Functional analysis of a *Campylobacter jejuni* alkaline phosphatase secreted via the Tat export machinery

Andries van Mourik, Nancy M. C. Bleumink-Pluym, Linda van Dijk, Jos P. M. van Putten and Marc M. S. M. Wösten

Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

Correspondence

Marc M. S. M. Wösten
M.Wosten@uu.nl

Bacterial alkaline phosphatases (PhoA) hydrolyse phosphate-containing substrates to provide the preferred phosphorus source inorganic phosphate (P_i). *Campylobacter jejuni* does not contain a typical PhoA homologue but contains a phosphatase that is regulated by the two-component system PhosS/PhosR. Here we describe the characterization of the enzyme, its secretion pathway and its function in the bacterium's biology. Phosphatase assays showed that the enzyme utilizes exclusively phosphomonoesters as a substrate, requires Ca^{2+} for its activity, and displays maximum activity at a pH of 10. Gene disruption revealed that it is the sole alkaline phosphatase in *C. jejuni*. The protein contained a twin-arginine motif (RR) at its N terminus, typical of substrates of the Tat secretion system. Substitution of the twin-arginine residues showed that they are essential for enzyme activity. *C. jejuni* genome analysis indicated the presence of four ubiquitously expressed Tat components that may form a functional Tat secretion system as well as 11 putative Tat substrates, including the alkaline phosphatase (PhoA^{Cj}) and the nitrate reductase NapA. Inactivation of *tatC* caused defects in both PhoA^{Cj} and NapA activity as well as a reduction in bacterial growth that were all restored by complementation *in trans* with an intact *tatC* copy. The atypical overall features of the PhoA^{Cj} compared to *Escherichia coli* PhoA support the existence in prokaryotes of a separate group of Tat-dependent alkaline phosphatases, classified as the PhoX family.

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INTRODUCTION

The human pathogen *Campylobacter jejuni* is one of the most important causes of bacterial gastroenteritis worldwide (Altekruse *et al.*, 1999). The natural habitat of *C. jejuni* is the intestine of warm-blooded animals and a wide variety of watery environmental sources (Diergaardt *et al.*, 2004; Rosef *et al.*, 2001). This diversity in ecological niche demands that the bacterium is able to adapt rapidly to changing environments, such as alterations in temperature or the availability of oxygen and nutrients. In recent years a number of signal transduction systems have been identified in *C. jejuni* that may contribute to bacterial adaptation (MacKichan *et al.*, 2004). One two-component signal transduction system important for bacterial survival is the PhosS-PhosR system, which senses changes in phosphate concentration and responds by altering the expression of a number of genes (the *pho* regulon) involved in phosphate acquisition and diverse metabolic processes (Wösten *et al.*, 2006).

For most bacterial species, inorganic phosphate (P_i) is the preferred source of phosphate. Most bacteria assimilate P_i

via specific uptake systems after hydrolysis of phosphate-containing substrates in the periplasm. An important enzyme involved in the hydrolysis of a variety of exogenous phosphate sources is alkaline phosphatase (PhoA). This metalloenzyme catalyses the non-specific hydrolysis of phosphomonoesters to an alcohol and P_i and thus makes P_i available for utilization by the bacterium. The production of PhoA is regulated by the phosphate concentration in the environment and upregulated during phosphate limitation. The enzyme is transported into the periplasm via the general secretory (Sec) system and becomes active after dimerization and binding of two Zn^{2+} and one Mg^{2+} cations (Torriani, 1990; Wang *et al.*, 2005).

Intriguingly, search of the whole genome of *C. jejuni* for *phoA* homologues suggested that the pathogen lacks the typical PhoA. Characterization of the *pho* regulon of *C. jejuni*, however, indicated that the bacterium possesses a phosphatase that is involved in phosphate assimilation (Wösten *et al.*, 2006). The corresponding gene (Cj0145) was upregulated during phosphate limitation and under the control of the PhosS-PhosR two-component system (Wösten *et al.*, 2006). To further decipher the nature of this seemingly atypical enzyme, which was provisionally

Abbreviation: PNPP, *p*-nitrophenyl phosphate.

designated PhoA^{Cj}, we further investigated the regulation, transport, activation and function of the enzyme. Here we provide evidence that the *C. jejuni* phosphatase deviates from the classical *Escherichia coli* PhoA in that it is transported over the cytoplasmic membrane via a previously unidentified twin-arginine translocation (Tat) secretion system, requires Ca²⁺ for its activity, and exclusively utilizes phosphomonoesters as a substrate.

METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *C. jejuni* strains were routinely cultured under microaerophilic conditions (5 % O₂, 10 % CO₂ and 85 % N₂) at 37 °C on plates containing blood agar base II (Oxoid) and 5 % horse blood lysed with 0.5 % saponin (Sigma) or in Heart Infusion broth (HI) (Oxoid). *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB medium at 37 °C. When appropriate, media were supplemented with ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹) or chloramphenicol (20 µg ml⁻¹).

