Role of the C-terminal region of dextranucrase from *Leuconostoc mesenteroides* IBT-PQ in cell anchoring

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dsrP, a gene that encodes a cell-associated dextranucrase produced by *Leuconostoc mesenteroides* IBT-PQ, was isolated, sequenced and expressed in *Escherichia coli*. From sequence analysis, seven repeat units in the N-terminal region were found, as well as five cell wall binding repeats in the C-terminal region. A model of the C-terminal domain of dextranucrase was built based on the solenoid structure of the cell wall binding domain already described in LytA. By experiments involving direct interactions of the enzyme with *L. mesenteroides* cells, as well as among the cells and the single C-terminal domain expressed in *E. coli*, evidence was obtained concerning the anchoring function of this region in cell-associated dextranucrase, a function which may be independent of its capacity to bind dextran.

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INTRODUCTION

Glucose polymers and their corresponding oligosaccharides can be synthesized through enzymic transglucosylation by a wide variety of glycanucrases capable of using sucrose as substrate. Among this group, dextranucrases (EC 2.4.1.5) are glucanucrases (GSs) able to produce dextran, a glucose polymer linked mainly through α 1-6 bonds, although α 1-3, α 1-6, α 1-4 and α 1-2 bonds are also found, in both the main chain and the branching linkages (Bozonnet *et al.*, 2002; Kralj *et al.*, 2002). Furthermore, glucose can also be transferred to molecules that can act as nucleophile acceptors; in the case of sugars such as maltose, a wide variety of low-molecular-mass glucose oligosaccharides are produced (Monsan & Paul, 1995).

Several GSs have been the subject of intensive research. They are mainly produced by *Streptococcus*, *Lactobacillus* and *Leuconostoc* species, where they may be both constitutive and sucrose-induced, and are obtained either as soluble proteins in the culture medium or as cell-associated proteins (Hamada & Slade, 1980; Kralj *et al.*, 2002; Monsan *et al.*, 2001).

On average, GS genes encode proteins of ~1600 aa, equivalent to a molecular mass of 160 kDa, and although no crystal structure is yet available, different functional domains have already been identified (van Hijum *et al.*,

2006). GSs belong to family 70 of glycoside hydrolases (<http://www.cazy.org>). A 36–40 aa signal peptide, characteristic of extracellular enzymes and bearing 50 % identity among dextranucrases, has been found in the N-terminal region. The signal peptide is followed by a variable region, which in some cases contains repeat sequences such as those known as A and TQQ repeats or T and E motifs (Kralj *et al.*, 2004; Bozonnet *et al.*, 2002). The catalytic domain contains ~1000 aa, including the three highly conserved carboxylic amino acids of the catalytic triad: two aspartic acid residues and one glutamic acid residue also present in α -amylases (Monchois *et al.*, 1999a; Matsuura *et al.*, 1984). It has been shown that in *Leuconostoc mesenteroides* NRRL B-512F dextranucrase (DsrS), the aspartic acid in position 551 is essential for catalytic activity, while glutamic acid 589 and aspartic acid 662 complement the catalytic triad (Monchois *et al.*, 1997). In fact, it has been proposed that the catalytic domain of the GSs from *L. mesenteroides* and *Streptococcus* species have the secondary structure of a circularly permuted α -amylase type (β/α)₈ barrel (MacGregor *et al.*, 1996). Tsai *et al.* (2000) have predicted a TIM-barrel of (α/β)₈ structure for the catalytic domain of *Streptococcus mutans* glucosyltransferase GtfB, based on the sequences and secondary structure alignment against the crystal structures reported for several α -amylases.

Finally, the C-terminal end of GSs consists of a 400–500 aa region containing a series of related but non-identical tandem repeats of 20–30 aa each, divided into four classes according to their sequence, and designated as A, B, C and D repeats (Russell, 1990). This region is involved in binding to the glucan in streptococcal and lactobacilli GSs,

Abbreviations: ChBR, choline binding region; CWBR, cell wall binding region; GBD, glucan binding domain; GS, glucanucrase; LTA, lipoteichoic acid.

