TonB-dependent maltose transport by *Caulobacter crescentus*

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We have shown previously that *Caulobacter crescentus* grows on maltodextrins which are actively transported across the outer membrane by the MalA protein. Evidence for energy-coupled transport was obtained by deletion of the *exbB exbD* genes which abolished transport. However, removal of the TonB protein, which together with the ExbB ExbD proteins is predicted to form an energy-coupling device between the cytoplasmic membrane and the outer membrane, left transport unaffected. Here we identify an additional *tonB* gene encoded by the *cc2334a* ORF, which when deleted abolished maltose transport. MalA contains a TonB box that reads EEVVIT and is predicted to interact with TonB. Replacement of valine number 15 in the TonB box by proline abolished maltose transport. Maltose was transported across the cytoplasmic membrane by the MalY protein (CC2283). Maltose transport was induced by maltose and repressed by the Mall protein (CC2284). In addition to MalA, MalY and Mall, the *mal* locus encodes two predicted cytoplasmic α -amylases (CC2285 and CC2286) and a periplasmic glucoamylase (CC2282). The TonB dependence together with the previously described ExbB ExbD dependence demonstrates energy-coupled maltose transport across the outer membrane. MalY is involved in maltose transport across the cytoplasmic membrane by a presumably ion-coupled mechanism.

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INTRODUCTION

In the genome of *Caulobacter crescentus*, 67 TonB-dependent outer-membrane proteins (OMPs) are predicted which consist of a β -barrel formed by 22 anti-parallel β -strands and a globular domain called cork, plug or hatch (Neugebauer *et al.*, 2005; Nierman *et al.*, 2001). In the crystal structures of outer-membrane transporters of *Escherichia coli* and *Pseudomonas aeruginosa*, the plug domain completely closes the pore formed by the β -barrel (Ferguson & Deisenhofer, 2004; Schalk *et al.*, 2004; Wiener, 2005). In *E. coli* and other *Gammaproteobacteria*, TonB-dependent OMPs transport ferric ions incorporated into siderophores and haem, and vitamin B₁₂ across the outer membrane (Andrews *et al.* 2003; Braun *et al.*, 1998; Postle & Kadner, 2003; Braun & Mahren, 2007). This transport is energized by the electrochemical potential of the cytoplasmic membrane (White *et al.*, 1973). Three proteins, TonB, ExbB and ExbD, are involved in transmitting energy from the cytoplasmic membrane to the outer-membrane transporters. TonB, ExbB and ExbD are inserted into the cytoplasmic membrane and extend into the periplasm. TonB interacts with the outer-membrane transporters, as

shown by genetic (Heller *et al.*, 1988; Schöffler & Braun, 1989), biochemical (Cadieux & Kadner, 1999; Ogierman & Braun, 2003) and crystallographic methods (Pawelek *et al.*, 2006; Shultis *et al.*, 2006). However, it is the release of substrates from their tight binding to the transporters and movement of the plug within the β -barrel of the transporters that requires an input of energy. Under the assumption that the 67 TonB-dependent proteins transport not only iron and vitamin B₁₂ but also other substrates, we studied the uptake of potential nutrients into *C. crescentus* which might be available in nutrient-poor inshore waters in which *C. crescentus* thrives. We selected starch, the third most abundant carbon source in nature. We have shown that *C. crescentus* CB15, the strain whose genome has been sequenced (Nierman *et al.* 2001), does not grow on starch since it lacks an extracellular amylase, but it does grow on starch degradation products (maltodextrins) as sole carbon sources (Neugebauer *et al.*, 2005). Uptake of maltodextrins across the outer membrane was mediated by the MalA protein, which is one of the 67 predicted TonB-dependent proteins in the bacterium. The initial transport rate of a *malA* insertion mutant was 1% of the wild-type rate. However, growth of the *malA* mutant was stimulated on nutrient agar with maltose, maltotriose and maltotetraose as sole carbon sources, but not with maltopentaose. The slow uptake of the smaller maltodextrins was sufficient to support growth of the *malA* mutant.

