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^{1}H, ^{15}N and ^{13}C resonance assignments of the C-terminal domain of PulL, a component of the *Klebsiella oxytoca* type II secretion system

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Declarations

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Conflicts of interest/Competing interests
The authors declare they have no conflict of interest.

Availability of data and material (data transparency)
The chemical shift values have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu/) under the accession number 50966.

Abstract

Type II secretion systems (T2SS) allow Gram-negative bacteria to transport toxins and enzymes from the periplasm to the external milieu, and are thus important for the pathogenicity of bacteria. To drive secretion, T2SS assemble filaments called pseudopili closely related to bacterial type IV pili. These filaments are non-covalent polymers of proteins that are assembled by an inner membrane complex called the assembly platform connected to a cytoplasmic ATPase motor. In the *Klebsiella oxytoca* T2SS, the PulL protein from the assembly platform is essential for pseudopilus assembly and protein secretion. However, its role in these processes is not well understood. To decipher the molecular basis of PulL function, we used solution NMR to study its structure and interactions with other components of the machinery. Here as a first step, we report the ^{1}H, ^{15}N and ^{13}C backbone and side-chain chemical shift assignments of the C-terminal periplasmic domain of PulL and its secondary structure based on NMR data.

Biological context

In Gram-negative bacteria, several complex machineries are dedicated to the secretion of proteins involved in important adaptive processes such as nutrient acquisition, motility, adhesion, respiration and virulence (Nivaskumar and Francetic, 2014). The type II secretion system (T2SS) is one of the most widespread and relevant from biomedical and environmental standpoints (Cianciotto and White, 2017). Together with the closely related type IV pili, T2SSs belong to the versatile superfamily of type IV filament assembly systems (Berry and Pelicic, 2015, Denise *et al.*, 2019). The T2SS is composed of 12-15 proteins organized in four subcomplexes: 1) an outer membrane secretin channel (Hardie *et al.*, 1996); 2) a periplasmic pseudopilus consisting of a non-covalent polymer of proteins (Sauvonnet *et al.*, 2000, López-Castilla *et al.*, 2017); 3) an inner membrane assembly platform (Py *et al.*, 2001); and 4) a cytosolic ATPase (Possot *et al.*, 1997, Camberg and Sandkvist, 2005, Patrick *et al.*, 2011). While the secretin channel allows passage of folded proteins across the outer membrane, the inner membrane subcomplex couples the energy of the ATPase motor to the pseudopilus polymerization, which is thought to drive protein transport (Nivaskumar and Francetic, 2014). Despite decades of studies, the molecular basis of this process remains elusive.

In this study, we focused on the T2SS from *Klebsiella oxytoca* that secretes pullulanase, the enzyme which degrades branched maltotriose polymers allowing their uptake and use as a nutrient (d'Enfert *et al.*, 1987). Pullulanase secretion requires pseudopilus assembly, which is orchestrated by the assembly platform proteins PulF, PulL and PulM and hexameric ATPase PulE (Possot *et al.*, 2000).

PulL protein comprises 398 residues forming an N-terminal cytoplasmic domain, a transmembrane helix and a C-terminal periplasmic domain. In the T2SS of *Vibrio cholerae*, the cytoplasmic N-terminal domain of the PulL homologue EpsL forms a stable complex with the N-terminal subdomain of the ATPase (Abendroth *et al.*, 2005). This interaction anchors the ATPase hexamer to the assembly platform complex. The C-terminal domain (CTD) of PulL interacts with the C-terminal domain of PulM in the periplasm (Possot *et al*, 2000). In several bacteria these domains have been shown to be each dimeric in solution (Abendroth *et al.*, 2004a, Abendroth *et*

al., 2004b, Fulara *et al.*, 2018) and to interact together as a heterodimer in bacterial two-hybrid and cysteine crosslinking assays (Lallemand *et al.*, 2013).

We report here the backbone and side-chain chemical shift assignments and secondary structure of the C-terminal domain of PulL. This assignment is the first necessary step towards the study of PulL structure and its dimerization. It will be further required for interaction studies with its partners to elucidate the role of PulL in the pseudopilus assembly mechanism. To our knowledge, this is the first NMR study of a protein from the T2SS assembly platform.