The GenBank/EMBL/DDBJ accession number for the entire *dsrP* gene sequence of *Leuconostoc mesenteroides* IBT-PQ is AY504865.

and has therefore been designated the glucan binding domain (GBD) (Shah *et al.*, 2004; Krajl *et al.*, 2004a). However, there is controversy about its function in *Leuconostoc* dextranucrases, as although several authors report strong binding of the C-terminal region to dextran (Miller *et al.*, 1986; Funane *et al.*, 1998), other authors are not able to find this interaction (Shah *et al.*, 2004).

The repeat units present in dextranucrase C-terminal regions resemble those found in the cell wall binding motif in the choline binding domain (ChBD) of autolysin from *Streptococcus pneumoniae*, as well as in other surface-located proteins from several bacteria (Janecek *et al.*, 2000). These modules bind choline to the lipoteichoic acid (LTA) that serves to anchor the target proteins to the cell surface. The crystal structure of a ChBD from autolysin LytA of *S. pneumoniae* has been determined, revealing a solenoid fold consisting exclusively of β -hairpins that stack to form a left-handed superhelix. DsrB, a glucosyltransferase from *L. mesenteroides* NRRL B-1299 and Gtfl GBD of *Streptococcus downei* are found among the proteins with high identity to the ChBD (Fernández-Tornero *et al.*, 2001). Recently, the crystal structure of the 255-residue C terminal of TcdA from *Clostridium difficile* bound to a synthetic derivative of a natural carbohydrate receptor, α -Gal-(1,3)- β -Gal-(1,4)- β -GlcNAcO(CH₂)₈CO₂CH₃, was determined (Ho *et al.*, 2005). This region also adopts a β -solenoid fold. Residues that interact with the carbohydrate are conserved in all seven putative binding sites in TcdA, suggesting a multivalent binding mode (Greco *et al.*, 2006).

Several streptococcal GSs and *Leuconostoc* dextranucrases are present in both the pellet and the supernatant fractions of cultures when strains are grown in the presence of sucrose; it is usually assumed that insoluble GSs are the result of binding to high-molecular-weight dextran that pellets together the enzyme and the cells. However, recent studies concerning the structures involved in cell anchoring have questioned this general hypothesis. For instance, Zahnley & Smith (2000) have shown that alternansucrase is bound to *L. mesenteroides* cells in the absence of polymer.

We have already reported the production and properties of a cell-associated dextranucrase produced by *L. mesenteroides* IBT-PQ, a strain isolated from pulque, a traditional pre-Hispanic alcoholic beverage (Chellapandian *et al.*, 1998). In the present study, we report the molecular and functional characterization of the *dsrP* gene that encodes this enzyme, as well as the role in cell wall anchorage of its C-terminal region. A C-terminal domain model was built based on the cell wall binding domain; to date, to the best of our knowledge, no model has been proposed for this region in dextranucrases.

METHODS

Bacterial strains, media and growth conditions. *L. mesenteroides* IBT-PQ and NRRL B-1299 bacterial strains were grown at 30 °C and 200 r.p.m., as previously reported (Chellapandian *et al.*, 1998), with

20 g l⁻¹ glucose or xylose instead of sucrose as carbon source, in order to avoid enzyme and polymer synthesis, as these interfere with DNA extraction. *Escherichia coli* DH5 α , the host for cloning, was grown at 37 °C and 250 r.p.m. in Luria-Bertani (LB) medium, supplemented where appropriate with 50 μ g kanamycin ml⁻¹ or 100 μ g ampicillin ml⁻¹ in order to maintain the plasmid construction. Agar (1.5 %, w/v) was added for growth in solid medium.

