598	99		None	Alkaline phosphatase, putative	Soluble	PP
43	170	2635	53 187	99		None	Hypothetical protein	Soluble	OM
44	170	2635	53 187	99		None	Hypothetical protein	Soluble	OM
45	902	4069	61 201	99	N	1749	Flagellar hook protein	Soluble	OM
46	902	4069	61 201	99	N	1749	Flagellar hook protein	Soluble	OM
47	3494	1781	47 605	99	N	1360	OmpA related	Soluble	PP ^g
48	3494	1781	47 605	99	N	1360	OmpA related	Soluble	PP ^g
49	210	2709	92 480	99	P	1629	TonB-dependent receptor	Soluble	OM ^g
50	2294	6732	25 481	99		None	Outer membrane protein28	Soluble	PP
51	3187	1159	34 218	98	M	399	Hypothetical protein	Soluble	SigPep ^h
52	1565	5361	58 824	98		None	PhoD	Soluble	SigPep ^h
53	3007	787	52 306	95		None	Hypothetical protein	Soluble	PP ^g
54	1318	4889	50 157	97	S	1538	Outer membrane protein TolC, putative	Soluble	SigPep ^h

Table 1. cont.

Spot	CC no. ^a	ORF ^b	Size (Da)	Conf ^c	COG GRP	COG ^d	Identification	Solubility (TM domains) ^e	Localization ^f
55	394	3081	48 857	93		None	Hypothetical protein	Membrane (1)	PP ^g
56	793	3854	27 926	94	N	1344	FliJN	Soluble	Fla ⁱ
57	1294	4831	57 383	97	T	642	Sensor histidine kinase	Membrane (2)	IM
58	1914	6001	22 360	99		None	Conserved hypothetical protein	Membrane (1)	PP
59	3392	1570	22 525	99		None	Conserved hypothetical protein	Membrane (1)	PP
60	2119	6393	20 287	95		None	Conserved hypothetical protein	Soluble	PP
61	3228	1248	30 509	98	S	1729	Hypothetical protein	Soluble	PP
62	1295	4833	71 268	99		None	3-Phytase	Soluble	PP
63	2819	440	61 207	99		None	TonB-dependent receptor	Soluble	I
64	288	2871	117 218	99		None	Hypothetical protein	soluble	OM ^h

a. CC no. refers to the gene symbol (<http://www.tigr.org/tigr-scripts/CMR2/GeneNameSearch.spl?db=gcc>).

b. ORF refers to the released genome sequence used by other groups (Grunenfelder *et al.*, 2001; Phadke *et al.*, 2001).

c. Confidence level as described previously (Karty *et al.*, 2002a).

d. COG groups are defined at <http://www.ncbi.nlm.nih.gov/COG/>.

e. Solubility as predicted by SOSUI (Phadke *et al.*, 2001). Number of trans-membrane domains is indicated in parentheses.

f. Localization as predicted by PSORT (Phadke *et al.*, 2001). OM, outer membrane; PP, periplasm; IM, inner membrane.

g. Translation start sites were reassigned as described in the text. The subcellular localization was determined using a combination of codon preference (GC bias; GCG), SIGNALP and PSORT algorithms.

h. SigPep indicates proteins containing an N-terminal signal peptide with a probability of >70%, with no strong prediction of subcellular location based on PSORT and without transmembrane domains based on SOSUI analysis (Hirokawa *et al.*, 1998; Mitaku *et al.*, 1999).

i. Flagellar proteins are not predicted to have general secretion signal peptides. These proteins are secreted using the type III secretion system. k. Gene may have a frameshift according to GC bias and has a signal peptide according to SIGNALP.

l. No signal peptide, transmembrane domains or subcellular localization was predicted.