Methods and experiments

Production and purification of isotope-labelled $PulL_{CTD}$

The pMalp2 vector was used for the expression of the C-terminal domain of PulL ($PulL_{CTD}$) containing 88 residues (residues 312 to 398 of PulL with an additional N-terminal serine). This vector directs production of a N-terminal Maltose Binding Protein (MBP)-tagged protein in the periplasm, followed by a His_6-tag and a TEV cleavage site. Uniformly ^{15}N labelled and $^{15}N/^{13}C$ double-labelled $PulL_{CTD}$ were produced in M9 minimal medium by using 1 g/L of $^{15}NH_4Cl$ and 4 g/L ^{13}C-glucose, as the sole nitrogen and carbon sources, respectively. Gene expression was induced with 1 mM IPTG overnight at 18 °C in *E. coli* BL21 (DE3) cells. The MBP-His_6-tag-$PulL_{CTD}$ labelled protein was purified from the supernatant after the sonication of the whole cells and centrifugation at 16000g, during 1 hour at 4°C. The purification was performed in three steps. The supernatant fraction was loaded onto a HisTrap HP column (*Cytiva*) previously equilibrated with 50 mM Tris–HCl, pH 8, 100 mM NaCl, 10 mM imidazole. Bound proteins were eluted with a linear imidazole gradient going from 10 to 500 mM. After a TEV cleavage overnight at 10°C, proteins were loaded on HisTrap HP column (*Cytiva*) and the flow-through corresponding to PuL_{CTD} without the tag was recovered and concentrated on a 3-kDa cutoff centricon device (*Cytiva*). The concentrated protein fraction was applied on a Sephacryl S-100 column (*Cytiva*) equilibrated with 50 mM HEPES, pH 7, 50 mM NaCl. Fractions containing the purified $PulL_{CTD}$ were pooled and concentrated to 0.45 mM with pH adjusted to 6.5 for NMR data acquisition. Protease inhibitor cocktail, EDTA-free (*Roche*) was added to all buffers during the purification.

NMR spectroscopy

NMR experiments were recorded at 25°C on a 600 MHz Avance III HD (*Bruker Biospin*) spectrometer equipped with a cryogenically cooled triple resonance ^{1}H [^{13}C /^{15}N] probe. The ^{1}H, ^{15}N and ^{13}C backbone and side chain resonance assignments (Cavanagh *et al.*, 1996) was carried out by using standard NMR experiments as implemented in TopSpin 3.6.1 (*Bruker Biospin*) and IBS libraries (Favier and Brutscher, 2019): Backbone and side chain resonances assignment was carried out by using standard NMR experiments: 2D ^{1}H-^{15}N-HSQC, ^{1}H-^{13}C-HSQC, and 3D HNCA, HN(CO)CA, HNCACB, CBCA(CO)HN, HNCO, HN(CA)CO, HCCH-TOCSY, C(CO)NH-TOCSY and H(CCO)NH-TOCSY. 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) signal was taken as 0 ppm for referencing proton chemical shifts and ^{15}N and ^{13}C chemical shifts were indirectly referenced to DSS (Wishart *et al.*, 1995). CcpNmr Analysis (Vranken *et al.*, 2005) was used for NMR data analysis. Analysis of the secondary structure was performed using HN, Hα, Cα, Cβ, CO, and N chemical shifts with the TALOS-N

prediction server (Shen and Bax, 2013).

Extent of assignments and data deposition

High quality NMR data were obtained for $PulL_{CTD}$ as illustrated by the $^{1}H–^{15}N$ HSQC spectrum (Figure 1). Backbone amide resonances were assigned for all non-proline amino acids except the first residue N312, I320 and A346, for which cross peaks were not detected in the ^{1}H-^{15}N HSQC. For the observable residues, 98% assignment of backbone and 96% for their corresponding side chain resonances were obtained. The chemical shift values have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu/) under the accession number 50966.