Abbreviation: OMP, outer-membrane protein.

A table of primers used for the construction of SL3508 is available with the online version of this paper.

Although *C. crescentus* belongs to the *Alphaproteobacteria*, analysis of its genome has identified genes which are 26, 40 and 46 % identical to the *E. coli* *tonB*, *exbB* and *exbD* genes, respectively. Inactivation of the *exbBD* genes impairs MalA-mediated maltose transport, but inactivation of the predicted *tonB* gene does not affect maltose, maltotriose and maltotetraose transport (Neugebauer *et al.*, 2005). This finding was unexpected since in hitherto studied *Gammaproteobacteria*, ExbB and ExbD function only together with TonB. We have predicted the presence of a second TonB protein which was identified in the present study and have shown it to be essential for maltose transport.

Synthesis of MalA is induced by growing cells on maltodextrins which strongly increases the maltodextrin transport rate. A gene with sequence homology to *lacI*-type repressor genes is present in the *mal* locus of *C. crescentus* which we designated *mall*. Here we show that deletion of *mall* increases maltose transport to the level of the maltodextrin-induced rate and complementation of the mutant by wild-type *mall* represses maltose transport. In addition, we assigned a transport function to the *malY* gene in the *mal* operon which encodes a protein homologous to the LacY-type transport proteins in the cytoplasmic membrane.

METHODS

Bacterial strains, plasmids and media. The *C. crescentus* and *E. coli* strains, and the plasmids used in this study are listed in Table 1. All *C. crescentus* strains are derived from the sequenced CB15 strain. Cells of *C. crescentus* were grown at 30 °C in PYE rich medium or in M2 salt minimal medium (Ely, 1991) supplemented with 0.3 % glucose or 0.3 % maltose. *C. crescentus* did not grow on tryptone/yeast extract medium (Difco), which was used to test contamination of cultures of slow-growing *C. crescentus* cells by fast-growing bacteria. Cells of *E. coli* were grown at 37 °C on tryptone/yeast extract medium. Antibiotics were used at the following concentrations ($\mu\text{g ml}^{-1}$) for *C. crescentus* (and in parentheses for *E. coli*): ampicillin, 50 on plates, 7.5 (50) in liquid culture; chloramphenicol, 2 (25); tetracycline, 2 on plates, 1 (12) in liquid culture; gentamicin, 50 on plates, 25 (50) in liquid culture; spectinomycin, 50 on plates, 25 (50) in liquid culture; streptomycin, 5 (30); kanamycin, (30); and nalidixic acid, (20). *C. crescentus* JS1003 is resistant to nalidixic acid, ampicillin and kanamycin. Growth promotion was tested on M2 salt agar plates on which 10^8 bacteria were seeded in 3 ml M2 salt soft agar. The nutrients to be tested (10 μl) were placed on sterile filter paper disks (6 mm in diameter). Bacterial growth was scored around the disks after 16 and 24 h incubation at 30 °C. Growth promotion was also determined on nutrient agar plates and in liquid culture with 0.1 and 0.3 % maltodextrins as sole carbon source. Maltose was purchased from Sigma-Aldrich Chemie and its purity was verified by HPLC analysis.

Recombinant DNA techniques. Isolation of plasmids, use of restriction enzymes, ligation, agarose gel electrophoresis, transformation, hybridization and Southern blotting followed standard methods (Sambrook *et al.*, 1989). PCR of *E. coli* DNA was started with 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 2 min annealing at 54 °C, 3 min elongation at 72 °C, and then 10 min at 72 °C. The GC-rich DNA was amplified similarly, except that the annealing

temperature started at 64 °C and was lowered in each cycle by 0.5 °C. The primers used for cloning by PCR are provided in Table S1 (available with the online version of this paper).