Construction of a *C. jejuni* tatC mutant. To inactivate *tatC* (Cj0578), the gene and its flanking genes were amplified with the primers TAT-1 and TAT-2 (Table 2) using *C. jejuni* 81116 chromosomal DNA as template. The resulting 2181 bp PCR product was cloned into the pGEM-T easy vector (Promega) to form plasmid pGEM578. Inverse PCR was performed to introduce a deletion of 424 bp in the *tatC* gene and to create a unique *Bam*HI restriction site by using the primers TAT-3BamHI and TAT-4BamHI (Table 2). After self-ligation, the plasmid was digested with *Bam*HI and ligated to the Km^r cassette gene of pILL550 (Labigne-Roussel *et al.*, 1987). Natural transformation (Wassenaar *et al.*, 1993) was used to introduce the resulting knockout plasmid pGEM578::Km into *C. jejuni* to obtain the 81116 *tatC*::Km mutant.

Construction of complementation plasmids. To complement the *phoA*^{Cj}::Cm (Wösten *et al.*, 2006) and *tatC*::Km mutants, two *E. coli*-*C. jejuni* shuttle conjugative expression plasmids, pMA1 and pMA2, were constructed. To this end, the *C. jejuni* *metK* (Cj1096) promoter was amplified with the primers metKFSPH1 and metKRNsiI (Table 2). After digestion with *Sph*I and *Nsi*I, the resulting 115 bp PCR product was ligated into *Sph*I- and *Nsi*I-digested pWM1007 (Miller *et al.*, 2000), yielding plasmid pWM1007-pr.metK. The *metK*

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
<i>C. jejuni</i> strains		
81116	Wild-type	Palmer <i>et al.</i> (1983)
NCTC 11168	Wild-type	Parkhill <i>et al.</i> (2000)
480	Wild-type	Wassenaar <i>et al.</i> (1993)
81116 <i>tatC</i> ::Km	81116 derivative <i>tatC</i> ::Km	This study
81116 <i>tatC</i> ::Km + pMA2- <i>tatC</i>	81116 derivative <i>tatC</i> ::Km + pMA2- <i>tatC</i>	This study
81116 <i>phoA</i> ^{Cj} ::Cm	81116 derivative <i>phoA</i> ^{Cj} ::Cm	Wösten <i>et al.</i> (2006)
81116 <i>phoA</i> ^{Cj} ::Cm + pMA1	81116 derivative <i>phoA</i> ^{Cj} ::Cm + pMA1	This study
81116 <i>phoA</i> ^{Cj} ::Cm + pMA1- <i>phoA</i> ^{Cj}	81116 derivative <i>phoA</i> ^{Cj} ::Cm + pMA1- <i>phoA</i> ^{Cj}	This study
81116 <i>phoA</i> ^{Cj} ::Cm + pMA1-GG <i>phoA</i> ^{Cj}	81116 derivative <i>phoA</i> ^{Cj} ::Cm + pMA1-GG <i>phoA</i> ^{Cj}	This study
81116 <i>phoA</i> ^{Cj} ::Cm + pMA1- <i>phoA</i> ^{Ec}	81116 derivative <i>phoA</i> ^{Cj} ::Cm + pMA1- <i>phoA</i> ^{Ec}	This study
<i>E. coli</i> strain		
PC2955	<i>relA1</i> ϕ 80 <i>dlacZAM15 phoA8 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS glnV44</i>	NCCB
Plasmids		
pGEM-T easy	PCR cloning vector, Amp ^r	Promega
pGEM578	pGEM-T easy containing <i>tatC</i>	This study
pILL550	Shuttle cloning vector	Labigne-Roussel <i>et al.</i> (1987)
pBluescript KS M13 +	Cloning vector, Amp ^r	Stratagene
pCB267	<i>lacZ</i> and <i>phoA</i> promoter probe vector	Schneider & Beck (1986)
pGEM145	pGEM-T easy containing <i>phoA</i> ^{Cj}	This study
pGEM145GG	pGEM145 containing a RR substituted <i>phoA</i> ^{Cj} gene	This study
pGEM <i>phoA</i>	pGEM-T easy containing <i>E. coli phoA</i>	This study
pGEM578-2	pGEM-T easy containing <i>tatC</i>	This study
pMA1	<i>C. jejuni/E. coli</i> shuttle conjugative expression vector, Km ^r	This study
pMA2	<i>C. jejuni/E. coli</i> shuttle conjugative expression vector, Cm ^r	This study
pWM1007	<i>C. jejuni/E. coli</i> shuttle vector	Miller <i>et al.</i> (2000)
pWM1007-pr.metK	pWM1007 containing the <i>metK</i> promoter	This study
pAV35	pBluescript II SK containing <i>E. coli</i> Cm ^r cassette	van Vliet <i>et al.</i> (1998)
pMA1- <i>phoA</i> ^{Cj}	pMA1 containing <i>phoA</i> ^{Cj}	This study
pMA1-GG <i>phoA</i> ^{Cj}	pMA1 containing a RR substituted <i>phoA</i> ^{Cj}	This study
pMA1- <i>phoA</i> ^{Ec}	pMA1 containing <i>E. coli phoA</i>	This study
pMA2- <i>tatC</i>	pMA2 containing <i>C. jejuni tatC</i>	This study