less abundant spots and a few of the highly abundant spots yielded poor quality mass spectra. The proteins that were not identified are indicated by a diamond on the stalk gel (Fig. 2A). Confidence values ranged from 93% to >99%, with 48 identifications at or above the 99% confidence level (Table 1). Eighteen spots corresponded to one of nine different TonB-dependent receptors. Protein spots corresponding to 11 different hypothetical proteins were found. Several of the hypothetical proteins were represented by multiple spots. Seven were represented by a single spot. In addition, four conserved hypothetical proteins were identified, each corresponding to a single spot. Two OmpA-related proteins, a dipeptidase, a TolC homologue, a sensor histidine kinase, GlpQ and Omp28 were identified. In addition, two alkaline phosphatases and a 3-phytase were identified. Multiple identifications of a single protein most often occurred from a series of spots with the same molecular weight but slightly different isoelectric points (pI). These patterns could be the result of post-translational modification, perhaps by phosphorylation (Cash *et al.*, 1999), or deamidation (Sarioglu *et al.*, 2000). It has also been hypothesized that these patterns could be artifacts of the TCA precipitation process (Antelmann *et al.*, 2001). These patterns have been observed by others, but have not been characterized (Blankenhorn *et al.*, 1999; Franzen *et al.*, 1999). All proteins identified, except for flagellar proteins, were also present in the membrane fraction 2D gel.

Several flagellar proteins were identified: FliJK (spots 29 and 30), FliJL (spots 26–28), FlgE (spots 3, 45–46), FliJM

(spots 31 and 33) and FliJN (spot 56). These proteins were found on the stalk gel as a consequence of the stalk preparation method (Poindexter, 1978; Poindexter and Hagenzieker, 1982). Flagella are shed during each cell cycle and sediment with shed stalks during the high-speed centrifugation. Flagella staining revealed that shed flagella were present in the purified stalk fraction (data not shown).

Discussion

In this report, we have used 2D gel analysis coupled with MALDI-TOF mass spectrometry to analyse the stalk proteome. Comparison of stalk proteins with proteins of other cellular fractions using 2D gel electrophoresis indicates that the stalk is mostly devoid of cytoplasmic proteins. The stalk proteome is very similar to the cellular membrane proteome, and the 2D gel pattern of the stalk proteome is very similar to that of the *C. crescentus* outer membrane described previously (Phadke *et al.*, 2001). The identity of stalk proteins indicated that the stalk 2D gel proteome is mostly composed of outer membrane and periplasmic proteins. It is not surprising that only one stalk protein identified is predicted to be an inner membrane protein. Inner membrane proteins are extremely difficult to resolve via isoelectric focusing, as they are too hydrophobic to stay in solution (Santoni *et al.*, 1999; 2000).

A combination of SOSUI (Hirokawa *et al.*, 1998; Mitaku *et al.*, 1999) and PSORT (Nakai and Kanehisa, 1991) algorithms were used to predict inner membrane proteins, and a combination of PSORT, SIGNALP (Nielsen *et al.*, 1997; Nielsen and Krogh, 1998), COGNITOR (Tatusov *et al.*,

Table 2. Corrected co-ordinates, predicted pI and molecular weight of the mature proteins.

CC	Co-ordinates ^a	pI ^b	Mass (kDa) ^b
0163	171 649–172 917	6.44	43 737
0210	225 121–227 526	5.61	85 580
0288	301 048–304 293 (c)	4.96	107 415
0394	415 406–416 755 (c)	8.14	43 881
2149	2 353 899–2356 214	5.15	80 738
2924	3 145 598–3 148 321 (c)	5.2	95 322
3007	3 226 382–3 227 866 (c)	5.19	45 996
3494	3 736 745–3 738 094 (c)	5.47	40 531

a. (c) indicates complementary strand.

b. pI and molecular weight were calculated using Compute pI/Mw (http://usexpassy.org/tools/pi_tool-ref.html) (Bjellqvist *et al.*, 1993; 1994; Wilkins *et al.*, 1998).

2000) and PSI-BLAST (Altschul *et al.*, 1997) was used to predict outer membrane proteins (Phadke *et al.*, 2001). Using these algorithms, 10 of the proteins identified on the stalk gel were predicted to be localized to the periplasm, six are predicted to be localized in the outer membrane, and one is predicted to be in the inner membrane. Twenty of the identified proteins had prediction values too low to be used to predict their localization.