Cloning of genes and mutated genes. The *tonB1*, *malY* and *mall* genes were amplified by PCR with appropriately designed primers from the *C. crescentus* chromosome and were then cloned into pBBR1-MCS2. Plasmid pBBtonB1 contains a 740 bp *SpeI*-*EcoRI* *tonB1* fragment, plasmid pBBmalY a 1.6-kb *SpeI*-*EcoRI* *malY* fragment, pBBmall a 1.1 kb *EcoRI*-*SpeI* *mall* fragment, plasmid pBBmalA a 3 kb *EcoRV*-*SpeI* *malA* fragment, and plasmid pBB2287 a 3 kb *EcoRV*-*SpeI* *malA* fragment with a TonB box mutation in pBBR1-MCS2. pSL2334a contains an Ω insertion in *cc2334a* (ΔtonB1) cloned with *SpeI* in pNPTS138Tet. The constructed plasmids were transferred by conjugation into mutants lacking the plasmid-encoded genes on the chromosome.

Construction of chromosomal *C. crescentus* mutants. The *tonB1*, *malY* and *mall* target genes were mutated by insertion of an Ω resistance cassette (Alexeyev *et al.*, 1995; Prentki & Krisch, 1984). The flanking genes of the target gene were amplified by PCR, ligated and cloned into the pDrive vector. The 2 kb Ω Spc^R - Str^R fragment of pHP45 Ω was cloned between the two flanking genes, and the DNA was amplified by PCR and cloned into pNPTS138Tet, which was derived from pNPTS138 by insertion of the 1.4 kb *MluI* Tet^R fragment of pBSL204. The following chromosomal Ω insertion mutants were cloned with *SpeI* into pNPTS138Tet: pSL2334a ΔtonB1 Ω in *cc2334a*, pSL2283 ΔmalY Ω in *cc2283*, pSL2284 Δmall Ω in *cc2284*. These plasmids were introduced into *E. coli* S17-1 λpir by transformation and transferred by conjugation on PYE nutrient plates into *C. crescentus* UJ2602. Growth of the donor strain was inhibited by nalidixic acid, and integration of the plasmid into the recipient genome was selected with streptomycin and spectinomycin. A second recombination deleted the target gene along with *sacB* and the Tet^R gene by growing cells on 3 % sucrose. The deletion of the target gene was verified by Southern blotting and DNA sequencing.

[¹⁴C]Maltose transport. Cells were grown in M2 medium supplemented with 0.3 % maltose as carbon source, harvested by centrifugation and washed once in M2 medium. The pellet was resuspended in 1.3 ml M2 medium containing 0.3 % glucose, and the optical density at 578 nm was adjusted to 0.5 (5×10^8 cells ml^{-1}). [¹⁴C]Maltose (specific activity 25.1 GBq mmol^{-1} ; Amersham Biosciences) was added to a final concentration of 0.2 μM . Samples of 0.2 ml were withdrawn after 0.2, 1, 2 and 3 min, collected on cellulose nitrate filters, washed twice with 5 ml M2 salt medium and dried. The radioactivity was determined in a liquid scintillation counter.

Sequence alignments. The *C. crescentus* genome was analysed and compared with other genomes using the Comprehensive Microbial Resource (<http://cmr.jcvi.org/tigr-scripts/CMR/CMrHomePage.cgi>), and the programs FASTA (www.ebi.ac.uk/fasta33/proteomes.html) and WU-BLAST2 (www.ebi.ac.uk/blast2).

RESULTS

Identification of the *tonB* gene involved in maltose transport

ORF *cc2327* in the sequenced genome of *C. crescentus* had previously been assigned as a putative *tonB* gene (Nierman *et al.*, 2001). The similar size of the encoded protein (240 residues) to the *E. coli* TonB protein (239 residues), the

