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2013-10


http://hdl.handle.net/10138/214799
https://doi.org/10.1107/S1744309113024676

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*Lactobacillus rhamnosus* GG, a widely used Gram-positive probiotic strain, is clinically well known for its perceived health-promoting effects. It has recently been shown to display proteinaceous pilus fibres (called SpaCBA) on its cell surface. Structurally, SpaCBA pili possess a characteristic three-pilin polymerized architecture, with repeating SpaA major pilins that form the backbone and two types of minor subunits (SpaB and SpaC). In this study, recombinant SpaA protein was purified, characterized and crystallized. The crystals diffracted to a resolution of 2.0 Å and belonged to space group \( \text{C}2 \), with unit-cell parameters \( a = 227.9, \ b = 63.2, \ c = 104.3 \ \text{Å}, \ \beta = 95.1^\circ \).

1. Introduction

Many bacteria assemble pili (or fimbriae) on their cell surface for facilitating host-cell adherence and promoting pathogenesis. While substantial structural information already exists for pili from Gram-negative bacteria, only recently has research effort been directed into pili from Gram-positive bacteria (for recent reviews, see Kang & Baker, 2012; Vengadesan & Narayana, 2011; Hendrickx *et al.*, 2011; Kline *et al.*, 2010; Waksman & Hultgren, 2009). The pili from Gram-positive bacteria are typified by a covalently bonded polymeric structure that consists of a major (or backbone) pilin subunit forming the shaft and two different minor (or ancillary) pilins, one situated at the pilus tip for adhesion and another at the pilus base for cell-wall anchoring (Ton-That *et al.*, 2004). Most structural investigations conducted on Gram-positive pili to date have focused primarily on various pathogenic species. Recently, it has emerged that pilus-like structures are also associated with a beneficial commensal Gram-positive bacterium, these being the so-called SpaCBA pili in the probiotic *Lactobacillus rhamnosus* GG strain (Kankainen *et al.*, 2009).

Within the *L. rhamnosus* GG genome, the SpaCBA pilus genes are organized into an operon that encodes a major pilin (SpaA), two minor pilins (SpaB and SpaC) and a pilin-specific sortase for catalyzing pilus assembly (Kankainen *et al.*, 2009). SpaCBA pili have a typical three-pilin architecture, with SpaA comprising the pilus backbone and SpaB and SpaC at the pilus base and tip, respectively (Kankainen *et al.*, 2009). Interestingly, in contrast to the pili from Gram-positive pathogens, SpaC and, to a lesser extent, SpaB are found sporadically throughout the SpaCBA pilus backbone (Reunanen *et al.*, 2012; Tripathi *et al.*, 2013), which might enhance adherence to the intestinal mucosa and epithelial layer and thereby then extend the relative longevity and transient colonization of *L. rhamnosus* GG cells in the gut.

In order to better understand the structural features of the pili from beneficial commensal bacteria, we have initiated a structural study of the pilin constituents of the SpaCBA pilus. Here, we describe the purification, crystallization and preliminary crystallographic analysis of the *L. rhamnosus* GG SpaA backbone-pilin subunit.

2. Materials and methods

2.1. Cloning, expression and purification

The construction of the recombinant clone for *L. rhamnosus* GG SpaA pilin protein has been described previously (von Ossowski *et al.*, 2012; Vengadesan & Narayana, 2011; Hendrickx *et al.*, 2011; Kline *et al.*, 2010; Waksman & Hultgren, 2009). The pili from Gram-positive bacteria are typified by a covalently bonded polymeric structure that consists of a major (or backbone) pilin subunit forming the shaft and two different minor (or ancillary) pilins, one situated at the pilus tip for adhesion and another at the pilus base for cell-wall anchoring (Ton-That *et al.*, 2004). Most structural investigations conducted on Gram-positive pili to date have focused primarily on various pathogenic species. Recently, it has emerged that pilus-like structures are also associated with a beneficial commensal Gram-positive bacterium, these being the so-called SpaCBA pili in the probiotic *Lactobacillus rhamnosus* GG strain (Kankainen *et al.*, 2009).