Table 2. Primers used in this study

Primer	DNA sequence (5'–3')
TAT-1	AAATTTAGAAGGCGGGCGTGT
TAT-2	GCAAAAATTCTAAAGGCTGTAAA
TAT-3BamHI	AAAGGATCCTTCATTGCGGTAAATAAGGC
TAT-4BamHI	AAAGGATCCTCAATTTTTTAATGGCAGGACC
phoAF1	GAGCTCAGGAGAAAAACAAATGGAAGAAGA
phoAF2	GAGCTCAGGAGAAAAACAAATGGAAGGAGGATTGTTT
phoAR	CCGCGGTATTTAGCTTCCTATCACTCCAC
phoAECF	GAGCTCAGGAGAAAAATAAATGAAACAAA
phoAECR	CCGCGGGGTATCAGGCGGCTTTCTTGAGG
TATcplforw.xbaI	TCTAGAGTGTAAATCATAGCTTGAAAG
TATcplrev1.sacI	GAGCTCCAAGATAAAAAACGAAAAATAAATG
metKFSphI	GCATGCAGTTGATTTTAACTAACTTTTGCT
metKRNsII	ATGCATAAAAAAGTCCTTTTCATTAAAAATG
pILL550-pWM1007F	CCAGATCTGATGATAAGCTGTCAAACATGAG
pILL550-pWM1007R	CCGAATTCAGCGACCGGCGCTCAGCTGG
T7	TAATACGACTCACTATAGGG
22CATDO	CTGGGATTTTATTTATTCAGCAAG

promoter, polylinker and the gene coding for green fluorescent protein (GFP) were amplified from pWM1007-pr.metK with the primers pILL550-pWM1007F and pILL550-pWM1007R (Table 2) and the 1.9 kb product was digested with *Bgl*II and *Eco*RI. The shuttle conjugative expression plasmid pMA1 was obtained by ligation of this 1.9 kb fragment into the *Bgl*II/*Eco*RI-digested plasmid pILL550. Plasmid pMA2 contains a chloramphenicol cassette (*Cm*^r) instead of the kanamycin resistance gene. For construction of this plasmid, the *Cm*^r of pAV35 (van Vliet *et al.*, 1998) was amplified with the primers T7 and 22CATDO (Table 2). The PCR product of 880 bp containing one *Eco*RI site was digested with *Eco*RI and ligated into an *Eco*RI- and *Swa*I-digested pMA1, resulting in plasmid pMA2.

To complement the *tatC*::Km mutant, the *tatC* gene was amplified with the primers TATcplforw.xbaI and TATcplrev1.sacI (Table 2). The restriction enzymes *Sac*I and *Xba*I were used to clone the 800 bp *tatC* PCR fragment into pMA2, generating the complementation plasmid pMA2-tatC.

Plasmid pMA1 was used to introduce the *phoA*^{Cj}, the GG*phoA*^{Cj} construct (a gene coding for a PhoA^{Cj} protein where the twin arginine residues are substituted by glycine residues) and an *E. coli phoA* gene into the *C. jejuni phoA*::*Cm* mutant. By using the primer combinations phoAF1/phoAR and phoAF2/phoAR (Table 2) and *C. jejuni* 81116 chromosomal DNA as template, *phoA*^{Cj} and GG*phoA*^{Cj} PCR products were generated. These PCR products of 1800 bp in size were digested with *Sac*I and *Sac*II and ligated into pMA1, resulting in the plasmids pMA1-*phoA*^{Cj} and pMA1-GG*phoA*^{Cj}, respectively. The *phoA* gene of *E. coli* was PCR amplified from pCB267 (Schneider & Beck, 1986) with the primers phoAECF/phoAECR (Table 2). The 1800 bp PCR product was digested with *Sac*I and *Sac*II and introduced into pMA1 to form plasmid pMA1-*phoA*^{Ec}. All PCR products in this study were obtained with the proofreading enzyme *Pfu* (Promega) according to the instructions of the manufacturer. Nucleotide sequences of the cloned PCR products were verified by sequencing both strands. Complementation plasmids were introduced into *C. jejuni* mutants via conjugation (Labigne-Roussel *et al.*, 1987).