Based on their predicted function, some of the proteins identified, such as TonB-dependent receptors, should be localized to the outer membrane. This led us to re-examine the assignment of the initiation codon in the databases. We used the CODONPREFERENCE program (Genetics Computer Group, Madison, WI, USA) to examine the GC bias in the area of the proposed start site of each of these proteins and found that, in some cases, the GC bias increased a significant distance after the assigned initiation codon, suggesting that the initiation site had been incorrectly assigned. In those cases, we then used SIGNALP to determine whether the most likely initiation site based on the GC bias analysis would result in the prediction of a signal peptide. Indeed, in eight of the 20 cases, the new initiation codon resulted in the strong prediction of a signal peptide by SIGNALP and a strong subcellular localization by PSORT. Furthermore, in the case of CC0163, CC1750, CC2925 and CC3392, the newly predicted N-terminal tryptic peptide was observed (Karty *et al.*, 2002a). In seven of the 20 cases, the original initiation codon resulted in a strong prediction of a signal peptide, but the resulting protein did not have strong prediction for subcellular localization. In one case, no new initiation codon was found, and the original protein sequence did not result in the strong prediction of a signal peptide, transmembrane domains or subcellular localization. The four remaining proteins are flagellar proteins, which are secreted using a type III secretion system. Table 2 indicates the new co-ordinates for each protein that had an incorrectly assigned initiation codon, as well

as the pI and molecular weight of the corrected mature protein, which were calculated using COMPUTE pI/Mw (http://usexpassy.org/tools/pi_tool-ref.html) (Bjellqvist *et al.*, 1993; 1994; Wilkins *et al.*, 1998).

In summary, 12 of the 39 stalk gel proteins identified are predicted to be outer membrane proteins, 14 are predicted to be periplasmic proteins, one is predicted to be an inner membrane protein, four are secreted by type III secretion systems, seven have signal peptides but do not have a strong prediction for subcellular localization, and one has no strong signal peptide prediction or subcellular localization. It is also particularly interesting that no cytoplasmic proteins were identified on the stalk 2D gel. This finding supports the hypothesis that the stalk has very little, if any, cytoplasmic protein. The mutant strain used for stalk purification sheds stalks as the result of aberrant cell division. However, to rule out the possibility that shed stalks leak cytoplasmic proteins upon shedding, we compared the proteins found in the supernatant media of wild-type and stalk-shedding mutant cultures and found no difference (data not shown). In addition, experiments have shown that the cytoplasmic proteins isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, β -galactosidase and green fluorescent protein are unable to diffuse from the cytoplasm of the cell to the stalk, whereas small molecules, such as fluorescein, are able to diffuse (J. Wagner and Y. V. Brun, unpublished results), further supporting the prediction that the stalk has a minimal, if any, cytoplasmic protein content.

The *Caulobacter* genome has been analysed (Phadke *et al.*, 2001) using the cluster of orthologous groups (COG) classification system (Tatusov *et al.*, 1997; 2001), which is based on position-specific score matrices of sequence similarity that predict the functional classification of each protein. The proteins identified from the stalk fraction fall into seven COG groups, which help to predict the likely function of the proteins (Table 3). The most abundant COG groups found among the stalk protein identifications were cell motility and secretion proteins (N) and inorganic ion transport and metabolism proteins (P). The presence of flagellar proteins is simply a result of the fact that they co-purify with stalks. Identification of 14 pro-

Table 3. Cluster of orthologous groups classification of proteins found in the stalk fraction of *Caulobacter crescentus*.

COG group	Function	No. identified on gel
N	Cell motility and secretion	6
C	Energy production and conversion	1
P	Inorganic ion transport and metabolism	8
R	General function prediction only	2
M	Cell envelope biogenesis, outer membrane	1
S	Function unknown	2
T	Signal transduction mechanisms	1
None	None	18

teins in the N and P groups supports the hypothesis that the stalk is involved in nutrient uptake. Thirty-eight per cent of the identified stalk proteins were hypothetical proteins. This is not surprising, as the *Caulobacter* genome was found to contain 721 proteins that were similar to proteins of unknown function (19.2%) and 1012 hypothetical proteins (26.9%), accounting for nearly 45% of its genome (Nierman *et al.*, 2001).