Within the *L. rhamnosus* GG genome, the SpaCBA pilus genes are organized into an operon that encodes a major pilin (SpaA), two minor pilins (SpaB and SpaC) and a pilin-specific sortase for catalyzing pilus assembly (Kankainen *et al.*, 2009). SpaCBA pili have a typical three-pilin architecture, with SpaA comprising the pilus backbone and SpaB and SpaC at the pilus base and tip, respectively (Kankainen *et al.*, 2009). Interestingly, in contrast to the pili from Gram-positive pathogens, SpaC and, to a lesser extent, SpaB are found sporadically throughout the SpaCBA pilus backbone (Reunanen *et al.*, 2012; Tripathi *et al.*, 2013), which might enhance adherence to the intestinal mucosa and epithelial layer and thereby then extend the relative longevity and transient colonization of *L. rhamnosus* GG cells in the gut.

In order to better understand the structural features of the pili from beneficial commensal bacteria, we have initiated a structural study of the pilin constituents of the SpaCBA pilus. Here, we describe the purification, crystallization and preliminary crystallographic analysis of the *L. rhamnosus* GG SpaA backbone-pilin subunit.
Purification of recombinant SpaA protein in dialysis buffer (see above) was concentrated to ~70 mg ml\(^{-1}\) as determined by UV absorbance at 280 nm using a theoretical extinction coefficient of 39 880 M\(^{-1}\) cm\(^{-1}\) (ProtParam tool on the ExPASy Proteomics Server; http://www.expasy.org) and used for crystallization trials. At first, 576 crystallization screening conditions were tested using the vapour-diffusion method at 295 K with a Mosquito Crystal (TTP LabTech Ltd) automated liquid microdispensing system. Micocrystals appeared under several screening conditions. Flower-like crystals of a size suitable for diffraction were observed after 3 weeks in drops containing 0.1 M sodium acetate and 25% PEG 2000 as well as those containing 0.2 M trisodium citrate and 20% PEG 3350. Further optimization of the latter condition produced the best crystals after equilibration for 4 d at 295 K. The drops consisted of 1 μl protein solution mixed with an equal volume of reservoir solution. The optimized reservoir solutions consisted of 0.35 M trisodium citrate and 14% PEG 3350.

2.3. Data collection

Initial X-ray diffraction data from SpaA crystals were collected to a resolution of 2.6 Å on laboratory X-ray equipment comprising a Xenocs GeniX\(^{3D}\) Cu HF (High Flux) microbeam X-ray generator operating at 50 kV and 0.6 mA equipped with a MAR 345 image-plate detector (MAR Research). Higher resolution data were collected to 2.0 Å on the BM14 beamline at the ESRF, Grenoble, France. The synchrotron data were collected from a single crystal soaked in 20% (v/v) PEG 400 cryoprotectant solution using a MAR 225 CCD area detector (MAR Research) over a range of 360\(^\circ\) with 0.25° oscillation steps, 182 mm crystal-to-detector distance and 10 s exposure. X-ray diffraction data were processed using the HKL-2000 suite of programs (Otwinowski & Minor, 1997).

2.4. Mass spectrometry analysis

The bands were excised from an SDS–PAGE gel and exposed to repeated dehydration and rehydration cycles, with a 2:1 mixture of acetonitrile (ACN) and 50 mM ammonium bicarbonate (ABC) for 5 min and then with 25 mM ABC for 2 min. The gel pieces were then incubated overnight with trypsin (20 μg ml\(^{-1}\) in 25 mM ABC) at 310 K. The trypsinated sample was analyzed using an ABSCIEX Triple TOF (time-of-flight) 5600 mass spectrometer system equipped with an ESI (electrospray ionization) source. Protein identification was achieved by the Mascot search engine (Matrix Science) using the NCBI nonredundant protein databases.

