Division protein interaction web: identification of a phylogenetically conserved common interactome between *Streptococcus pneumoniae* and *Escherichia coli*

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The ability of each of the 11 *Streptococcus pneumoniae* division proteins to interact with itself and with each of the remaining proteins was studied in 66 combinations of protein pairs, using a bacterial two-hybrid system. Interactions (homo- or hetero-dimerizations) were detected between 37 protein pairs, whereas 29 protein pairs did not interact. In some cases, positive interactions of the *S. pneumoniae* proteins were confirmed by co-immunoprecipitation experiments in *Escherichia coli*. Comparison between the *S. pneumoniae* division protein interaction web and that of *E. coli*, the only micro-organisms for which the whole division interactome has been described systematically, was also performed. At least nine division proteins, ZapA, FtsZ, FtsA, FtsK, FtsQ/DivIB, FtsB/DivIC, FtsL, FtsI and FtsW, are believed to have a conserved function between these bacteria and thus we may say that a significant part of the interactions are conserved. Out of 45 protein pairs tested in both bacteria, 30 showed the same behaviour: 23 interacted while seven did not. In agreement with these results, cross-interactions between *S. pneumoniae* proteins and the corresponding *E. coli* orthologues were observed. Taken together, these results suggest a phylogenetically conserved minimal common interactome of the division proteins.

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INTRODUCTION

The division machinery of Eubacteria is made through the formation of a macromolecular complex containing at least twelve proteins, which assemble with a defined dependence hierarchy at the site of division. The organization of the genes coding for division proteins is generally conserved, and many different bacteria, including Gram-negative and Gram-positive rods and cocci, possess a chromosomal region similar to that found at the 2 min region of the *Escherichia coli* chromosome (Yura *et al.*, 1992; Ayala *et al.*, 1994). This region contains many genes whose products are involved in division and cell wall biosynthesis and is therefore known as the *dcw* (division and cell wall) cluster (Ayala *et al.*, 1994; Vicente & Errington, 1996). This cluster, which is very similar in organization in many Gram-negative bacteria, shows various differences in Gram-positive bacteria, particularly in cocci (Tamames *et al.*, 2001).

Despite the differences in *dcw* cluster organization, the major proteins involved in the division process appear to be conserved. In particular, at least eight proteins are present in almost all cell-wall-containing Eubacteria – FtsZ, FtsA, FtsK, FtsQ/DivIB, FtsB/DivIC, FtsL, FtsW and FtsI – although additional proteins may also be present, depending on the bacterial species (Margolin, 2000; Errington *et al.*, 2003; Harry *et al.*, 2006).

It has been shown that in many species, the cell division proteins, organized by the tubulin-like FtsZ, assemble at the division site to form a molecular division machinery, the septosome or divisome. FtsZ, in fact, directs the cytokinesis process, constituting a scaffold for the other division proteins, which, as determined for the best-characterized rod-shaped model organisms *E. coli* and *Bacillus subtilis*, are recruited hierarchically at the mid-cell to form the septal machinery (Errington *et al.*, 2003; Margolin, 2003; Weiss, 2004; Goehring & Beckwith, 2005; Harry *et al.*, 2006; Vicente *et al.*, 2006; Erickson, 2007). As proposed recently, the assembly of the divisome components

Abbreviation: THA, two-hybrid assay.

is a multistage process, which proceeds in a concerted way (Goehring *et al.*, 2006; Vicente & Rico, 2006).

In *E. coli*, the early event is marked by the interactions of FtsZ with FtsA and ZipA, which assemble in the cytoplasmic membrane (for review see Vicente *et al.*, 2006). After the addition of FtsK, which completes the formation of the cytoplasmic ring, a periplasmic connector formed by the complex FtsQ–FtsB–FtsL is added (Buddelmeijer & Beckwith, 2004), followed by the proteins involved in manufacturing the septal peptidoglycan, FtsW and FtsI. Finally, FtsN is recruited to the septosome (Vicente *et al.*, 2006).

Despite the differences observed in some aspects of the division mechanism in different bacteria, one common thread is the role of FtsZ as a cell division organizer. Moreover, the similar conservation of many division proteins suggests that they should all be recruited at the Z-ring by a common mechanism.

