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Expression, crystallization and preliminary X-ray diffraction studies on the complete choline-binding domain of the major pneumococcal autolysin

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The major pneumococcal autolysin (LytA), a virulence factor of this bacterium, is composed of an amino-terminal catalytic domain plus a carboxyl-terminal choline-binding domain (ChBD). This C-terminal domain, responsible for anchorage to the cell wall, is a tandem of six imperfect 20-residue repeats whose precise ends have been difficult to establish by sequence methods. The reported crystal structure of a shortened C-terminal fragment of the protein suggested that it might contain an additional repeat and thus an additional choline-binding site (ChBS). The complete recombinant choline-binding domain of LytA has now been overexpressed in soluble form using a secreting *Escherichia coli* strain which facilitates purification with a higher yield. It has been crystallized at room temperature using MPD as the main precipitant. The crystals belong to space group $P2_1$ and diffract to beyond 3.2 Å resolution on a synchrotron-radiation source. The molecular-replacement solution indicates that a new ChBS which fits the topology of the solenoid structure is formed in the N-terminal region.

1. Introduction

The increasing number of antibiotic resistant strains (Campbell & Silberman, 1998) and the suboptimal clinical efficacy of the available vaccines (Lipsitch, 1999) hamper control of *Streptococcus pneumoniae* (pneumococcus), the leading cause of life-threatening infectious diseases such as pneumonia, meningitis and bacteremia. Substantial attention is currently focused on virulence-related pneumococcal proteins common to all serotypes, such as the 15-member family (Tettelin *et al.*, 2001) of choline-binding proteins (ChBPs), which stand out because they are involved in pathogenic processes such as adhesion to host cells, nasopharyngeal colonization and bacterial sepsis (Hollingshead & Briles, 2001). ChBPs are responsible for a wide range of different functions, which all depend on the non-covalent attachment of their highly conserved choline-binding domain (ChBD) to the choline moieties of both teichoic and lipoteichoic acids on the cell surface (Fischer, 2000). This peculiar manner of displaying proteins at the cell surface, also described for other Gram-positive bacteria (Wren, 1991), is essential for bacterial virulence (Gosink *et al.*, 2000).

The major pneumococcal autolysin (LytA), the first and one of the better characterized ChBPs, is a 36.6 kDa modular enzyme comprising an amino-terminal catalytic domain plus the carboxyl-terminal ChBD. The N-terminal domain catalyses the hydrolysis of the *N*-acetylmuramoyl-L-alanine bond in the pneumococcal peptidoglycan backbone (Mosser & Tomasz, 1970). LytA is thereby responsible for cellular autolysis, through which it mediates the release of toxic substances (such as the pore-forming toxin pneumolysin and cell-wall degradation products) that damage endothelial and epithelial host cells (Berry & Paton, 2000) and thus favour bacterial invasion. The 136 carboxyl-terminal residues of LytA (C-LytA), thought to encompass the complete ChBD of LytA, were crystallized in complex with choline and the crystal structure at 2.6 Å resolution was recently reported by our group (Fernández-Tornero *et al.*, 2001). The primary sequence of this fragment contains six imperfect 20-residue motifs known as choline-binding repeats (ChBRs). The multiple alignment of the ChBRs constituting the primary sequence of the shortened C-LytA showed that highly conserved residues are also present in an additional repeat at its N-terminus, especially the aromatic core in strands I and II and the glycine residue at position 5 of the alignment (Fernández-Tornero *et al.*, 2001). In order to confirm that this motif is likely to fold as a loop/hairpin unit with the same characteristics as those observed for all the other motifs in the solenoid structure, a fragment with 19 additional residues at the N-terminus (Glu169–Val188) has now been cloned, expressed and crystallized.