Cloning and expression of the *L. mesenteroides* IBT-PQ dextranucrase gene. Molecular biology methods were carried out according to Sambrook & Russell (2001). Based on sequence homology between conserved regions located in the catalytic core of different GS genes of Gram-positive bacteria, degenerate primers PQ+1 [5'-AATGAT(A/G)TTGATAATTCAAATCCA-3'] and PQ-1 [5'-A(A/G)CATAACGATCA(G/T)(C/T)AAAAGCATA-3'] were designed, and were used for PCR with chromosomal DNA of *L. mesenteroides* strain IBT-PQ as template, resulting in the isolation of a single fragment of 1505 bp. Sequence analysis confirmed its GS identity. In order to obtain the 3' region of the gene, Southern hybridization of chromosomal DNA of *L. mesenteroides* IBT-PQ digested with *Clal*, using the 1505 bp amplified PCR fragment as probe, was carried out. Results revealed a hybridizing fragment of about 3200 bp. The chromosomal DNA of *L. mesenteroides* IBT-PQ digested with *Clal* was then ligated to yield a circular DNA. This sample was used as template for a PCR with diverging primers PQ-2 (5'-CATATTCAGCTTCTGTGGTTGGTG-3') and PQ+2 (5'-GAGTTGGGGAATTACGAACCTTCG-3'), yielding an amplification product of ~3200 bp. Based also on the 1505 bp sequence, primers PQ-3 (5'-AAGTTTAACAAGTAATACATCCC-3') and PQ+3 (5'-GATAGTGATGTCTCACAAGC-3') were designed in order to isolate the 5' region by inverse PCR. In the same way, *L. mesenteroides* IBT-PQ genomic DNA was digested with *KpnI* and ligated, yielding circular DNA that was used as the template for PCR, obtaining an amplification product of ~3500 bp. Finally, a 5909 bp fragment containing the whole gene was amplified with primers PQ+4 (5'-CTTGTTCCTCAAAGCGCTAATC-3') and PQ-4 (5'-ACAGAACTTGACCCAGATGTG-3'). The G+C content was found to be similar to that of other *Leuconostoc* genes. The sequence of the entire *dsrP* gene was submitted to GenBank.

The *dsrP*-encoding gene and its C-terminal region (343 aa) were cloned in an *E. coli* expression vector under the control of the inducible promoter (araBAD). Using primers PQ+5 (5'-AGAAATAGAAATGTAACAAGCGT-3') and PQ-5 (5'-GCTTTTAATCAGCTCTCCAGA-3'), the *dsrP* gene was amplified, while the C-terminal region was amplified using the primers GBDDsrPfw (5'-CGCGGATCCGGGATGACATATTATTCTACAAGTGG-3') and GBDDsrPrev (5'-CCCAAGCTTGCTTTTAATCAGCTCTCCAGAATTGTC-3'). Both fragments were cloned in the pBAD-TOPO Thiofusion vector (Invitrogen), which adds thioredoxin in the N-terminal and a His-tag in the C-terminal regions, leading to plasmids pBAD-*dsrP* and pBAD-*dsrP*CTR. The expression was carried out in *E. coli* TOP10, after optimization of arabinose concentration. The Thio-DsrS-His was produced using the construction and procedure reported by Moulis *et al.* (2006b), and was kindly provided by Professor Magali Remaud-Simeon, INSA-Toulouse, France.

Preparation of recombinant DsrP, DsrS and GBD-DsrP in *E. coli*. *E. coli* Top10 harbouring the aforementioned constructions was grown to OD₆₀₀ 0.5. The cells were induced with 0.02 % (w/v) arabinose, incubated for 4 h and harvested by centrifugation (5000 g at 4 °C for 10 min). The resulting pellet was washed twice with 50 mM sodium acetate buffer, pH 5.2, and broken by ultrasonication with four 10 s pulses of 10 μ m with 3 min intervals. Cell debris was removed by centrifugation, and the cell extracts of DsrP and DsrS were assayed for activity. The initial rate of the dextranucrase reaction was measured at 30 °C in 50 mM sodium acetate buffer,

pH 5.2, in the presence of 0.3 M sucrose. The dextranase activity was measured by following the release of reducing sugar from sucrose by the 3,5-dinitrosalicylic acid (DNS) method. One activity unit (U) was defined as the amount of enzyme producing one micromole of fructose equivalent per minute. The amount of glucose produced by hydrolytic activity was found to be negligible as determined by HPLC. Denatured samples of the extracted proteins were electrophoretically separated on 6% polyacrylamide gels and stained with Coomassie Brilliant Blue. The proteins of the cellular extract were separated on SDS-PAGE gels, followed by pre-incubation in 50 mM sodium acetate buffer, pH 6.5, and 1% Tween 80. *In situ* polymer synthesis was then induced by overnight incubation in the same buffer supplemented with 0.3 M sucrose (Figures & Edwards, 1979) in order to identify the enzymes. Two SDS-PAGE activity gels were carried out in parallel, and after incubation with sucrose, the buffer was changed to 50 mM sodium acetate buffer, pH 6.0, and one of the two gels was treated with dextranase (Sigma) at 37 °C for 12 h, in order to identify those bands where dextran had been synthesized. GBD DsrP in cell extracts was analysed by Western blotting with anti-His antibodies. The Western blots were performed using the immunoblot detection method described by Coligan *et al.* (2007). Detection of C-terminal His-tags was carried out using antibodies from the QIAexpress anti-His HRP conjugate kit (Qiagen) and visualized with tetramethylbenzidine (TMB; Zymed).