The identification of TonB-dependent receptors in the stalk of *Caulobacter* suggests that the stalk plays a role in sensing nutrients and importing them into the cell. The *Caulobacter* genome contains 65 genes that encode predicted TonB-dependent receptors, possibly compensating for the lack of OmpF-type outer membrane porins, which allow passive diffusion of nutrients (Nierman *et al.*, 2001). This is nearly twice as many TonB-dependent receptors as any other characterized genome; the *Pseudomonas aeruginosa* genome contains 34 (Stover *et al.*, 2000; Nierman *et al.*, 2001), and no other sequenced bacterial genome contains more than 13 (<http://www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG1629>). TonB-dependent receptors facilitate the import of molecules into the periplasm. The receptors are found in the outer membrane of cells and are coupled to TonB, which transduces energy from the ExbB–ExbD complex in the inner membrane to the receptor. It is thought that most of these receptors function as ligand-gated channels, opening in the presence of ligand to allow access of the nutrient to the periplasmic space (Moeck and Coulton, 1998; Postle, 1999). Some of these receptors are exploited as binding sites by bacteriophage (Luria and Delbruck, 1943; Matsushiro, 1963) and colicins (Lazdunski *et al.*, 1998), whereas some are transport proteins involved in high-affinity transport for iron (Frost and Rosenberg, 1975; Hantke and Braun, 1975; Williams, 1979), vitamin B12 (Bassford *et al.*, 1976) and other molecules that are too large to diffuse freely through general porins. It seems unlikely that *Caulobacter* contains such a high number of proteins dedicated to iron uptake. It has been postulated that a large number of TonB-dependent receptors in *Caulobacter* are likely to be involved in the transport of a number of other nutrients and macromolecules into the cell, because many of *Caulobacter's* TonB-dependent receptors do not fall into the classical operon structure characteristic of iron-metabolizing outer membrane proteins (Phadke *et al.*, 2001). Others have reported the characterization of proteins that have sequence similarity to TonB-dependent receptors; these proteins are outer membrane proteins involved in the utilization of starch, maltooligosaccharides and chondroitin sulphate in *Bacteriodes thetaiotamicon* (Cheng *et al.*, 1995; Reeves *et al.*, 1996).

Two OmpA-related proteins identified on the stalk gel could also be involved in nutrient uptake. One of them,

CC0925, is similar to 276 amino acids of the Oar precursor protein in *Myxococcus xanthus* (Martinez-Canamero *et al.*, 1993). This protein is a 115 kDa membrane protein required for development in *Myxococcus* that shares many short segments similar to OmpA. The other OmpA-related protein, CC3494, has two conserved domains – an OmpA domain and an opacity domain. Opacity proteins are inner membrane proteins related to porins and are used for adhesion to hosts in some organisms (Knepper *et al.*, 1997).

Another outer membrane protein identified on the stalk gel is TolC, a large membrane channel with a *trans*-periplasmic tunnel domain (Koronakis *et al.*, 2000). This unique structure allows for the export of molecules without requiring the formation of a periplasmic intermediate (Andersen *et al.*, 2000; Koronakis *et al.*, 2000). TolC and its homologues are involved in type I secretion and act as outlet ducts for large molecules, including toxins and antibiotics, in some bacteria (Sharff *et al.*, 2001). This suggests that the stalk may be involved in the secretion of various types of molecules. Members of the TolC family are also known to allow colicins to enter the cell (Sharff *et al.*, 2001) and may also be exploited by various bacteriophage as a receptor to gain entry (German and Misra, 2001).

Phosphate is the limiting nutrient in the environments in which *Caulobacter* is found (Paerl, 1982). The identification of two alkaline phosphatases in the stalk is consistent with one of the hypothesized functions of the stalk: phosphate uptake. Stalks elongate when phosphate is limiting, increasing the surface area available for phosphate uptake (Felzenberg *et al.*, 1996). The presence of alkaline phosphatases would allow the uptake of organic phosphate by the stalk. The strain used in this study, YB2811, contains a *pstS::Tn5* mutation resulting in the induction of the Pho regulon. Thus, all proteins that are induced by the Pho regulon should be present in protein preparations, with the exception of PstS, which is absent because of mutation, and other members of the PstSCAB complex, which may be destabilized in the absence of PstS.