Alkaline phosphatase assay. Alkaline phosphatase activity was determined as previously described (Wösten *et al.*, 2006), except that EDTA was omitted from the lysis buffer. In short, alkaline

phosphatase activity of a bacterial culture grown in defined medium (Leach *et al.*, 1997) containing 1.6 mM *P*_i (high) or 0.08 mM *P*_i (low) was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (PNPP) (Sigma). The units of alkaline phosphatase were calculated using the formula $10^3 \times [A_{420} - (1.75 \times A_{550})] / t \times OD_{600} \times V$.

Nitrite assay. Nitrate reductase activity was determined as previously described (Sellars *et al.*, 2002). Briefly, *C. jejuni* strains were grown overnight in HI medium containing 50 mM potassium nitrate. To the culture supernatants 1% (w/v) sulphanilamide dissolved in 1 M HCl and 0.02% (w/v) naphthylethylenediamine were added and after 15 min absorbance was measured at 540 nm. Nitrite concentrations, adjusted to the cell density, were determined by reference to a standard curve.

Growth experiments. Overnight cultures grown in HI medium or defined medium were diluted to a starting OD₆₀₀ of 0.05 and grown at 37 °C under microaerophilic (5% O₂) or oxygen-limited (0.3% O₂) conditions using the anoxomat system (MART Microbiology BV). Bacterial growth and cell density were monitored by measuring OD₆₀₀ and counting c.f.u. at intervals.

Localization of the alkaline phosphatase activity. A 10 ml *C. jejuni* culture grown with 0.08 mM *P*_i was subjected to cellular fractionation as described by Myers & Kelly (2005).

RESULTS

Regulation of PhoA^{Cj} phosphatase activity

Inactivation of gene Cj0145 encoding PhoA^{Cj} in *C. jejuni* strain 81116 resulted in a complete loss of bacterial phosphatase activity (Fig. 1) (Wösten *et al.*, 2006). To ensure that this phenotype was caused by disruption of PhoA^{Cj}, we complemented the *phoA*^{Cj}::*Cm* mutant *in trans* with the shuttle plasmid pMA1-*phoA*^{Cj}. This plasmid constitutively expresses the *C. jejuni phoA* gene via the *metK* promoter. Plasmid pMA1-*phoA*^{Cj} restored the

Fig. 1. Phosphatase (PhoA^{Cj}) activity of *C. jejuni* strains during phosphate starvation. Alkaline phosphatase activity in bacterial whole-cell lysates was measured for strain 81116 wild-type, the *phoA^{Cj}::Cm* mutant strain, and the *phoA^{Cj}::Cm* mutant strain containing the complementation plasmid pMA1-PhoA^{Cj}. Bacteria were grown in defined medium containing 1.6 or 0.08 mM P_i . Standard errors based on four independent experiments are shown.

phosphatase activity when bacteria were grown in low-phosphate medium (Fig. 1, filled bars), indicating that PhoA^{Cj} is responsible for the phosphatase activity.

To investigate whether additional components induced under conditions of low P_i were required for PhoA^{Cj} activity, we compared the enzyme activity for the wild-type, mutant and complemented strains after growth in low- and high-phosphate conditions. Whereas for the wild-type enzyme activity clearly varied with the availability of phosphate in the medium, this regulatory effect was lost in the complemented strain in which PhoA^{Cj} was constitutively expressed (Fig. 1). This indicates that, at least when the gene is expressed from a multicopy plasmid, no additional components present under low-phosphate conditions are necessary for the PhoA^{Cj} activity.

pH optimum of *C. jejuni* phosphatase activity

To assess whether PhoA^{Cj} is an alkaline phosphatase, we measured the phosphatase activity at different pH values. Wild-type 81116 and the *phoA^{Cj}::Cm* mutant were grown in defined medium under low-phosphate conditions and lysed in Tris/HCl buffer containing SDS and lysozyme with different pH values. Maximum phosphatase activity was obtained at pH 10 (Fig. 2a). No phosphatase activity was observed for the *phoA^{Cj}::Cm* mutant over the entire pH range (data not shown), indicating that PhoA^{Cj} is the sole phosphomonoesterase of *C. jejuni*.

PhoA^{Cj} activity requires Ca^{2+}

Assessment of the conservation of alkaline phosphatase activity among *C. jejuni* strains revealed considerable

variation in enzyme activity (Fig. 2b), although they all contained an intact *phoA^{Cj}* gene (data not shown). One explanation for this diversity may be the variable presence of enzyme cofactors. As alkaline phosphatases of other species require divalent cations for their activity, we tested *C. jejuni* phosphatase activity in bacteria lysed in the absence of the metal chelator EDTA. All *C. jejuni* strains tested showed a strong increase in alkaline phosphatase activity in the absence of EDTA (Fig. 2b), indicating that PhoA^{Cj} needs a cation for its activity.