Streptococcus pneumoniae is a developing model for studying cell division in Gram-positive pathogens. Since the *dcw* cluster was identified (Massidda *et al.*, 1998), considerable progress has been made in understanding the basic mechanism of septum formation in this Gram-positive coccus. In particular, a number of cell division proteins have now been identified in *S. pneumoniae* and shown to localize at the mid-cell to form the septal machinery, consistent with what is known for the rod-shaped model bacteria (Errington *et al.*, 2003; Weiss, 2004; Goehring & Beckwith, 2005; Vicente *et al.*, 2006). These proteins include the cell division initiator proteins, such as FtsZ and FtsA, required at the early stages of the process (Massidda *et al.*, 1998; Morlot *et al.*, 2003; Lara *et al.*, 2005), some of the later players FtsQ/DivIB, FtsB/DivIC, FtsL, FtsW, PBP2x and PBP1A (Massidda *et al.*, 1998; Morlot *et al.*, 2003, 2004; Noirclerc-Savoye *et al.*, 2005; Zapun *et al.*, 2008), which constitute the septal markers in *S. pneumoniae* cells, and most recently DivIVA, which appears to be required for septum completion and pole maturation (Fadda *et al.*, 2007).

Despite this progress many aspects of cell division in *S. pneumoniae* still remain to be clarified, in particular the sequence of the events necessary for septum formation, including the order of recruitment of the proteins to the cell division site. In addition, very little is known about the complex pattern of interactions among the different players that constitute the divisome.

In model organisms, particularly *E. coli*, the progression of knowledge has been achieved through the establishment of the requirement of a protein for the sequential localization of the others that follow. In *S. pneumoniae*, the unavailability of specific thermosensitive or conditional division mutants has so far limited the possibility of establishing precisely the hierarchical order of recruitment, if any, and the interdependence of the division proteins at the mid-cell. Moreover, although interaction between the strep-

tococcal FtsA and FtsZ has been detected (Lara *et al.*, 2005) and biochemical evidence for a trimeric complex, formed by FtsQ/DivIB, FtsL and FtsB/DivIC, has been reported (Noirclerc-Savoye *et al.*, 2005), no other information is available on protein–protein interaction between the other players.

To clarify the different and common pathways of the division mechanism in *S. pneumoniae*, we studied the division protein interaction network, using a bacterial two-hybrid assay, applied previously to study protein–protein interaction among the cell division players in *E. coli* (Di Lallo *et al.*, 2003) and, more recently, to identify the interactive partners of the *S. pneumoniae* DivIVA protein (Fadda *et al.*, 2007). Moreover, we compared the streptococcal division interaction web with that of *E. coli* and found that a significant number of the interactions were conserved. Consistently, several cross-interactions between *S. pneumoniae* proteins and the corresponding *E. coli* orthologues were observed. Taken together, these results indicate the presence of a minimum division interactome in common between these two, phylogenetically distinct, micro-organisms.

METHODS

Bacterial strains and growth conditions. The *S. pneumoniae* Rx1 strain, a non-encapsulated well-characterized laboratory strain, was used as a source of DNA, as described previously (Fadda *et al.*, 2007). *E. coli* K-12 and derivatives, used and generated in this work, are listed in Table 1. Luria–Bertani (LB) broth or agar was used for bacterial culture and plating and SM (salt solution) was used for bacterial dilutions as described by Miller (1972). The following antibiotics (Sigma) were added to the culture media when required: ampicillin (50 µg ml⁻¹), chloramphenicol (34 µg ml⁻¹) and kanamycin (30 µg ml⁻¹).

DNA manipulation. Standard procedures were used for small-scale plasmid preparations, endonuclease digestion, ligation, agarose gel electrophoresis, elution of DNA fragments from agarose and bacterial transformation (Sambrook *et al.*, 1989). PCR was carried out using the *Taq* DNA polymerase kit (Promega), according to the recommendations of the manufacturer. Synthetic oligonucleotides for *S. pneumoniae* and for *E. coli*, used for gene amplification in the construction of plasmids for the two-hybrid assay, are reported in Fadda *et al.* (2007) and D’Ullisse *et al.* (2007), respectively. The upstream and downstream primers, containing the restriction sites for *SalI* and *BamHI* (underlined), for amplification of *pbp2B* were spnSFbp2B (5′-GCGTCGACCATGAGAAAATTTAACAGCCATTC-3′) and spnBRbp2B (5′-CGCGGATCCGCAATAGGTGTTGGATAA-AGCA-3′); the start and stop codons, respectively, are indicated in bold.