One stalk protein, the synthesis of which might be regulated by the Pho regulon is GlpQ, a glycerophosphoryldiester phosphodiesterase, which is part of the glycerol utilization regulon in *Bacillus subtilis* and *Escherichia coli*. GlpQ is involved in the hydrolysis of deacylated phospholipids to glycerol-3-phosphate and has been shown to be highly upregulated in phosphate-starved *B. subtilis* (Antelmann *et al.*, 2000). It has been suggested that the glycerol-3-phosphate produced by GlpQ is a substrate for alkaline phosphatases (Antelmann *et al.*, 2000). In fact, *Caulobacter* is able to use glycerol-3-phosphate as a sole source of carbon and phosphate (B. Gold and Y. V. Brun, unpublished results). The presence of both GlpQ and

alkaline phosphatase in the stalk indicates that stalks should be able to produce carbon and phosphate from deacylated phospholipids. The identification of 3-phytase is also interesting, as this enzyme belongs to a group of phosphatases that is capable of hydrolysing phytate [myo-inositol (1,2,3,4,5,6)-hexakisphosphate] to a series of lower phosphate esters of myo-inositol and phosphate. Phytate can store phosphate and is capable of chelating important minerals such as calcium, magnesium, zinc and iron (Greiner *et al.*, 1997; Kerovuo *et al.*, 2000).

Several of the proteins identified in this study have been identified previously as proteins that are differentially expressed throughout the cell cycle (Grunenfelder *et al.*, 2001). Specifically, two hypothetical proteins, CC3392 and CC2119, identified on the stalk gel are upregulated early in the cell cycle before the swarmer-to-stalked transition. In addition, the transcription of two TonB-dependent receptors identified on the stalk gel is also cell cycle regulated. CC3146 is upregulated in G2, whereas CC1750 is upregulated in G1 (Grunenfelder *et al.*, 2001).

In summary, the presence of a large number of transporters, phosphatases and other outer membrane proteins in the stalk supports the hypothesis that the stalk plays a role in import and perhaps export of molecules. Stalk elongation, by increasing the surface area of the cell, increases the number of transport proteins and, thus, the uptake of nutrients. Mutational analysis of hypothetical proteins, as well as the various other proteins identified in this study, may lead to better insight into the function of the stalk. The presence of TonB-dependent receptors in the stalk suggests that nutrients can be transported from the environment into the periplasm of the stalk. If the stalk is involved in nutrient uptake, TonB, ExbB and ExbD should be required to transduce energy to the TonB-dependent receptors, but these proteins have not yet been identified on the stalk gel. This could be because these proteins are associated with the inner membrane (Skare *et al.*, 1993) and do not focus well during isoelectric focusing. If nutrients are transported into the core of the stalk, we would expect to see ABC transporters in the inner membrane of the stalk as well, but these proteins are probably absent from the 2D gels because of their hydrophobic nature. If ABC transporters, ExbB and ExbD are present in the stalk, ATP would be required to provide energy and should be present in the core of the stalk. Small molecules such as fluorescein can diffuse from the cell cytoplasm into the stalk, suggesting that the same is possible for ATP (J. Wagner and Y. V. Brun, unpublished data). Further SDS-PAGE analysis of the stalk proteome in comparison with the whole proteome will undoubtedly yield more information about the stalk, as inner membrane proteins present in one or both can be identified. Such identifications should determine whether nutrients are transported via ABC transporters to the core of the

stalk and subsequently to the body of the cell or, alternatively, are transported via the stalk periplasm to the periplasm of the body of the cell and subsequently across the inner membrane.