Preparation of wild dextranases (DsrP and DsrE) and non-induced *L. mesenteroides* IBT-PQ cells for binding assays. The cell-associated wild enzymes from *L. mesenteroides* IBT-PQ and NRRL B-1299 were obtained from 500 ml cultures in LM medium (Chellapandian *et al.*, 1998) with sucrose as inducer. After 4–6 h of culture, the cells were centrifuged and extensively washed with 50 mM phosphate buffer, pH 6.5. The enzyme was then extracted by treatment of the pellet recovered in 12.5 ml 8 M urea at 25 °C for 1 h with occasional gentle shaking, as previously described (Olivares-Illana *et al.*, 2003). After centrifugation, the extract was dialysed against 10 mM phosphate buffer, pH 6.5, and analysed by SDS-PAGE. In order to prepare *L. mesenteroides* IBT-PQ cells devoid of the enzyme, cultures were prepared in LM broth supplemented with xylose as carbon source. After 8 h of growth, the cells were harvested and washed twice with 50 mM phosphate buffer, pH 6.5.

Binding assays. Binding assays were carried out in 5 ml experiments by placing into contact 0.5 mg ml⁻¹ of purified wild dextranase (DsrP or DsrE), recombinant DsrS or the His-tagged C-terminal region (not purified) with 2.5 mg ml⁻¹ of cells at 4 °C with shaking for 12 h. After incubation, the cells were pelleted by centrifugation and washed twice with 50 mM phosphate buffer, pH 6.5. Cells and supernatants were then subjected to electrophoresis and assayed for dextranase activity. In the case of GBD DsrP, the His-tag C-terminal region was detected by Western blotting with anti-His antibodies.

Molecular modelling. The DsrP protein sequence was submitted to BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>), Geno3d (<http://geno3d-pbil.ibcp.fr>) (Combet *et al.*, 2002) and SWISS-MODEL (<http://www.expasy.org/swissmod/SWISS-MODEL.html>) (Schwede *et al.*, 2003; Guex & Peitsch, 1997; Peitsch, 1995) to search for structural homologues. The alignments and models retrieved were used to construct a molecular model of DsrP C-terminal domains. Computer procedures were performed on a Silicon Graphics IRIS Solid Impact workstation. Modelling, graphic display, energy minimization, molecular dynamics and analysis were carried out with the program INSIGHT II, and with the HOMOLGY, BIOPOLYMER, DISCOVER and DOCKING packages (Accelrys). We used the default parameters for minimization (steepest, derivative 0.001, charges, CVFF force field) and molecular dynamics (equilibration 100, history 10, charges, temperature 300.00, time step 1.00, CVFF force field), except for the

number of iterations for minimization and steps for dynamics, in which we used 200 and 20 000 (20 psec), respectively. All minimizations and molecular dynamics were carried out with the molecule soaked with a 5 Å water layer.

RESULTS AND DISCUSSION

Molecular characterization of *L. mesenteroides* strain IBT-PQ dextranase gene

Based on sequence homology between conserved regions located in the catalytic core of different GS genes of Gram-positive bacteria, degenerate primers were designed and used in a PCR with chromosomal DNA of *L. mesenteroides* strain IBT-PQ as template. The resulting fragment was used as probe to obtain the 3' region by Southern blotting and the 5' region by inverse PCR, as described in Methods. One complete ORF was found in the compiled sequence of 4365 bp, and encoded a putative GS gene that we designated *dsrP*. A potential promoter sequence upstream from *dsrP* (79 bp from the start codon) was identified, with a -35 sequence (CTGACA) separated by 23 bp from a -10 sequence (TATGAT). Also, an ATG start codon preceded by an 8 bp upstream putative RBS (AGGGAA) was observed. A search of the entire nucleotide sequence revealed one incomplete ORF situated upstream of *dsrP* on the opposite strand. This ORF was preceded by a putative RBS (TGGGTC). From a BLAST search of the translated amino acid peptide sequence, an identity of 21% with the transcription regulator *rgg* from GtfD of *Streptococcus gordonii* was found (Vickerman *et al.*, 2001).