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and phenotype*	Reference or source
<i>C. crescentus</i>		
CB15	ATCC 19089, wild-type strain	Jenal & Fuchs (1998)
NA1000	<i>syn1000</i> , synchronizable derivative of <i>C. crescentus</i> CB15	Evinger & Agabian (1977)
JS1003	NA1000 <i>rsaA</i> ::KSac Kan ^R cassette; Amp ^R	Edwards & Smith (1991)
HB2003	JS1003 <i>malA</i> ::Ω cassette; Spc ^R Str ^R	Neugebauer <i>et al.</i> (2005)
UJ2602	CB15 <i>drsaA</i>	U. Jenal, Biozentrum Basel
SL2283	UJ2602 <i>malY</i> ::Ω cassette; Spc ^R Str ^R	This study
SL2284	UJ2602 <i>malI</i> ::Ω cassette; Spc ^R Str ^R	This study
SL2334a	UJ2602 <i>tonB1</i> ::Ω cassette; Spc ^R Str ^R	This study
S17-1 λ pir	<i>hsdR</i> ⁻ <i>hsdM</i> ⁺ RP4-2-Tc::Mu Kan::Tn7 (Tmp ^R Str ^R) Tra ⁺ λpir	Alexeyev <i>et al.</i> (1995)
Plasmids		
pHP45Ω	Ω cassette (Spc ^R Str ^R) Amp ^R	Prentki & Krisch (1984)
pBSL204	R6K <i>oriV</i> RP4 <i>oriT</i> ; Amp ^R Tet ^R	Alexeyev <i>et al.</i> (1995)
pNPTS138	Km ^R derivative of pLITMUS with <i>sacB</i> and <i>oriT</i>	Jenal & Fuchs (1998)
pNPTS138Tet	pNPTS138; Tet ^R Kan ^R	Neugebauer <i>et al.</i> (2005)
pBBR1-MCS2	Broad-host-range cloning vector; Kan ^R	U. Jenal, Biozentrum Basel
pDRIVE	PCR Cloning vector; Amp ^R	Qiagen
pSL2283	pNPTS138Tet Δ <i>malY</i> <i>cc2282</i> Ω <i>cc2284</i> ; Kan ^R Tet ^R Spc ^R Str ^R	This study
pSL2284	pNPTS138Tet Δ <i>malI</i> <i>cc2283</i> Ω <i>cc2285</i> ; Kan ^R Tet ^R Spc ^R Str ^R	This study
pSL2334a	pNPTS138Tet Δ <i>tonB1</i> <i>cc2333</i> Ω <i>cc2335</i> ; Kan ^R Tet ^R Spc ^R Str ^R	This study
pBB2287	pBBR1-MCS2 <i>ccmAla tonB</i> -box mutation; Kan ^R	This study
pBBmalA	pBBR1-MCS2 <i>cc2287</i> ; Kan ^R	This study
pBBmalY	pBBR1-MCS2 <i>cc2283</i> ; Kan ^R	This study
pBBmalI	pBBR1-MCS2 <i>cc2284</i> ; Kan ^R	This study
pBBtonB1	pBBR1-MCS2 <i>cc2334a</i> ; Kan ^R	This study

*Amp, Ampicillin; Kan, kanamycin; Spc, spectinomycin; Str, streptomycin; Tet, tetracycline; Tmp, trimethoprim.

26 % sequence identity with the *E. coli* TonB protein, and two features characteristic of *E. coli* TonB – a hydrophobic N-proximal sequence that could span the cytoplasmic membrane and the accumulation of proline residues in the central segment – supported the assignment (Fig. 1). However, inactivation of this gene did not affect mal-

todextrin transport (Neugebauer *et al.*, 2005). We therefore re-examined the genome and identified an additional gene homologous to *tonB* that mapped adjacent to and was transcribed in the same direction as *exbB exbD* (Fig. 2a). The degree of amino acid sequence identity of the predicted protein to the *E. coli* TonB protein (Fig. 1) is

Fig. 1. Comparison of the amino acid sequences of the *C. crescentus* and the *E. coli* TonB proteins. CC2334 has been renamed CC2334a (TonB1) and CC2327 is proposed to be TonB2. Dark grey shading, identical residues in all three proteins; light grey shading, identical residues in two proteins.