Plasmids construction and bacterial two-hybrid assay. Recombinant plasmids (listed in Table 1) were constructed by cloning the entire genes of interest into the *SalI* and *BamHI* restriction sites of pCI_{P22} and pCI₄₃₄ vectors (Di Lallo *et al.*, 2001). The DNA of the gene of interest was obtained by PCR amplification using specific oligonucleotides carrying compatible restriction sites at the ends. All the different combinations of recombinant plasmids coding for the chimeric repressors, obtained as described above, were cotransformed into the recipient *E. coli* strain R721, carrying the 434/P22 chimeric operator. β-Galactosidase activity was assayed as described by Miller (1972). Bacterial cultures were grown at 34 °C

Table 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant genotype	Source
<i>E. coli</i> strains		
71/18	<i>supE thy Δ(lac proAB)/F' (proAB⁺ lacI^f lacZΔM15)</i>	Lab collection
DH5α	<i>supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi1 relA1</i>	Lab collection
R721	71/18 <i>glpT::OP₄₃₄/P₂₂lacZ</i>	Di Lallo <i>et al.</i> (2001)
Plasmids		
pTTQ18 <i>gfp</i>	pTTQ18 derivative harbouring <i>gfp</i> gene	D'Ulisse <i>et al.</i> (2007)
pBAD33 <i>gst</i>	pBAD33 derivative harbouring <i>gst</i> gene	D'Ulisse <i>et al.</i> (2007)
pTTQ18 <i>gfp</i> -spnftsZ	pTTQ18 <i>gfp</i> derivative harbouring <i>ftsZ</i> gene of <i>S. pneumoniae</i>	Fadda <i>et al.</i> (2007)
pTTQ18 <i>gfp</i> -spnftsW	pTTQ18 <i>gfp</i> derivative harbouring <i>ftsW</i> of <i>S. pneumoniae</i>	This work
pTTQ18 <i>gfp</i> -spnbp2x	pTTQ18 <i>gfp</i> derivative harbouring <i>pbp2x</i> of <i>S. pneumoniae</i>	This work
pTTQ18 <i>gfp</i> -spnftsA	pTTQ18 <i>gfp</i> derivative harbouring <i>ftsA</i> of <i>S. pneumoniae</i>	This work
pBAD33 <i>gst</i> -spndivIC	pBAD33 <i>gst</i> derivative harbouring <i>divIC</i> of <i>S. pneumoniae</i>	This work
pBAD33 <i>gst</i> -spnftsW	pBAD33 <i>gst</i> derivative harbouring <i>ftsW</i> of <i>S. pneumoniae</i>	This work
pBAD33 <i>gst</i> -spnftsK	pBAD33 <i>gst</i> derivative harbouring <i>ftsK</i> of <i>S. pneumoniae</i>	This work
pBAD33 <i>gst</i> -spnftsL	pBAD33 <i>gst</i> derivative harbouring <i>ftsL</i> of <i>S. pneumoniae</i>	This work
pCl _{P22} -ecftsZ	pCl _{P22} derivative harbouring <i>E. coli ftsZ</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -ecftsZ	pCl ₄₃₄ derivative harbouring <i>E. coli ftsZ</i>	Di Lallo <i>et al.</i> (2003)
pCl _{P22} -ecftsA	pCl _{P22} derivative harbouring <i>E. coli ftsA</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -ecftsA	pCl ₄₃₄ derivative harbouring <i>E. coli ftsA</i>	Di Lallo <i>et al.</i> (2003)
pCl _{P22} -ecftsK	pCl _{P22} derivative harbouring <i>E. coli ftsK</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -ecftsK	pCl ₄₃₄ derivative harbouring <i>E. coli ftsK</i>	Di Lallo <i>et al.</i> (2003)
pCl _{P22} -ecftsQ	pCl _{P22} derivative harbouring <i>E. coli ftsQ</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -ecftsQ	pCl ₄₃₄ derivative harbouring <i>E. coli ftsQ</i>	Di Lallo <i>et al.</i> (2003)
pCl _{P22} -ecftsL	pCl _{P22} derivative harbouring <i>E. coli ftsL</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -ecftsL	pCl ₄₃₄ derivative harbouring <i>E. coli ftsL</i>	Di Lallo <i>et al.</i> (2003)
pCl _{P22} -ecftsW	pCl _{P22} derivative harbouring <i>E. coli ftsW</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -ecftsW	pCl ₄₃₄ derivative harbouring <i>E. coli ftsW</i>	Di Lallo <i>et al.</i> (2003)
pCl _{P22} -eczapA	pCl _{P22} derivative harbouring <i>E. coli zapA</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -eczapA	pCl ₄₃₄ derivative harbouring <i>E. coli zapA</i>	Di Lallo <i>et al.</i> (2003)
pCl ₄₃₄ -ecftsB	pCl ₄₃₄ derivative harbouring <i>E. coli ftsB</i>	D'Ulisse <i>et al.</i> (2007)
pCl _{P22} -ecftsB	pCl _{P22} derivative harbouring <i>E. coli ftsB</i>	D'Ulisse <i>et al.</i> (2007)
pCl _{P22} -spnftsZ	pCl _{P22} derivative harbouring <i>S. pneumoniae ftsZ</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnftsZ	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae ftsZ</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnftsA	pCl _{P22} derivative harbouring <i>S. pneumoniae ftsA</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnftsA	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae ftsA</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnzapA	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae zapA</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnzapA	pCl _{P22} derivative harbouring <i>S. pneumoniae zapA</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnftsK	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae ftsK</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnftsK	pCl _{P22} derivative harbouring <i>S. pneumoniae ftsK</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spndivIB	pCl _{P22} derivative harbouring <i>S. pneumoniae divIB</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spndivIB	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae divIB</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spndivIC	pCl _{P22} derivative harbouring <i>S. pneumoniae divIC</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spndivIC	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae divIC</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnftsL	pCl _{P22} derivative harbouring <i>S. pneumoniae ftsL</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnftsL	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae ftsL</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnftsW	pCl _{P22} derivative harbouring <i>S. pneumoniae ftsW</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnftsW	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae ftsW</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnbp2X	pCl _{P22} derivative harbouring <i>S. pneumoniae pbp2X</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnbp2X	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae pbp2X</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnbp1A	pCl _{P22} derivative harbouring <i>S. pneumoniae pbp1A</i>	Fadda <i>et al.</i> (2007)
pCl ₄₃₄ -spnbp1A	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae pbp1A</i>	Fadda <i>et al.</i> (2007)
pCl _{P22} -spnbp2B	pCl _{P22} derivative harbouring <i>S. pneumoniae pbp2B</i>	This work
pCl ₄₃₄ -spnbp2B	pCl ₄₃₄ derivative harbouring <i>S. pneumoniae pbp2B</i>	This work