The *dsrP* gene, which encodes a protein of 1454 aa, with a deduced molecular mass of 160 586 Da and a pI of 4.5, was cloned and expressed. The highest dextranase activity was obtained when *E. coli* TOP10 (pBAD-*dsrP*) cells were incubated with 0.02% (w/v) arabinose, added at OD₆₀₀ 0.5. Under these conditions, after 4 h, the activity reached 0.06 U (mg protein)⁻¹. The polymers produced by recombinant DsrP, the wild-type enzyme produced by *L. mesenteroides* IBT-PQ, and the industrial strain *L. mesenteroides* NRRL B-512F used as a control, were readily digested after incubation of SDS-PAGE activity gels with dextranase, as described in Methods, demonstrating that they were all dextrans.

Alignment of the amino acid sequence of DsrP with that of other GSs by BLAST revealed clear similarities. The putative protein structure of DsrP contains (i) an N-terminal signal sequence of 41 aa, (ii) a relatively large variable N-terminal domain of 250 aa, (iii) a catalytic domain of 820 aa, and (iv) a C-terminal domain of 343 aa. The highest similarity (89% identity and 90% similarity) at the amino acid level was found with DsrT from *L. mesenteroides* NRRL B-512F. The gene *dsrT* encodes a dextranase-like protein that is truncated after the catalytic domain by the deletion of five nucleotides. The *dsrT* gene product does not synthesize dextran, but the insertion of the five nucleotides CAGAT in the *dsrT* gene (*dsrT5*) and its expression in *E. coli* resulted

61	D_ATTT_DKS	68
71	TNDKATTTADTS	82
83	TNDKATTTADTS	94
95	TNDKATTTADTS	106
107	TNDKATTTADIS	118
119	TNNKATTTADTS	130
131	TNNKAATTADTS	142
Consensus	TNDKATTTADTS	

Fig. 1. N-terminal region repeat units of the GS DsrP. Bold type indicates conserved amino acids.

in a 210 kDa enzyme (DsrT5) with dextransucrase activity (Funane *et al.*, 2000).

The deduced N-terminal amino acid sequence of DsrP contains a putative secretion peptide with a predicted signal peptidase cleavage site between amino acids 40 and 41 (<http://www.cbs.dtu.dk/services/SignalP/>). Within the deduced N-terminal variable region of DsrP, a series of seven repeat units with a mean length of 12 aa, similar to motif T present in DsrT described by Bozonnet *et al.* (2002), was found (Fig. 1). The same kind of repeat units with a different consensus (motif S) were also identified by those authors in DsrE, while in GTF33, nine repeats of 14 aa were found and designated TTQ repeats by Krajl *et al.* (2004b). Repeat units with a different consensus have also

been found in fructansucrases, such as inulosucrase IslA from *Leuconostoc citreum* (Olivares-Illana *et al.*, 2003), designated motif I, in levansucrase LevC (motif C) from *L. mesenteroides* ATCC 8293 (Olvera *et al.*, 2007), and in fructansucrase FtfA (motif F) from *Lactobacillus sanfranciscensis* (Tieking *et al.*, 2005).

The putative catalytic domain of DsrP showed high similarity (about 50 % identity and 60 % similarity) to other known *Streptococcus* and *Leuconostoc* GS catalytic domains. In DsrP this domain is found between amino acids 292 and 1111, and contains the essential conserved amino acids required for enzymic activity. Analysis of DsrP sequences suggests that the amino acids of the catalytic triad correspond to aspartic acids 473 and 589, and glutamic acid 511.