2. Materials and methods

2.1. Cloning, expression and purification

DeepVent DNA polymerase (New England Biolabs) and the oligonucleotide primers 5′-GTTAACGAGAACGGCTTGACGATTGAA-3′ and 5′-CGCATACTATTAAGCTTTTTCAAGACC-3′ were used to amplify the sequence encoding for the complete choline-binding domain from the pGL80 plasmid DNA. The resulting 526 bp *Hpa*I- and *Hin*dIII-flanked fragment was cloned into the pRHO vector and transformed into the secreting *E. coli* BL21(DE3)-SS mutant strain (Fernández-Tornero, unpublished data). This expression system secretes the cloned protein into the culture medium, thereby facilitating the subsequent purification as no resuspension and sonication of the bacterial pellet is required. Cultures for induction were inoculated from a 5 ml 16 h LB culture and grown in minimal medium supplemented with 0.4% casein-amino acids and 100 µg ml^{-1} of the selective antibiotic. Isopropyl-thio-β-D-galactoside was added to a final concentration of 0.5 m*M* when the optical density at 600 nm had reached 0.8. After 16 h growth at 310 K, the supernatant was harvested by centrifugation and loaded onto a DEAE-Sephacel column for purification of the complete ChBD of LytA as described previously (Sánchez-Puelles *et al.*, 1990). Elution of the domain from the column with 150 m*M* choline chloride ensures that the polypeptide corresponds to the active form of the enzyme (García *et al.*, 1985). The purity of the protein sample was assessed by SDS–PAGE analysis. The purified protein was concentrated to 12 mg ml^{-1} using a 3 kDa molecular-weight cutoff centrifugation membrane (PAL-Filtron).

2.2. Crystallization and data collection

Crystallization trials were performed using the sitting-drop vapour-diffusion method at 295 K. The search for suitable crystallization conditions was conducted with 96-condition Hampton Screens 1 and 2 (Jancarik & Kim, 1991), with drops assembled using 1 µl protein and 1 µl reservoir solution. Small needles were obtained from several conditions with different alcohols and PEGs, the longest with MPD and *tert*-butanol. Conditions were optimized in a grid screen for 40–70% MPD at pH 7.0. Fine-tuning of the condition yielded single rod-shaped crystals in 60% MPD pH 6.8, 0.1 *M* HEPES. The largest rod has dimensions of 1 × 0.1 × 0.05 mm (Fig. 1).

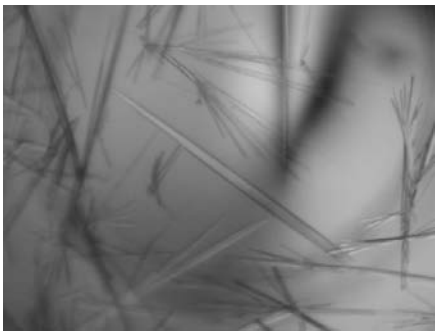

Figure 1
Crystals of the complete ChBD of LytA, approximate maximum dimensions 1 × 0.1 × 0.05 mm, grown for one week using 60% MPD as the main precipitant. The crystals are easily separated with a glass needle.

For the diffraction experiments, crystals were flash-frozen in a stream of nitrogen gas at 100 K. The crystallization solution was used as cryoprotectant. Data were collected on a MAR 345 imaging-plate detector using an X-ray synchrotron source (λ = 0.8453 Å) at beamline BW7B-DESY (Hamburg, Germany). Diffraction was visible to 2.8 Å, but only data to 3.2 Å were suitable for subsequent processing using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997).