The nature of the cation that activates the PhoA^{Cj} was sought by selective addition of cations to bacteria grown overnight in the presence of 1 mM EDTA (Fig. 2c). Enzyme activity was measured 30 min after the addition of the cation(s) to the culture. Alkaline phosphatase activity in the EDTA-treated strains was almost fully restored by the addition of 10 mM Ca^{2+} , while other ions including Mn^{2+} , K^+ , Mg^{2+} , Zn^{2+} (Fig. 2c) or Na^+ , Cu^{2+} , Co^{2+} , Ni^{2+} , Mo^{6+} , VO_4^{3-} , Fe^{2+} or Fe^{3+} or combinations thereof (data not shown), did not restore enzyme activity.

Substrate specificity of *C. jejuni* PhoA^{Cj}

The main function of bacterial alkaline phosphatases is to release P_i from various exogenous organophosphate compounds for use in bacterial growth. To assess which type of compounds may serve as a substrate for PhoA^{Cj}, we tested various potential phosphate donors for their ability to support growth of *C. jejuni* 81116 and the *phoA^{Cj}::Cm* mutant strain. Measurement of the optical density and c.f.u. counting of overnight cultures showed that the wild-type strain but not the mutant grew with 1.6 mM of the organophosphate monoesters, glucose 6-phosphate (G6P) and glycerol 3-phosphate (G3P) as sole phosphate source, while no growth was observed with the phosphodiester cAMP or DNA (Fig. 2d).

PhoA^{Cj} requires a twin-arginine sequence for enzyme activity

A large number of bacterial alkaline phosphatases are secreted via the Sec-dependent pathway. Analysis of the PhoA^{Cj} protein revealed that it contains a conserved twin-arginine domain near the N terminus, characteristic of proteins that are exported by the Tat secretion machinery (Stanley *et al.*, 2000). To address whether PhoA^{Cj} might be transported by the *C. jejuni* Tat system, we replaced the coding sequence for two arginine residues with two glycine residues in the *phoA^{Cj}* located on plasmid pMA1-*phoA^{Cj}*, containing the constitutively expressed *metK* promoter. The resulting plasmid pMA1-GGphoA^{Cj} was introduced into *phoA^{Cj}::Cm* mutant strain and alkaline phosphatase activity was measured under high- and low-phosphate concentrations. This strain lacked alkaline phosphatase activity under conditions of phosphate limitation, while minimal activity (less than 5 % of wild-type) was observed under high-phosphate conditions (Fig. 3a). For the control

Fig. 2. Biochemical characteristics of PhoA^{Cj}. (a) The pH optimum of PhoA^{Cj} activity was determined after lysis of *C. jejuni* 81116 in buffer of the indicated pH using PNPP as substrate. Standard errors based on four independent experiments are shown. (b) EDTA inhibits PhoA^{Cj} activity. PhoA^{Cj} activity was measured for three *C. jejuni* strains grown in defined medium containing 0.08 mM P_i. Bacteria were lysed in the presence or absence of 1 mM EDTA and PhoA^{Cj} activity was measured using PNPP as substrate. Standard errors based on four independent experiments are shown. (c) PhoA^{Cj} requires Ca²⁺ as cofactor. *C. jejuni* strain 480 transformed with a plasmid containing *phoA*^{Cj} downstream of the constitutive *metK* promoter was grown overnight in HI medium in the absence or presence of EDTA. Then, bacteria were incubated with the indicated cation(s) (each 10 mM) for 30 min to monitor their effect on alkaline phosphatase activity. Standard errors based on four independent experiments are shown. (d) Substrate specificity of PhoA^{Cj}. *C. jejuni* strain 81116 and the *phoA*^{Cj}::Cm mutant were grown overnight in defined medium containing the indicated phosphate sources. Optical densities as well as the corresponding c.f.u. ml⁻¹ are shown. Bacterial cultures were diluted to OD₆₀₀ between 0.1 and 0.5 to enable accurate measurement of OD₆₀₀. Data correspond to one representative of three independent experiments.

strain carrying the plasmid with the original *phoA*^{Cj} (pMA1-PhoA^{Cj}), high levels of enzyme activity were present independent of the phosphate concentration in the medium (Fig. 3a). These results strongly suggest that PhoA^{Cj} is secreted via a thus far unidentified *C. jejuni* Tat secretion machinery.

PhoA^{Cj} is transported via the *C. jejuni* Tat secretion system

Complementary evidence that PhoA^{Cj} is secreted via the Tat pathway was sought by inactivation of the system. Genomic analysis suggests that *C. jejuni* has a Tat system, as four of its putative components, *tatA/E* (Cj1176), *tatB* (Cj0578), *tatC* (Cj0579) and *tatD* (Cj0644), are present in the genome of the sequenced strain NCTC 11168 (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000) (Fig. 3b).