The C terminal in DsrP is formed by 343 aa, starting from position 1112. Five 65 aa imperfect sequences were identified in this region (Fig. 2a). Within these sequences, five type A repeats were found, according to the consensus sequences proposed by Russell (1990), also shown in Fig. 2(a). However, an alternative description of this region is possible from the consensus with the choline binding region (ChBR) or cell wall binding region (CWBR) of LytA reported by Fernández-Tornero *et al.* (2001). A comparison between DsrP C-terminal repeats and LytA CWBR is shown in Fig. 2(b). In this case, five putative repeats were also identified. CWBR repeats have been reported in DsrE from *L. mesenteroides* NRRL B-1299 (Bozonnet *et al.*,

(a)	
1124	KDTFIQDDKSNWYYFDKNGYMTYG--FQTVNDNN--YYFLPNGIELQDAILED SKGDVYYFN-QY GKQT ¹¹⁸⁷
1188	IDGYYMLANKTWRYFDKNGVMANAGLTTVTVDGQKHIIQYFDKNGIQVKGTSVKDADGKLR YFDTDSGEMV ¹²⁵⁷
1258	TNRFGKTDGTSWYFGADGIAVTG--AQTISGQK--LFFDADGQQIKGKEATDKKGKMHYYDADSGEMT ¹³²²
1323	TNRFEKLSDGSWMYFNKKGNVVTG--AQVINGQH--LFFESNGNQVKGREYATDGMRYDADSGDMV ¹³⁸⁷
1388	TNRFERISDGSWAYFGANGVAVTG--AQNINGQQ--LYFDTKGHQVKGAAVAQADGSQKYDANS GELIKS ¹⁴⁵
(b)	
LytA	
198	KFEK.ING.TWYYFD.SSG
219	RWRKHTDG.NWYWFD.NSG
240	GWKKIAD..KWYYFN.EEG
260	GWVKYKD..TWYYLDAKEG
282	AFIQSADGTGWYYLK.PDG
DsrP	
1126	TFIQDDKS.NWYYFD.KNG
1190	GYYMLANK.TWRYFD.KNG
1260	RFGKTDG.TSWYFG.ADG
1325	RFEKLSDG.SWMYFN.KKG
1390	RFERISDG.SWAYFG.ANG

Fig. 2. (a) A repeats (in boxes) and putative ChBRs (in bold type) found within the 65 aa imperfect repeat sequences in the DsrP C-terminal region. (b) Comparison between repeat sequences of the DsrP C-terminal domain and those of LytA ChBR (consensus in bold type).

2002), another cell-associated GS, in DsrB from *L. mesenteroides* and in Gtfl from *S. downei* (Fernández-Tornero *et al.*, 2001). Different functions have been suggested for the GS C-terminal domain, including roles in glucan polymerization, in glucan structure, in the transfer of products from the catalytic site, and in the cell-surface localization of GSs. However, the precise role of the C terminal remains unknown (Janecek *et al.*, 2000; Kato & Kuramitsu, 1991).

DsrP C-terminal region molecular model

A DsrP C-terminal domain model was constructed based on the known structures of the CWBR of LytA and ToxA (GenBank accession nos 1GVMB and 2F6EA) (Fernández-Tornero *et al.*, 2001, 2002; Ho *et al.*, 2005; Greco *et al.*, 2006). Both the Geno3D and the SWISS-MODEL programs retrieved equivalent structural models from C-terminal domain sequences. Although the DsrP C-terminal domain is longer than that of LytA CWBR, it was easy to model, as it may be described as a modular sequence of LytA CWBR motifs. All the different repeats were independently modelled and later joined into a single structure. This model was soaked with a 5 Å layer of water, energy-minimized and improved using molecular dynamics. This resulted in a DsrP C-terminal domain model with several