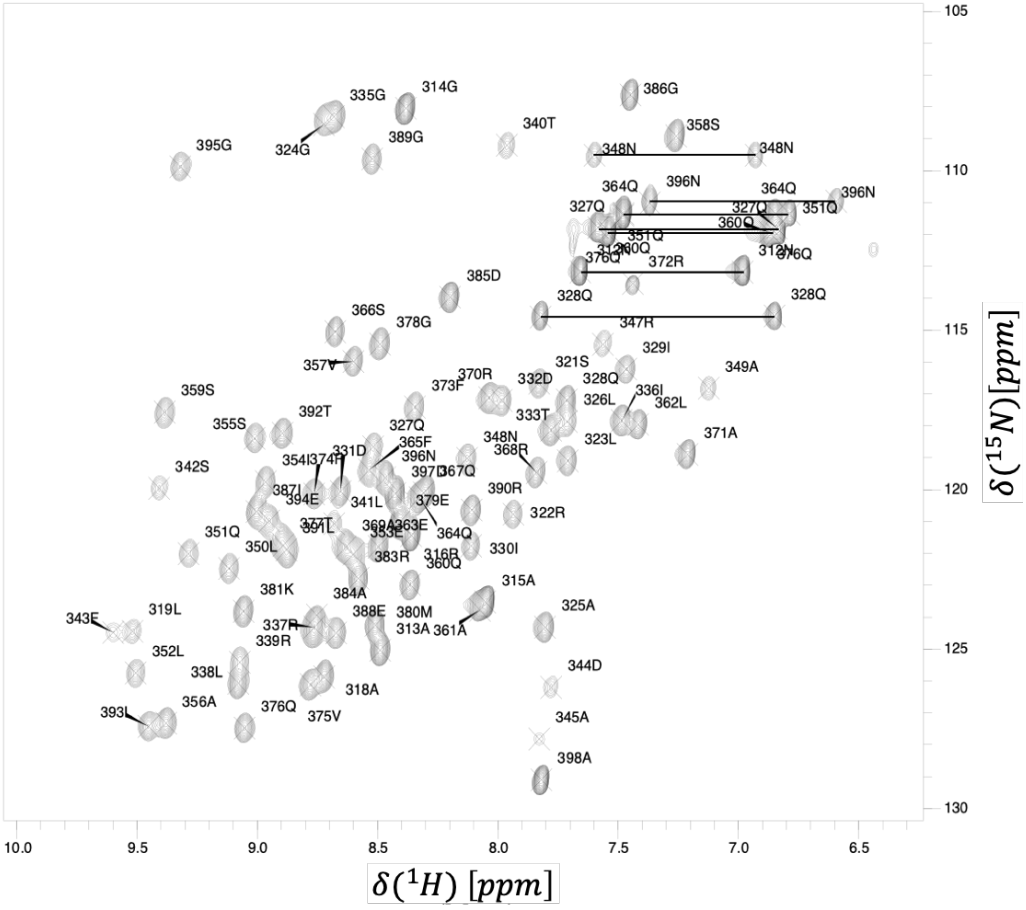

Figure 1: **^{1}H-^{15}N HSQC spectrum of $PulL_{CTD}$,** the C-terminal periplasmic domain of PulL, a protein of the T2SS assembly platform from *K. oxytoca*. The spectrum was recorded at 600MHz on a 0.45 mM ^{15}N-labeled protein sample, in 50 mM HEPES buffer, pH 6.5, 50 mM NaCl, 5% D_2O (v/v), 25°C. The resonance assignments for $^{1}H–^{15}N$ backbone amide peaks are depicted using sequence number and one-letter residue code. Side chain NH_2 peaks of Asn (N) and Gln (Q) are connected by horizontal lines.