Fig. 2. (a) Arrangement of the *exbB*, *exbD* and *tonB1* genes in *C. crescentus*. ORFs with the prefix cc are predicted from the genome of *C. crescentus*, except for *cc2334a*, which encodes TonB1 and replaces the erroneously assigned *cc2334* (Nierman *et al.*, 2001). However, the possibility that this region is transcribed in both directions is not excluded. The arrows indicate the direction of transcription. (b) Genes of the *mal* locus assigned to ORFs in the genome of *C. crescentus*. We have assigned functions here and previously (Neugebauer *et al.*, 2005). The arrows indicate the direction of transcription.

low (23 %), but higher than that of the previously studied CC2327 TonB protein (19 %) (Fig. 1). The N-terminal fragment extending into the cytoplasm in *C. crescentus* TonB is longer (24 residues) than in *E. coli* TonB (11 residues). The hydrophobic, presumably transmembrane region contains a histidine residue which is conserved in the two *C. crescentus* TonB proteins; this residue (number 20) plays an essential role in *E. coli* TonB activity (Larsen *et al.*, 2007; Larsen & Postle 2001; Traub *et al.*, 1993). Since this newly identified *tonB* gene is at a locus that was previously assigned to another gene with opposite polarity (*cc2334*), we refer to it here as *cc2334a* (*tonB1*) (Fig. 2). The *exbB* *exbD* *tonB1* genes are densely packed. The distance between *exbB* and *exbD* is 5 bp, and between *exbD* and *tonB1* 10 bp, suggesting that they are cotranscribed from a promoter upstream of *exbB*.

Inactivation of *cc2334a* by an Ω insertion in *C. crescentus* SL2334a abolished maltose transport. Complementation of this mutant with the wild-type gene on plasmid pBBtonB1 restored transport (Fig. 3, Table 2). These results indicate that gene *cc2334a* is involved in maltose transport.

Another possible candidate for a *tonB* gene on the *C. crescentus* genome is *cc3508*, which is larger (401 residues), but 41 % identical to *cc2327* in the homologous region. A large TonB protein of probably 440 residues (there are other potential start codons) is found in *Rhizobium leguminosarum* and is involved in the uptake of haem and iron siderophores (Wexler *et al.*, 2001). Inactivation of

Fig. 3. $[^{14}\text{C}]$ Maltose transport into wild-type and *tonB* mutants. ♦, *C. crescentus* UJ2602; ■, *tonB1* mutant SL2334a; ▲, SL2334a complemented by plasmid-encoded wild-type *tonB1*. Cells were grown on maltose.

cc3508 and inactivation of both *cc3508* and *cc2327* together did not affect maltose transport (data not shown).

In *E. coli*, TonB binds to the TonB box of outer-membrane transporters (Pawelek *et al.* 2006; Shultis *et al.* 2006). The L8P replacement in the TonB box DTLVV of the BtuB transporter abolishes vitamin B₁₂ transport (Heller *et al.*, 1988), and the replacements I9P and V11D in the TonB box DTITV of the FhuA transporter abolish ferrichrome transport (Schöffler & Braun, 1989). To examine whether MalA interacts with TonB via its predicted TonB box EEVVIT similar to the interactions observed in *E. coli*, the valine at position 15 of MalA was replaced by proline (V15P), resulting in plasmid pBB2287. The chromosomal *malA* mutant HB2003 was transformed with pBB2287 *malA*(V15P). The transformant did not transport maltose (Table 2). Complementation with plasmid-borne wild-type

Table 2. Transport of $[^{14}\text{C}]$ maltose into *tonB1* and *mal* mutants

Cells were grown in 0.3 % maltose, unless marked uninduced in which case they were grown in 0.3 % glucose. The 1 min values in the linear range of the transport curves were used for comparison.

Strain	Transport (%)
IS1003 wild-type	100
SL2334a <i>tonB1</i>	3
SL2334a pBBtonB1	77
HB2003 pBB2287 <i>malA</i> (V15P)	3
HB2003 pBBmalA	63
SL2283 <i>malY</i>	1
SL2283 pBBmalY	89
SL2284 <i>malI</i> (uninduced)	102
SL2284 pBBmalI	1

malA restored maltose transport to 63 % of that of the wild-type strain (Table 2). These results suggest an interaction of MalA with TonB, similar to the interactions between energy-coupled transporters and TonB in *E. coli*.