similarities to LytA CWBR but also with an important overall difference. LytA CWBR is an asymmetric heterodimer linked through a hydrophobic patch in each CWBR repeat, forming a structure similar to a boomerang (Fernández-Tornero *et al.*, 2001), while the DsrP C-terminal model is a continuous extended structure with a shape similar to a cigar, bent at its centre by $\sim 20^\circ$ (Fig. 3). As in LytA CWBR, there is a sequence regularity represented by the repeat units found in the DsrP C-terminal domain that fits well with the 'modular' character of the left-handed helix of CWBR in LytA. The DsrP C-terminal domain sequence has several imperfect repeats with a mean length of ~ 65 aa. These structural repeats are K¹¹²⁴-T¹¹⁸⁷, I¹¹⁸⁸-T¹²⁵⁸, N¹²⁵⁹-T¹³²³, N¹³²⁴-T¹³⁸⁸ and N¹³⁸⁹-S¹⁴⁵⁴ (Fig. 2a). Within these structural motifs, A repeats, usually present in these regions (Janecek *et al.*, 2000; Shah *et al.*, 2004), are found. Moreover, other repeat units found in this type of protein, such as IDGYYFD + N + G (von Eichel-Streiber *et al.*, 1992) and the Y-G repeats (Giffard & Jacques, 1994), are also contained in these structural motifs (Fig. 3). We also found several repeats in the DsrP sequence, with a YFDANG consensus, forming part of an equivalent β -hairpin structure in the model. All these repeats reflect the cyclic modular nature of the DsrP C-terminal model. Five regions similar to cell wall binding sites found in LytA CWBR were identified in the

Fig. 3. Upper panel, ribbon representation of the DsrP C-terminal region consisting of five 65 aa imperfect sequences; lower panel, localization of the Y-G and A repeats within the C-terminal repeats in the first two imperfect sequences. The figure was obtained with Insight II.

Fig. 4. Ribbon representation of the putative CWBR repeat binding pocket of DsrP, showing the residues probably involved in binding. The colour shows the type of amino acid residue: blue, basic; red, acidic; green, polar; white, non-polar. The figure was obtained with VMD.

DsrP C-terminal domain. In LytA, these sites serve to attach the protein through teichoic acids and LTAs to the cell wall of *S. pneumoniae* (Fernández-Tornero *et al.*, 2001, 2002). Repeats similar to cell wall binding pockets are formed in our model by a combination of five residues, mainly hydrophobic. This is illustrated in Fig. 4 for the fifth site, which is formed by F¹³⁹¹, W¹³⁹⁹, L¹⁴²⁰, Q¹⁴²⁸ and S¹⁴⁴⁸. In the same way, the cell wall binding pockets in LytA are formed by a combination of hydrophobic residues (F¹⁹⁹, W²⁰⁶, Y²²⁹, M²³⁷ and E²⁵⁴ for the first CWBR repeat in LytA) (Fernández-Tornero *et al.*, 2001).

Evidence in favour of our model prediction is provided by circular dichroism studies performed on the GBD region of GtfI from *S. downei*, in which a high β -sheet and loop content without any proportion of α -helix was found, in agreement with the LytA CWBR structure and with our model (Monchois *et al.*, 1999a).

Although the functionality of these sites remains to be clarified (see below), the model suggests that the C-terminal DsrP binds to the cell wall in *L. mesenteroides*, as all five putative cell wall binding sites are oriented to the same face of DsrP (Fig. 3). The hydrophobic and hydrophilic patterns in the DsrP C-terminal model are very similar to those found in LytA (not shown). The protein has hydrophilic residues located in the cleft formed by the β -hairpins of the

solenoid that join the glucan unit of the teichoic acid/LTA, as suggested by Fernández-Tornero *et al.* (2001). As glucan could also be linked to the hydrophilic residues in this region, this domain could therefore have the double function of joining DsrP to the *L. mesenteroides* cell wall and to the glucan matrix. In fact, this double function of the C-terminal domain in GSs has already been proposed for GtfI of *S. mutans* (Kato & Kuramitsu, 1991).

DsrP anchoring to the cell wall

In order to explore the ability of DsrP to anchor the *L. mesenteroides* cell wall, cells devoid of DsrP were produced in xylose cultures to avoid induction. Afterwards, the non-induced cells were placed in contact with a partially purified preparation of DsrP. Other GSs were also included in this experiment: DsrE from *L. mesenteroides* NRRL B-1299, which is also a cell-associated enzyme that contains CWBR repeats in the C-terminal domain; and DsrS from *L. mesenteroides* NRRL B-512F as a negative control, on the basis that it lacks CW repeats. After intensive washing it was found that both DsrP and DsrE were retained and active on the surface of *L. mesenteroides* cells, as shown in Fig. 5(a, b), while DsrS was not retained (Fig. 5c). This was demonstrated basically by enzyme activity assays of the cells and the enzyme solution, before and after contact among them. These results suggest that DsrE and DsrP CWBR repeat units are involved in cell attachment. As DsrE and DsrP also contain repeat units in the N-terminal region (E and T motifs, respectively), binding could also result from attachment mediated by interactions through this region. In fact, in a similar experiment, LevC, a levansucrase from *L. mesenteroides* ATCC 8293 that has repeats (C motif) in the N terminus, did not attach to the cell wall.