Secondary structure

Secondary structure elements of $PulL_{CTD}$ were determined based on resonance assignments by calculating the deviation of Cα, Cβ and C′ chemical shifts from the corresponding random coil chemical shifts (ΔδCα, ΔδCβ and ΔδC′, Figure 2A). Residues with positive or negative values on ΔδCα and ΔδC′ are classified as helical or β-strand structures, respectively. For ΔδCβ, positive and negative values correspond to β-sheet and α-helix, respectively (Figure 2A). To complete these data, a TALOS-N analysis was also performed using the HN, Hα, Cα, Cβ, C′ and N chemical shifts (Shen and Bax, 2013). As shown in Figure 2B, the TALOS prediction agrees with the secondary chemical shifts results. Both predictive approaches revealed that $PulL_{CTD}$ presents two helical segments (α1: I320-D331 and α2: S359-A369) and 4 β-strands (β1: G335-S342, β2: L350-A356, β3: F373-K381 and β4: G386-G395), distributed in the α1–β1–β2–α2–β3–β4 sequential order (Figure 2).

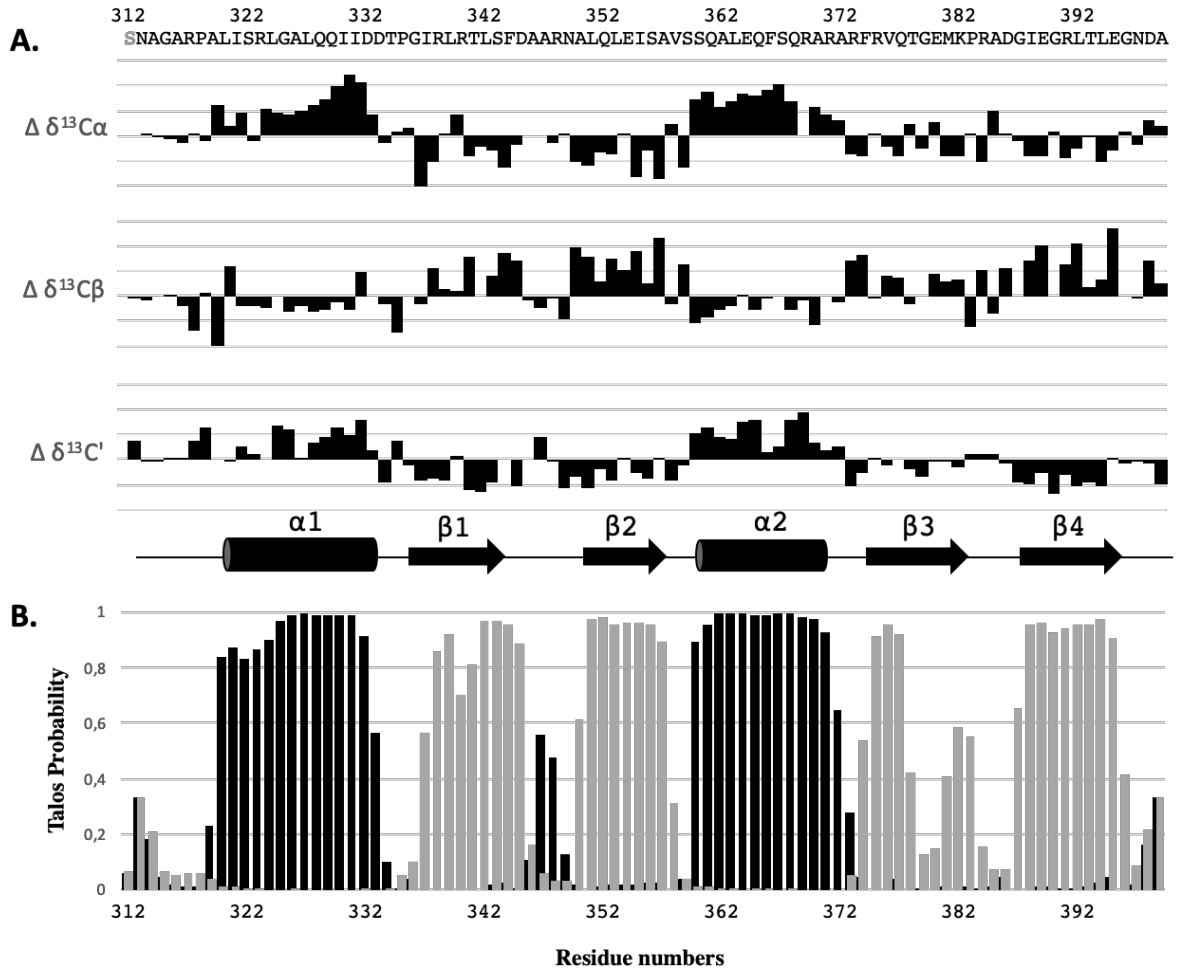

Figure 2: **$PulL_{CTD}$ secondary structure predictions based on the NMR assignment data**. **A.** Secondary chemical shifts from Cα, Cβ, C′. The same scale (grey line = 2 ppm) is used for the three plots. **B.** TALOS-N secondary structure probability of each residue to be involved in an α-helix (*black bars*) or in a β-strand (*grey bars*). The $PulL_{CTD}$ secondary structure, as a consensus of both predictive approaches, is represented between the panels. α-helices and β-strands are displayed as *cylinders* and *arrows*, respectively. The sequence of $PulL_{CTD}$ is shown on *top*.