MalY is required for maltose transport

The gene cluster encoding the maltose transport system (Fig. 2b) contains a gene (*cc2283*) homologous to sugar permease genes (Busch & Saier, 2002), such as the *lacY* permease gene of *E. coli*. Therefore, we named this gene *malY*. Inactivation of *malY* by Ω insertion in *C. crescentus* SL2283 abolished maltose transport, and complementation of this mutant with plasmid pBBmalY largely restored maltose transport (Table 2). We conclude that *malY* encodes a permease that catalyses transport of maltose across the cytoplasmic membrane.

MalI functions as a repressor of maltose gene transcription

We have previously shown that synthesis of the MalA protein and transport of maltose, maltotriose and maltotetraose are induced when *C. crescentus* is grown in a medium containing maltose as sole carbon source (Neugebauer *et al.*, 2005). Analysis of the *mal* locus identified a gene (*cc2284*) that encodes a protein with homology to the LacI repressor family of proteins. We therefore assumed that a repressor was involved in the regulation of maltose transport. We named this gene *malI* and inactivated it by Ω insertion. In the absence of maltose in the growth medium, the *C. crescentus* *malI* mutant SL2284 transported maltose at the same rate as the wild-type after induction with maltose (Table 2). Complementation of the *malI* mutant by wild-type *malI* encoded on the medium-copy plasmid pBBmalI repressed maltose transport (Table 2). The complemented mutant did not transport maltose even when maltose was added to the medium, and it did not grow on maltose, which indicates that repression by overproduced MalI could not be overcome by maltose. The overproduced repressor probably completely inhibited *mal* transport gene transcription, resulting in insufficient amounts of maltose in the cells to inactivate the repressor.

DISCUSSION

Our demonstration that *tonB1* is required for the transport of maltose into *C. crescentus* complements the former finding of the dependence of maltose transport on *exbB* *exbD*. In addition, inactivation of maltose transport by the MalA(V15P) mutation suggests binding of MalA via its TonB box to TonB1, as has been demonstrated for *E. coli* transporters and TonB. Together these data indicate that maltose transport by *C. crescentus* occurs by an energy-coupled mechanism, as used for transport of ferric siderophores into *E. coli* and nearly all other

Gammaproteobacteria. The findings reported here extend energy-coupled transport across the outer membrane to an organism that belongs to the *Alphaproteobacteria* and to maltodextrins. The previously examined *tonB* gene, designated *tonB* in the *C. crescentus* genome (Nierman *et al.*, 2001), has a rather high sequence homology to *tonB1* (proteins are 41 % identical). It is likely that it displays a TonB function in TonB-dependent uptake systems other than maltose transport and therefore we have named it *tonB2* (TonB2). More than one functional *tonB* gene was identified in a number of organisms; some of these *tonB* genes can replace each other, whereas others display specific functions (Blanvillain *et al.*, 2007; Chu *et al.*, 2007; Wandersman & Delepelaire, 2004; Wyckoff *et al.*, 2007; Zhao & Poole, 2000).

We have proposed that many Gram-negative bacteria take up a variety of scarce substrates other than ferric siderophores and vitamin B₁₂ by energy-coupled transport across the outer membrane (Neugebauer *et al.*, 2005). The 67 predicted outer-membrane transporters of *C. crescentus* probably transport a large variety of substrates under the nutrient-poor conditions in the freshwater lakes in which *C. crescentus* thrives. Tight binding to outer-membrane transporters extracts these substrates from the medium, but this also requires a structural, energy-consuming change in the transporters to release the substrates from their binding sites and movement of the plug within the β -barrel to enable diffusion of the substrates through the opened pore into the periplasm. In fact, MalA tightly binds maltose with a K_d of 0.2 μ M (Neugebauer *et al.*, 2005), which is 1000-fold lower than the K_d of the LamB porin of *E. coli* (Benz *et al.*, 1987).