To test the functionality of the Tat system in *C. jejuni*, we inactivated the putative *tatC* homologue by insertion of a kanamycin-resistance cassette. A severe growth defect as measured by optical density and c.f.u. counting was observed for the *tatC*::Km mutant compared to the wild-type 81116 strain (Fig. 3c). This defect was rectified by introduction of plasmid pMA2-*tatC* harbouring a constitutively expressed *C. jejuni* *tatC* gene.

Further evidence of the functionality of the *C. jejuni* Tat system was obtained by measurement of nitrate reductase activity. A search of the *C. jejuni* proteome for putative Tat substrates based on the presence of the specific Tat recognition consensus sequence (RRxFLK) (Lee *et al.*, 2006) indicated 11 potential Tat substrates (Table 3) (Dilks *et al.*, 2003), including NapA. In other bacterial species this Tat substrate reduces nitrate to nitrite, which may be used by the bacterium as an alternative electron acceptor

Fig. 3. *C. jejuni* possesses a functional Tat secretion system. (a) Alkaline phosphatase assay showing that the twin-arginine residues are essential for PhoA^{Cj} activity. Alkaline phosphatase activity was measured for the *phoA*^{Cj} :: Cm mutant, and mutants containing plasmid pMA1 (empty vector), pMA1-*phoA*^{Cj} (containing intact PhoA^{Cj}) or pMA1-GG*phoA*^{Cj} (intact PhoA^{Cj} where the twin-arginine residues were replaced by glycine residues) after growth under high- or low-phosphate conditions. (b) Organization of the Tat genes in the genome of *C. jejuni* NCTC 11168. The length of the gene arrows corresponds to the length of the genes. (c) Growth of strain 81116 (■), the *tatC* mutant (*tatC* :: Km) (▲) and complemented *tatC* mutant carrying plasmid pMA2-*tatC* (▼) in HI medium under microaerophilic conditions. Growth curves are from one representative of three independent experiments. The optical densities as well as the corresponding c.f.u. ml⁻¹ are presented. Bacterial cultures were diluted to OD₆₀₀ between 0.1 and 0.5 to enable accurate measurement of OD₆₀₀. The actual c.f.u. counts fully reflected optical densities for each strain for the entire duration of the incubation, indicating that bacterial growth was measured. (d) Nitrite reductase assay demonstrating that the enzyme required a functional TatC. Nitrite accumulation was measured in culture supernatants of 1 × 10⁹ c.f.u. of *C. jejuni* strain 81116, the *tatC* :: Km mutant and the mutant containing complementation plasmid pMA2-*tatC* grown in HI with 50 mM potassium nitrate. Standard errors based on four independent experiments are indicated.

(Pittman & Kelly, 2005). Analysis of nitrate reductase activity indicated that *C. jejuni* 81116 exhibited this enzyme activity, while the *tatC* :: Km mutant did not (Fig. 3d). This defect was restored by complementation of the *tatC* :: Km mutant with pMA2-*tatC*.

To unequivocally demonstrate that PhoA^{Cj} is transported via the Tat system, we assessed the phosphatase activity under low-phosphate conditions for the parental strain, the *tatC* :: Km mutant and the pMA2-*tatC* complemented *tatC* mutant. Inactivation of TatC resulted in very low alkaline phosphatase activity compared to the parent strain. The defect was fully rectified after introduction of an intact copy of the *tatC* gene (Fig. 4a). To exclude that the lack of

enzyme activity in the TatC mutant was caused by a more general protein secretion defect, we introduced into the *tatC* :: Km mutant strain a plasmid carrying the *E. coli* PhoA, which utilizes the Sec secretion pathway (Palmer & Berks, 2003). This enzyme was active in this background, indicating that the Sec system in the *C. jejuni* *tatC* mutant was still functional (data not shown). These results, in conjunction with the requirement of the twin-arginine motif, indicate that PhoA^{Cj} is transported across the cytoplasmic membrane by the Tat secretion pathway. Like the *E. coli* PhoA, the *C. jejuni* PhoA^{Cj} remains cell associated, as measurement of enzyme activity in the culture supernatant indicated that virtually all the activity remained associated with the cell fractions (Fig. 4b). The