2.5. Dynamic light scattering

Polydispersity and the oligomeric state of purified SpaA were analyzed by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd) equipped with a 633 nm laser and a temperature-controlled measuring chamber. SpaA protein (1 mg ml\(^{-1}\)) in 20 mM HEPES pH 7.0, 150 mM NaCl, 1 mM EDTA was used in DLS measurements. Before measuring the scattering, the protein solution was passed through a 0.2 μm filter. DLS measurements were performed using polystyrene cuvettes at 298 K and the experimental data obtained were analyzed using the Zetasizer software (Malvern Instruments Ltd).
related to intramolecular isopeptide bonds that stabilize the pilin structures (Kang et al., 2007). Such an interpretation was given for a similar feature in an earlier report (von Ossowski et al., 2010).

When screened for crystal growth, purified recombinant SpaA protein could be crystallized at room temperature by hanging-drop vapour diffusion under several conditions. Further optimization of one of these conditions to 0.35 M trisodium citrate, 14% PEG 3350 led to the best crystals (Fig. 2a), which diffracted to 2.6 Å resolution on a home X-ray source and 2.0 Å resolution at the ESRF (Fig. 2b).

SpaA crystals belonged to the monoclinic space group $C2$, with unit-cell parameters $a = 227.9$, $b = 63.2$, $c = 104.3 \text{ Å}$, $\beta = 95.1^\circ$ (Table 1). Calculation of the Matthews coefficient ($V_M = 2.44 \text{ Å}^3 \text{Da}^{-1}$) (Matthews, 1968) suggests the presence of five molecules in the asymmetric unit, which corresponds to a solvent content of 50%. Since SpaA has a limited sequence identity of less than 25% to known pilin structures in the Protein Data Bank (PDB), our initial attempts to obtain phases by molecular replacement were not successful. Moreover, as methionine or cysteine residues are not present in SpaA, phase calculation by selenium or sulfur single-wavelength anomalous dispersion (SAD) was not feasible. We are instead currently attempting to solve the phasing problem through the use of heavy-atom derivatization.

This investigation was supported by the Regional Centre for Biotechnology (RCB) and the Department of Biotechnology (DBT). VK and DS thank Professor Dinakar Salunke for reviews and support. VK and DS acknowledge Dr Hassan Belrhali and Dr Babu Manjashetty at the ESRF BM14 beamline, Madhava Rao at the in-house X-ray facility and Vishakha Choudhary at the Mass Spectrometry facility for their help during data collection. Recombinant cloning performed at the University of Helsinki was financed by an Academy of Finland general research grant (118165) and the Center of Excellence in Microbial Food Safety (CoE-MiFoSa) research program (141140), which was initiated and funded as part of the Research Program on Nutrition, Foods and Health (ELVIRA) by the Academy of Finland.

### References


### Table 1

Data-collection and processing statistics for SpaA.

| No. of crystals | 1 |
| Beamline | BM14, ESRF |
| Wavelength (Å) | 0.97625 |
| Detector | MAR CCD 225 |
| Crystal-to-detector distance (mm) | 182 |
| Rotation range per image (°) | 0.25 |
| Total rotation range (°) | 360 |
| Exposure time per image (s) | 10 |
| Resolution range (Å) | 40-2.0 (2.03–2.00) |
| Space group | $C2$ |
| Unit-cell parameters (Å, °) | $a = 227.9$, $b = 63.2$, $c = 104.3$, $\beta = 95.1$ |
| Total No. of measured intensities | 642704 |
| Unique reflections | 94497 |
| Multiplicity | 6.8 (4.5) |
| Mean $I/\sigma(I)$ | 34.2 (3.5) |
| Completeness (%) | 95.2 (75.1) |
| $R_{merge}$ (%) | 8.7 (38.1) |