Experimental procedures

Bacterial strain and growth conditions

We obtained a stalk-shedding strain of *C. crescentus*, NY111d1 (Poindexter, 1978). A *pstS::miniTn5* mutation (*pstS::miniTn5lacZ1100*, YB767) (Gonin *et al.*, 2000) was introduced into NY111d1 by transduction with ϕ CR30 (Ely and Johnson, 1977), and transductants were selected on PYE (Poindexter and Bazire, 1964) with $20\ \mu\text{g ml}^{-1}$ nalidixic acid and $5\ \mu\text{g ml}^{-1}$ kanamycin. The resulting strain, YB2811, yielded a greater quantity of stalk material with little contaminating cellular material. To isolate stalks and cell bodies, cells were grown at 30°C for 3–5 days in Hutner base–imidazole–buffered–glucose–glutamate (HIGG) minimal media (Poindexter, 1978) containing $120\ \mu\text{M}$ phosphate. For all other purposes, cells were grown at 30°C in PYE liquid medium.

Stalk protein preparation

Stalks were collected as described previously (Poindexter and Hagenzieker, 1982) with modifications. Cultures of cells were centrifuged at $17\ 000\ g$ for 25 min at 4°C to pellet cells. The supernatant culture was recentrifuged at $47\ 800\ g$ for 30 min at 4°C to pellet stalks. Pellets were resuspended in rehydration solution [8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS; Sigma)]. Serine and cysteine protease inhibitor cocktail (Roche, Complete, mini, EDTA free) was prepared as directed and added to a final concentration of 0.5%. The stalks were lysed by freezing in a dry ice–EtOH bath and thawing at room temperature. This was repeated four times to ensure the lysis of all stalks. The proteins were precipitated as described previously with modifications (McCann *et al.*, 1998). Briefly, 50% (v/v) trichloroacetic acid (TCA) was added to a final concentration of 10%, and the protein was incubated on ice for at least 30 min. The proteins were pelleted by centrifuging at $17\ 000\ g$ for 20 min at 4°C. The pellet was washed with 35 ml of 10% TCA, 35 ml of 5% TCA and 35 ml of acetone, respectively, and centrifuged as described previously between each wash. After the acetone wash, the pellets were allowed to dry for 5–10 min until they appeared flaky and waxy but not overly dry. The pellets were resuspended in rehydration solution. Modified Lowry assays (Dulley and Grieve, 1975; Markwell *et al.*, 1978) were performed on all protein preparations in order to determine protein concentration.

Soluble stalk proteins were separated from membrane-bound stalk proteins. Stalks were collected as described above, resuspended in 10 mM Tris-HCl, pH 7.4, containing 0.2 mM EDTA and protease inhibitor, lysed by the freeze–thaw method and ultracentrifuged at $154\ 000\ g$ in a Beckman SW41-Ti rotor for 2 h at 10°C to pellet membranes. The resulting supernatant was precipitated with TCA and resuspended in rehydration solution as described above.

Cell body protein preparation

The cell pellet from the stalk preparation was treated to separate soluble and membrane proteins. First, the cell pellet was resuspended in 10 mM Tris-HCl, pH 7.4, with protease inhibitor, and the cell suspension was passed through a French press four times to lyse cells. The unlysed cells were removed from the suspension by centrifuging at 1900 *g* for 10 min at 4°C. The resulting supernatant fraction was ultracentrifuged. The pellet, containing the membrane proteins, was resuspended in rehydration solution, precipitated with TCA and resuspended in rehydration solution as described above, yielding the whole-cell membrane fraction. The supernatant after ultracentrifugation was precipitated with TCA and resuspended in rehydration solution, yielding the whole-cell soluble fraction.

Microscopy

DNA staining of cell bodies and stalks was performed as described previously (Porter and Feig, 1980) with modifications. Briefly, stalks or cell bodies were incubated in 5 µg ml⁻¹ DAPI for 10 min at room temperature and washed with M2 salts (Johnson and Ely, 1977) five times, centrifuging in a bench-top centrifuge at high speed between each wash. Stained stalks and cell bodies were resuspended in Slow Fade A antifade reagent (Molecular Probes). Flagella were visualized in the stalk preparation using flagella stain (Carr Scarborough Microbiologicals). Cells, stalks and flagella were visualized using a Nikon Eclipse E800 light microscope with a 100× Plan Apo oil objective. Images were captured using a Princeton Instruments cooled CCD camera model 1317 and the METAMORPH imaging software package version 4.5 (Universal Imaging).