Table 3. Putative Tat substrates of *C. jejuni* NCTC 11168

Gene designation	Predicted function	Position of the twin-arginine motif
Cj0005c	Putative molybdenum-containing oxidoreductase	MKQNDQKENRRDFLKNIG
Cj0145	<i>phoA^{Cj}</i> Alkaline phosphatase	MERRFLKGS
Cj0264c	Molybdopterine-containing oxidoreductase	MLDRRKFLKIGA
Cj0379c	Hypothetical protein	MLITPEKLYKQRRNFLKGA
Cj0414	Putative oxidoreductase subunit	MQDNIIDRRSFFKLGL
Cj0437	<i>sdhA</i> Succinate dehydrogenase flavoprotein subunit	MGEFSRRDFIKTAC
Cj0780	<i>napA</i> Periplasmic nitrate reductase	MNRRDFIKNTA
Cj1358c	Putative periplasmic cytochrome C	MIILRRKILKTS
Cj1511c	<i>fdhA</i> Putative formate dehydrogenase large subunit	MSSVGENIKLTRSFLKMAA
Cj1513c	Possible periplasmic protein	MKNRRREFLKSA
Cj1516	Putative periplasmic oxidoreductase	MNRRNFLKFNA

enzyme activity for the *E. coli* PhoA in *C. jejuni* was primarily found in the periplasmic fraction, while active *C. jejuni* PhoA was mostly found in the membrane fraction.

DISCUSSION

Most bacterial species contain a number of proteins to assimilate the essential element phosphate (P_i) from the environment (Wanner, 1996). Here we provide evidence that the *C. jejuni* alkaline phosphatase (PhoA^{Cj}) is a key enzyme in the acquisition of P_i . The enzyme differed from the typical PhoA present in most bacterial species as it required calcium as a cofactor and is transported into the periplasm via the Tat secretion pathway. The transport of PhoA^{Cj} via the Tat system is the first functional evidence of the existence of this pathway in *C. jejuni*.

Key evidence that *C. jejuni* Cj0145 encodes the only functional alkaline phosphatase in *C. jejuni* was the successful complementation *in trans* of the mutant strain with an intact copy of this gene. We placed the intact *phoA^{Cj}* gene on a shuttle plasmid under a constitutive promoter rather than its own PhosS-PhosR-regulated promoter to investigate whether additional phosphate-regulated factors were required for the enzyme activity. The strong enzyme activity in the complemented strain measured under high-phosphate conditions (Fig. 1) indicates that the *C. jejuni* phosphatase can act independently of other phosphate-regulated molecules. The enzyme utilizes calcium as a cofactor. This became evident when multiple *C. jejuni* strains were tested for phosphatase activity. In some strains, enzyme activity was only observed when EDTA, which was used to lyse the bacteria, was omitted from the lysis buffer or when an excess of calcium was added to the EDTA-containing buffer. The need for a divalent cation as a cofactor is not unusual, as alkaline phosphatases from other bacterial species also require divalent cation(s) to hydrolyse different types of phosphate compounds (Monds *et al.*, 2006; Wang *et al.*, 2005; Wu *et al.*, 2007). *E. coli* PhoA requires Mg^{2+} and Zn^{2+} as cofactors. The requirement of Ca^{2+} as cofactor has thus far only been reported for PhoA^{Vc} of *Vibrio cholerae* (Roy *et al.*,

1982) and PhoX of *Pasteurella multocida* (Wu *et al.*, 2007). In *P. multocida* an aspartic acid together with a stretch of hydrophobic amino acids near the C terminus of the protein are essential for Ca^{2+} binding and phosphatase activity (Wu *et al.*, 2007). Analysis of the PhoA^{Cj} protein sequence indicates the presence of a similar motif.

Bacterial alkaline phosphatase provides bacteria with P_i by reducing different phosphoester compounds available in the environment. The alkaline phosphatase of *P. multocida* (Wu *et al.*, 2007) can cleave both phosphomonoester and phosphodiester bonds. Our results indicate that the *C. jejuni* enzyme is able to utilize phosphomonoesters such as glucose 6-phosphate and glycerol 3-phosphate, but not phosphodiesters (Fig. 2d). This may limit the ecological niches of the bacterium.

Alkaline phosphatases generally exert the hydrolysis of phosphomonoester-containing substrates in the bacterial periplasm. *E. coli* PhoA and most of its homologues in other bacterial species are transported across the cytoplasmic membrane by the main protein secretion system, the Sec system (Palmer & Berks, 2003). Analysis of the protein sequence of PhoA^{Cj} indicated the presence of a typical twin-arginine (Tat) consensus motif in the N terminus. This RRxFLK motif, in which the twin-arginine is highly conserved (Stanley *et al.*, 2000), led to the discovery that the *C. jejuni* phosphatase exploits the Tat secretion machinery to gain access to the periplasm. Evidence that the Tat system serves as transport machinery for PhoA^{Cj} includes the lack of enzyme activity after substitution of the twin-arginine residues (Fig. 3a) and after inactivation of the *tatC* gene, which results in dysfunction of the Tat system (Fig. 4a). The absence of enzyme activity in these strains even in the presence of calcium (data not shown) implies that the enzyme is not active in the cytosol, consistent with its assumed function. Although *phoA^{Cj}* was transcribed, PhoA^{Cj} is probably not transported through the *E. coli* Tat system, because no difference was observed in alkaline phosphatase activity between *E. coli* wild-type and a *tatC* mutant (data not shown). Furthermore, the *E. coli* *tatC* mutant could not be complemented with an intact *C. jejuni* *tatC* (data not shown). This may indicate that, in