We observed significant differences in the peak intensities in the $^{1}H–^{15}N$-HSQC spectrum. Some signals are very weak leading also to weak peaks or absence of peaks in the 3D experiments. As shown in Figure 3, two regions (α1, β1-β2) of the protein displayed significantly weaker signal intensity compared to the others. This spectral behaviour can be due to conformational exchange of the residues in these regions. Since there is evidence that $PulL_{CTD}$ and its homologs are dimeric in solution (Fulara *et al.*, 2018, Abendroth *et al.*, 2004a), the regions with low signal intensity might be at the dimer interface or affected by the dimerization. Also, in the HSQC spectrum, no peaks corresponded to the residues 320 and 346, suggesting that these residues can be at the dimer interface. Determination of the 3D structure will be performed as a subsequent step to validate this hypothesis.

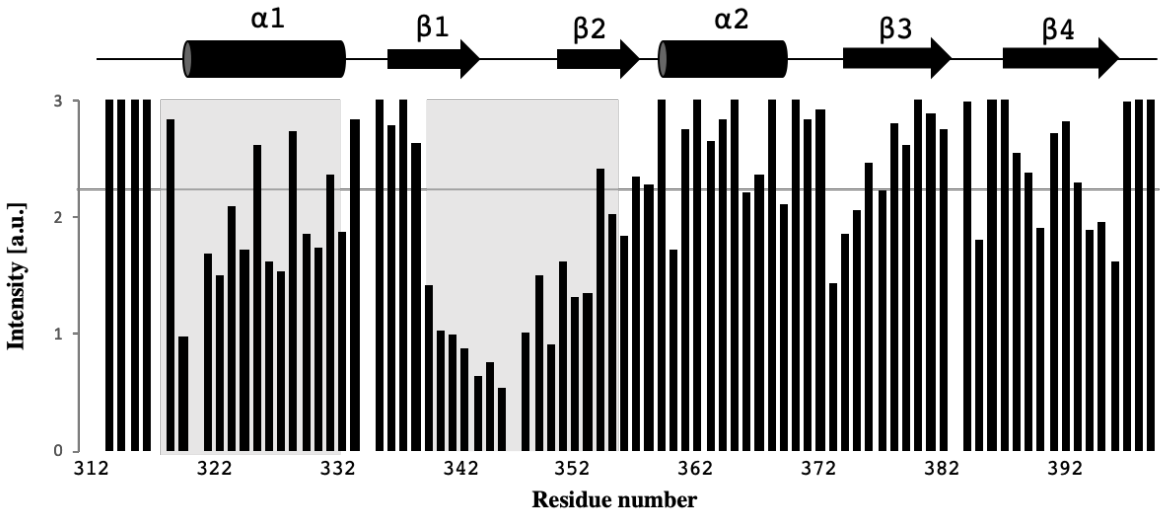

Figure 3: ^{1}H-^{15}N-HSQC peak intensity (arbitrary unit a.u.) as a function of residue numbers. The two regions with lower peak intensity than the average (*grey line*) are highlighted with *grey boxes*.

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