Maltose uptake by *C. crescentus* is mechanistically different from the well-studied maltose uptake system in *E. coli*. In *C. crescentus*, maltodextrins are actively transported by MalA across the outer membrane. In *E. coli*, maltodextrins enter the periplasm by facilitated diffusion through the LamB protein. In *C. crescentus*, maltodextrin transport across the cytoplasmic membrane is mediated by a protein that is predicted to be energized by proton motive force. In *E. coli*, maltodextrin transport across the cytoplasmic membrane is mediated by an ABC transporter consisting of four proteins. In *C. crescentus*, transcription of the maltose transport genes is controlled by a repressor, whereas in *E. coli* it is controlled by an activator. Two maltose-cleaving α -amylases encoded in the *mal* loci occur in the cytoplasm and are common to both organisms, but their mode of action might differ. We did not find a function for the periplasmic MalS protein which is predicted to be a glucoamylase. Inactivation of *malS* by Ω insertion did not reduce the growth rate of *C. crescentus* on maltose, maltotriose and maltotetraose (data not shown). For maltodextrins up to this size, degradation by MalS to maltose is not required. It may degrade larger maltodextrins provided that they are transported into the periplasm by MalA for which uptake of maltodextrins up to maltohexaose has been shown (Neugebauer *et al.* 2005).

Recently, an explanation has been given for the long-known observation that *C. crescentus* forms much longer stalks in phosphate-limiting media (Wagner *et al.*, 2006). The stalks serve to collect phosphate, which then diffuses into the common periplasm of the stalk and the cell body where, in the cell body, the ABC transporter for phosphate resides. However, no phosphate-specific energy-coupled transporter has been identified in the outer membrane of the stalk, and the ExbB protein is not found in the cytoplasmic membrane of the stalk, but rather in the cytoplasmic membrane of the cell body (Wagner *et al.*, 2006). These results suggest that phosphate diffuses through the outer membrane of the stalk. Diffusion is favoured by the long and thin shape and the small volume of the stalk. This seems to be an adaptation to an environment where nutrient uptake is limited by diffusion, along with the adaptations of strong binding to outer-membrane transporters and subsequent energy-coupled transport across the outer membrane. In the experiments reported in this paper cells may have formed stalks. It is unlikely that maltose porins exist in the outer membrane of stalks since maltose transport in a *malA* mutant is only 1 % of the *malA* wild-type.

An energized outer-membrane transport for nickel ions has been characterized in *Helicobacter pylori* (Schauer *et al.*, 2007). Ni^{2+} uptake at pH 5 was strongly reduced in an *frpB4* mutant (*frpB4* encodes an OMP). Likewise Ni^{2+} uptake at pH 5 was strongly reduced in an *exbB exbD tonB* mutant. Transcription of *frpB4* and *exbB exbD tonB* was under the control of the Ni^{2+} -responsive NikR repressor (Davis *et al.*, 2006; Ernst *et al.* 2006).

Additional evidence for energy-coupled transport of nutrients other than ferric siderophores and vitamin B₁₂ across the outer membrane comes from the analysis of genomes which, for example, predict 107 paralogues of SusC, an OMP required for growth of *Bacteroides thetaiotaomicron* on maltodextrins. SusC is predicted to consist of a β -barrel with a plug inside (Reeves *et al.*, 1996), similar to the crystal structures of energy-coupled outer-membrane transporters of *E. coli* and *P. aeruginosa*. For *Xanthomonas campestris*, 48 TonB-dependent outer-membrane transporters are predicted, and it has been shown that for sucrose transport, the SuxA OMP is required (Blanvillain *et al.*, 2007). Synthesis of SuxA is induced by growing cells on sucrose. The assumed TonB dependence could not be verified because individual knockout of eight potential *tonB* genes did not affect sucrose transport; this might be explained by cross-complementation by one or more *tonB* genes.

The maltodextrin transport system is the only transport system in *C. crescentus* for which the outer- and inner-membrane transport proteins have been identified and for which its mode of action has been characterized to such an extent that energization via the proton motive force can reasonably be derived. We propose that this system is representative of many substrate transport systems and supports the prediction of 67 predicted outer-membrane transporters in *C. crescentus*.

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