in LB medium supplemented with 0.1 mM IPTG to OD₆₀₀ 0.5, and the residual β -galactosidase activity was evaluated for each strain. *E. coli* R721 without plasmids and *E. coli* R721 with plasmids pC₄₃₄434 and pC_{P22}434 were used as negative and positive controls, respectively. Residual β -galactosidase activity of less than 50 % was judged to indicate that there was repression and hence a protein–protein interaction, whereas when the activity was greater than 50 %, the interaction was uncertain or null. The use of these values has been discussed previously (Di Lallo *et al.*, 2003; D’Ulisse *et al.*, 2007; Fadda *et al.*, 2007).

Construction of GFP and GST derivatives for co-immunoprecipitation studies. The division proteins fused with GFP (green fluorescent protein of *Aequorea victoria*) were obtained by in-frame cloning of the PCR-produced DNA fragments of the corresponding gene into *Pst*I and *Hind*III sites downstream of the *gfp* gene previously inserted into the *Eco*RI and *Pst*I sites in the plasmid pTTQ18 to give the plasmid pTTQ18-*gfp* (D’Ulisse *et al.*, 2007; Fadda *et al.*, 2007; Table 1). Analogously, the GST (glutathione S-transferase of *Schistosoma japonicum*) derivatives were obtained by cloning the gene of interest into *Sal*I and *Hind*III sites downstream of the *gst* gene inserted into the *Sac*I and *Sal*I sites of the plasmid pBAD33 to give the plasmid pBAD33-*gst* (D’Ulisse *et al.*, 2007; Table 1).