In LytA, the CWBR repeats recognize choline in the LTA that serves to anchor the protein to *S. pneumoniae* cells. In order to determine whether the CWBR repeats in DsrP have a similar function, the binding experiments described above were performed in the presence of choline, phosphorylcholine and PAF-18 (an antagonist of choline). Contrary to what was expected, the binding of DsrP to the cell wall was not inhibited in the presence of choline or the two analogous compounds. Similarly, DsrP was not retained by DEAE-cellulose, an affinity support commonly used for the purification of choline-binding proteins. These results suggest that the attachment of DsrP to the *L. mesenteroides* cell wall is not mediated by the LTA choline moieties. In fact, the cell envelope of *L. mesenteroides* contains only traces of choline (<2%), phosphatidyl glycerol (50%) being the major component of LTA phospholipids.

The DsrP C terminal is involved in anchoring to the *L. mesenteroides* cell wall

In order to demonstrate that DsrP is bound to the *L. mesenteroides* cell wall through the C-terminal domain, a

Fig. 5. Binding assays of dextransucrases with (a) DsrP, (b) DsrE and (c) DsrS. Lanes: 1, molecular weight marker; 2, partially purified dextransucrase; 3, non-induced *L. mesenteroides* cells; 4, washed non-induced cells after contact with dextransucrase; 5, supernatant after contact with dextransucrase.

Fig. 6. Western blot analyses of the His-tagged DsrP C-terminal fragment placed in contact with non-induced *L. mesenteroides* IBT-PQ cells. Lanes: 1, molecular weight marker; 2, non-induced *L. mesenteroides* IBT-PQ cells; 3, His-tagged DsrP C-terminal fragment in solution; 4, His-tagged DsrP C-terminal fragment in solution after contact with non-induced cells; 5, non-induced *L. mesenteroides* IBT-PQ cells after contact with the His-tagged DsrP C-terminal fragment; 6, His-tagged DsrP C-terminal fragment in solution after contact with non-induced cells in the presence of 10% Dextran T10; 7, non-induced *L. mesenteroides* IBT-PQ cells after contact with His-tagged DsrP C-terminal fragment cells in the presence of 10% Dextran T10.

His-tagged DsrP C-terminal fragment of 343 aa (38 kDa) was expressed in *E. coli*. Contact experiments similar to those performed with the whole protein were carried out with the single C-terminal region of DsrP. As shown in Fig. 6, the attachment of the DsrP C-terminal domain to the non-induced *L. mesenteroides* IBT-PQ cells was detected by Western blot analysis using anti-His antibodies, demonstrating that this domain binds to the cell wall. This is probably the function of the C-terminal domain in other cell-associated GSs, such as alternansucrase, DsrE (which in a previous experiment was demonstrated to bind *L. mesenteroides* IBT-PQ cells) and GtfB from *S. mutans*, which contains CWBR repeats in its C-terminal domain.

It has been suggested that dextran associates with the A repeats in dextransucrase: Funane *et al.* (1998) isolated four peptides bound to glucan from *L. mesenteroides* NRRL B-512F dextransucrase, one of these peptides having an A repeat sequence. Based on the reaction products of truncated versions of DsrS, Moulis *et al.* (2006a) have suggested that A repeats interact with dextran during polymer synthesis, and possibly support the anchoring of the growing polymer to the enzyme surface. Considering this additional function of the C-terminal domain, an experiment was performed to find out if cell-anchoring and dextran binding properties are mutually exclusive. Cell-contact assays of the His-tagged DsrP C-terminal region were therefore performed in the presence of 10% Dextran T10, in order to determine whether dextran inhibits cell

wall anchoring. It was found that in spite of the dextran, the C-terminal region retained the ability to anchor to the cell surface, demonstrating that the protein cell wall binding sites do not interact with dextran, as suggested in the structural model. This may be the consequence of different sites for glucan and cell wall binding. Further experiments are required to demonstrate the nature of the interactions among DsrP, glucan and the cell wall.

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