First-dimension isoelectric focusing (IEF)

Protein samples were adjusted to 400 µg ml⁻¹ with rehydration solution (100 µg of protein was loaded on each strip). Dithiothreitol (DTT; 2.8 mg ml⁻¹), pH 3–10 non-linear (NL) IPG buffer (0.5% final concentration) (Amersham Pharmacia Biotech) and a trace amount of bromophenol blue dye were added to each sample before loading. Samples were prepared for IEF on the IPGphor apparatus (Amersham Pharmacia Biotech) as directed by the manufacturer, and were focused on 13 cm 3–10 NL Immobiline drystrips (Amersham Pharmacia Biotech). Strips were rehydrated with sample overnight (10 h) and focused using a series of voltage increases: 500 Vh at 500 V, 1000 Vh at 1000 V, 25 000 Vh at 5000 V and 50 000 Vh at 8000 V.

Second dimension and gel staining

IEF strips were equilibrated in equilibration buffer [50 mM Tris, pH 8.8, 30% (v/v) glycerol, 6 M urea, 2% SDS, trace of bromophenol blue] containing 10 mg ml⁻¹ DTT for 20 min with slight agitation and transferred to equilibration buffer containing 25 mg ml⁻¹ iodoacetamide (Fluka) for 20 min with slight agitation. The strips were rinsed briefly with water. The strips

were placed on top of 12% SDS–polyacrylamide second-dimension gels with a small (2–3 mm) layer of 0.5% low melt agarose (SeaPlaque; FMC BioProducts) containing bromophenol blue. A sample application piece (Amersham Pharmacia Biotech) with a prestained protein ladder (Amersham LifeScience) was added to the basic end of the gel. Another layer of 0.5% agarose was overlaid to cover the strips. Gels were run at 35 mA per gel, constant current, at 10°C for 4 h in running buffer [25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS]. Gels were stained overnight in Colloidal blue stain (Novex) and destained in water for at least 8 h. The gels were scanned using a UMAX Powerlook III scanner with MAGICSCAN version 4.4 software (UMAX), and images were compared using IMAGEMASTER 2D gel analysis software version 4.01 (Amersham Pharmacia Biotech).

Protein preparation, mass spectrometry and data analysis

Spot analysis was performed as described previously (Karty *et al.*, 2002b). Briefly, spots were cut from the second-dimension gels using cut-off P200 pipette tips. The spots were transferred to Eppendorf tubes or 96-well microtitre plates for processing. Gel spots were first destained by adding 100 µl of 100 mM NH₄HCO₃ in 50% acetonitrile and washed with distilled water. The spots were incubated for 5 min in acetonitrile, the acetonitrile was discarded, and the bands were centrifuged under vacuum in a Speedvac (Savant). The spots were then digested with 16.7 µg ml⁻¹ trypsin (Sigma). Peptides were eluted in 0.1% (v/v) trifluoroacetic acid and washes of acetonitrile. The resulting extract was dried under vacuum, and the protein from each spot was rehydrated with distilled water.

Peptides were prepared as described previously (Karty *et al.*, 2002b) with α -cyano-4-hydroxycinnamic acid (α -cyano; Sigma). Positive ion mass spectra were recorded using a Bruker Reflex III MALDI-Reflectron TOF mass spectrometer with a 1 GHz digitizer rate. All mass spectra were internally calibrated with three or four trypsin autolysis peaks (805.4 Da, 1153.6 Da, 2163.1 Da and 2273.2 Da) using a linear fit. Data were analysed and confidence values for the identifications were established as described previously (Karty *et al.*, 2002b). Briefly, confidence levels were assigned using Monte Carlo simulation of peptide mass mapping experiments. Theoretical mass spectra were generated using predicted *Caulobacter* tryptic peptides. These mass spectra were analysed using the same algorithm that was used for analysing experimental data. Over 5.5 million theoretical mass spectra were analysed to generate the confidence limits.

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