Co-immunoprecipitation experiments. To prepare the extracts for co-immunoprecipitation experiments, cultures of *E. coli* DH5 α , harbouring the recombinant plasmids containing the genes of interest fused in-frame to GST and GFP, were grown in LB supplemented with the appropriate antibiotics to OD₆₀₀ 0.3–0.4. The expression of the tagged genes was induced by adding 0.1 mM IPTG (for the GFP-fused genes) and 0.2 % arabinose (for the GST-fused gene) and the culture was grown for 4 h. The culture was then centrifuged, washed and resuspended (1/300, v/v) in lysis buffer (1 mM EDTA pH 8, 25 mM HEPES pH 7.6, 0.1 mg lysozyme ml⁻¹), cooled on ice for 30 min, and lysed by freezing and thawing. The protein concentration, determined by the Bradford method after cell lysis, was adjusted to 10 mg ml⁻¹ and 1 ml aliquots of lysed samples were centrifuged at 50 000 g for 45 min. Soluble fractions were obtained by recovering the supernatant, and membrane fractions were prepared as described by Buddelmeijer & Beckwith (2004). Immunoprecipitation experiments were similar to those described by Duong & Wickner (1997). The beads (Protein A-Sepharose) were divided into aliquots of 50–100 μ l in microcentrifuge tubes; 10 μ l anti-GFP (1:100) (Santa Cruz Biotechnology) or anti-GST (1:100) (Santa Cruz Biotechnology) mouse monoclonal antibodies were added to each tube. The samples were incubated overnight at 4 °C with gentle mixing on a suitable shaker. After centrifugation, samples were washed with 1 ml washing buffer (20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 0.1 % Triton X-100 and 10 %, v/v, glycerol), then 0.1–1 ml cell lysate was added to each tube. Samples were incubated from 90 min to overnight at 4 °C, with gentle mixing on a suitable shaker. Immunoprecipitated complexes were collected by centrifugation at 3000 g for 2 min at 4 °C.

Western blotting. Electrophoresis and immunoblotting were performed as described by Laemmli (1970) and Towbin *et al.* (1979), respectively. The nitrocellulose membranes were probed with anti-GST antibodies (Santa Cruz Biotechnology) (1:2000) or with mouse anti-GFP monoclonal antibodies (1:1000) (Santa Cruz Biotechnology), depending on the specific GFP- or GST-fused protein used in the experiment, and detected with the ECF fluorescence-based detection system (Amersham, Pharmacia). The specific polyclonal antibodies raised against *S. pneumoniae* division proteins DivIB, FtsL, FtsK, FtsW, PBP2x, PBP1A and PBP2B used in Western blot experiments were generously supplied by A. Zapun and T. Vernet of the Institut de Biologie Structurale, CNRS, Grenoble, France.

RESULTS

Identification of *S. pneumoniae* division protein interaction network

A bacterial two-hybrid assay (THA), developed previously in our laboratory and used successfully to describe the *E. coli* division protein interaction web (Di Lallo *et al.*, 2003), was applied to dissect the analogous pathway in *S. pneumoniae*. In this assay, the interaction between two proteins, encoded by the corresponding genes cloned in the two recombinant plasmids, is tested by their ability to form a chimeric lambdaoid repressor. When the gene products interact they shut down the expression of a chromosomal copy of the reporter gene, *lacZ*, whose synthesis is governed by a hybrid promoter/operator region, recognized by the chimeric repressor consisting of the N-terminal domain of the lytic phage repressors from either of two lambdaoid phages 434 and P22 fused in-frame with the pair of division proteins whose interaction is under investigation (Di Lallo *et al.*, 2001). In particular, we tested the cell division proteins that are known or are thought to participate in divisome formation in *S. pneumoniae*, like FtsZ, FtsA, ZapA, FtsK, FtsQ/DivIB, FtsB/DivIC, FtsL, FtsW, PBP2x and PBP1A. In addition, we tested PBP2B, an essential monofunctional transpeptidase, the gene of which in *S. pneumoniae* is located in the *dcw* cluster with *ftsA* and *ftsZ* (Massidda *et al.*, 1998).