Fig. 4. Localization of PhoA^{Cj} and the role of the *C. jejuni* Tat secretion system. (a) Alkaline phosphatase assay demonstrating that PhoA^{Cj} requires a functional TatC. PhoA^{Cj} activities for *C. jejuni* strain 81116, the *tatC*::Km mutant and the mutant complemented with plasmid pMA2-tatC were determined after 8 h growth in defined medium containing 0.08 mM P_i. Standard errors based on four replicates are shown. (b) Alkaline phosphatase assay showing that PhoA^{Cj} is associated with the cellular fraction. PhoA^{Cj} activities for strain 81116 and the *phoA*^{Cj}::Cm mutant complemented with plasmid pMA1-phoA^{Ec} containing the *E. coli phoA* were measured after overnight growth in defined medium containing 0.08 mM P_i. The culture supernatant, periplasm, cytoplasm and membrane were separated to determine the localization of active alkaline phosphatase. The values corresponding to 100 % were 386 units of alkaline phosphatase for 81116 and 120 units for the *phoA*^{Cj}::Cm mutant containing pMA1-phoA^{Ec}. The experiment was repeated four times with similar results.

contrast to the Sec systems, at least some components of the Tat systems of *C. jejuni* and *E. coli* are not compatible.

The transport of PhoA^{Cj} via the Tat system is the first reported evidence that this secretion pathway is functional in *C. jejuni*. Genome analysis predicts at least 11 putative Tat substrates in *C. jejuni* strain NCTC 11168 (Table 3) (Dilks *et al.*, 2003). Besides PhoA^{Cj}, we provide evidence that nitrate reductase NapA, a well-documented Tat substrate in other bacterial species (Ding & Christie, 2003; Lavander *et al.*, 2006; Weiner *et al.*, 1998), requires

the Tat system of *C. jejuni* (Fig. 3d). The lack of nitrate reductase activity in the TatC mutant but gain-of-function after introduction of an intact copy of the gene clearly demonstrates that TatC, which is an essential component of the Tat system (Bogsch *et al.*, 1998; Jongbloed *et al.*, 2000), is required for NapA function. Considering the nature of most predicted Tat substrates, which are mostly cofactor requiring enzymes involved in electron transport (Table 3), it seems plausible to assume that the main function of the Tat secretion system in *C. jejuni* is to translocate redox cofactor-containing proteins that contribute to the assembly of the electron transport chain and energy conservation under oxygen-limited conditions (Palmer & Berks, 2003). Why the alkaline phosphatase of *C. jejuni* also exploits this secretion pathway, rather than the classical Sec system often used by PhoA of other species, awaits further investigation.

The atypical use of the Tat system to transport alkaline phosphatase across the cytoplasmic membrane has also been demonstrated for *Pseudomonas fluorescens* and *Thermus thermophilus* (Monds *et al.*, 2006; Angelini *et al.*, 2001) and is assumed for *V. cholerae* and *P. multocida* (Wu *et al.*, 2007). *P. fluorescens*, however, also possesses Tat-independent alkaline phosphatases (Monds *et al.*, 2006). Interestingly, the sole alkaline phosphatase PhoA^{Cj} of *C. jejuni* is 52 % identical to the *V. cholerae* PhoA^{Vc} protein, and 49 % and 41 % identical to the PhoX proteins of *P. fluorescens* and *P. multocida*, respectively. Furthermore, PhoA^{Vc} of *V. cholerae* and PhoX of *P. multocida* are activated by Ca²⁺, as we found for the *C. jejuni* enzyme. Similarly, the pH optimum of PhoA^{Cj} (~pH 10, Fig. 2a) is comparable to that of the *P. multocida* homologue (Wu *et al.*, 2007), which is higher than that for *E. coli* PhoA (pH 8.5) (Nesmeyanova *et al.*, 1981). Because of their shared dependence on the Tat secretion system, the alkaline phosphatases of *V. cholerae*, *P. fluorescens* and *P. multocida* have been proposed to form a separate group of enzymes, designated the PhoX family (Wu *et al.*, 2007). The sequence similarity and functional characteristics of the *C. jejuni* PhoA^{Cj} indicate that PhoA^{Cj} also belongs to this group and therefore we propose to rename PhoA^{Cj} as PhoX. This further supports the designation of a novel PhoX family of Tat-dependent alkaline phosphatases.

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