3. Results and discussion

We have expressed the complete choline-binding domain of LytA in the extracellular medium of *E. coli* cultures using a novel T7 secretion vector (pRHO). This allowed us to obtain higher yields of properly folded soluble polypeptide (up to 10 mg per culture volume) and facilitated the subsequent purification step on a DEAE Sephacel column (Pharmacia). The purified protein was very stable and could be stored at 277 K at a protein concentration of 12 mg ml^{-1} in 50 m*M* Tris–HCl pH 6.5 buffer containing 150 m*M* choline chloride. Crystals of recombinant full-length C-LytA were obtained by the sitting-drop vapour-diffusion method using MPD as the main precipitant. The autoindexing procedure performed with *DENZO* indicated that the crystals belong to the monoclinic system, with unit-cell parameters a = 51.9, b = 30.3, c = 113.6 Å, β = 96.4°. The space group was determined to be $P2_1$ on the basis of systematic absences. The asymmetric unit contains two molecules of full-length C-LytA, giving a crystal volume per protein mass (V_M) of 2.3 Å^3 Da^{-1} and a solvent content of 47.2% (Matthews, 1968). The R_{sym} was 6.3% and was 19.5% for the highest resolution shell (3.37–3.2 Å). The data-collection statistics are presented in Table 1.

Table 1
Data collection and processing statistics.

Values for the highest resolution shell are shown in parentheses.

Wavelength (Å)	0.8453
Space group	$P2_1$
Unit-cell parameters (Å,°)	a = 51.9, b = 30.3, c = 113.6, β = 96.4
Resolution (Å)	35.0–3.2 (3.37–3.2)
V_M (two molecules per asymmetric unit) (Å^3 Da^{-1})	2.35
Solvent content (%)	47.2
R_{merge}† (%)	6.3 (19.5)
Completeness (%)	99.6 (99.6)
Measured reflections	19526 (2830)
Unique reflections	6081 (895)
Average $I/\sigma(I)$	8.8 (3.4)

† $R_{merge} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$.

A molecular-replacement solution using the shortened C-LytA (PDB code 1hcx; Fernández-Tornero *et al.*, 2001) as the search model has been obtained using the program *AMoRe* (Navaza, 1994). Close inspection of the electron-density maps did not allow us to trace a good number of the amino-acid side chains and discontinuities at some specific points were observed. However, owing to the repetitive nature of the β-spiral staircase solenoid structure, it was possible to build the peptide backbone of the newly appended ChBR (residues Glu169–Val188).

Although the crystals of shortened and complete C-LytA were grown under different conditions (PEG 4K *versus* MPD), the functional dimer constituting the asymmetric unit of both crystal forms, made by a tail-to-tail coupling of the monomers, is conserved in its basic architecture. Fig. 2 shows the crystal packing in the orthorhombic crystals of the shortened C-LytA

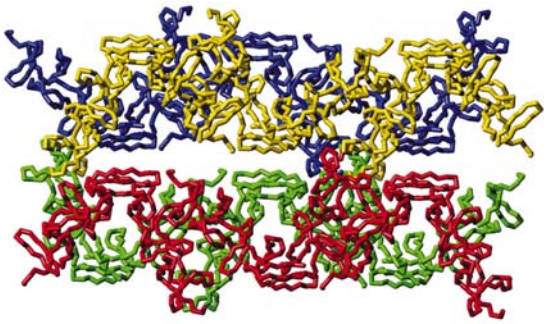

Figure 2
Molecular packing of the shortened ChBD of LytA (Fernández-Tornero *et al.*, 2001) viewed along the b axis of the crystal. The NCS axis that relates the monomers is nearly parallel to the crystallographic c axis.

(Fernández-Tornero *et al.*, 2001), where dimers run along the *b* axis in rows as alternating V-shaped pieces. The solution derived from the molecular replacement shows that the building blocks in the rows of the $P2_1$ crystals are equivalent, but adjacent rows are slightly displaced along the *c* axis.

Our results suggest that the 19 additional residues constitute an extra ChBR and that a new choline-binding site is formed at the interface between the new hairpin and the following one. This new choline-binding site is likely to increase the affinity of the protein for the pneumococcal cell wall, as the combination of several weak binding sites has been shown to provide high affinity (Hajduk *et al.*, 1997). Although this structure is currently being refined, we have also focused our efforts on searching for better diffracting crystals using the glass capillary liquid-diffusion method.

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