Since *S. pneumoniae* fusion proteins were expressed in a heterologous system, we first tested the correct expression of those proteins for which specific antibodies were available, namely FtsZ, FtsA, FtsL, FtsQ/DivIB, PBP1A, PBP2B and PBP2x, by Western blot experiments (data not shown). Although the Western blot results cannot be generalized for the other *S. pneumoniae* proteins for which antibodies were not available, since all the proteins tested interact with at least one division partner, as shown in Table 2, such interaction should account for correct expression of the recombinant protein.

THA results are presented as the ratio between the β -galactosidase units (Miller units) produced by the strain harbouring the two recombinant plasmids and that of the same strain with plasmid vectors alone, as a control. We interpret β -galactosidase percentages lower than 50 % to represent an interaction between the two proteins under investigation whereas percentages over 50 % indicate a lack of interaction. Values close to 50 % could represent weak or transient interactions. The rationale for this cut-off is reported in previous work (Di Lallo *et al.*, 2003; D’Ulisse *et al.*, 2007; Fadda *et al.*, 2007).

The results reported in Table 2 highlight that, out of 66 combinations among the *S. pneumoniae* cell division proteins tested, 37 show homo or hetero-dimerizations, while 29 did not interact, either with themselves or with each other. Of the streptococcal cell division proteins shown in Table 2, 18 (i.e. 14 positive interactions and four non-interactions) were consistent with those established

Table 2. Homo- and hetero-dimerization of 11 *S. pneumoniae* known or putative cell division proteins

Sixty-six possible combinations of recombinant plasmids were tested to reconstruct a functional 434-P22 chimeric repressor able to bind the chimeric operator O-P_{434/P22} of *E. coli* strain R721, where it controls the expression of the *lacZ* reporter gene. Each of these plasmids carries the N-terminal domain of the 434 or P22 phage repressor fused in-frame with the coding sequence of one of the 11 streptococcal cell division genes. The effective binding to the operator sequence is deduced by the repression of β -galactosidase synthesis, evaluated by the ratio between the Miller units of β -galactosidase produced by strain R721 carrying each pair of plasmids and that of R721 without plasmids (Di Lallo *et al.*, 2003). The assay was performed as described in Methods. The results represent the means of at least five independent determinations, in which standard deviation did not exceed ± 4 . The residual β -galactosidase activity of the positive and negative controls was 5 % and 100 %, respectively. Values of less than 50 % (in bold) indicate an interaction between the two proteins under investigation. Similar results were obtained when the genes were cloned in the reciprocal vectors (not shown).

pCl ₄₃₄	pCl _{P22}										
	FtsA	FtsZ	ZapA	FtsK	FtsQ/ DivIB	FtsB/ DivIC	FtsL	FtsW	PBP2x	PBP1A	PBP2B
FtsA	19										
FtsZ	22	18									
ZapA	9	5	9								
FtsK	32	13	38	36							
FtsQ/ DivIB	58	27	5	23	30						
FtsB/ DivIC	71	16	17	61	81	35					
FtsL	26	17	14	30	15	58	29				
FtsW	53	31	27	31	40	34	20	55			
PBP2x	22	54	55	72	88	64	30	95	30		
PBP1A	73	53	55	84	77	67	22	53	86	100	
PBP2B	100	18	25	100	96	33	22	53	100	52	84

previously for *E. coli*, by two different THAs (Di Lallo *et al.*, 2003; Karimova *et al.*, 2005) and co-immunoprecipitation experiments (D'Ulisse *et al.*, 2007). Some of these interactions, like those of the *S. pneumoniae* FtsA with itself and with FtsZ, were expected, confirming and extending our previous results (Lara *et al.*, 2005), by using a yeast two-hybrid system, and thus providing additional reliable positive controls. Moreover, the predicted FtsZ self-interaction, which could not be determined before, due to the activation of the reporter gene of single transformants producing the FtsZ protein when fused to the GAL4-binding domain (Lara *et al.*, 2005), was detected. Besides FtsA and FtsZ, other self-interacting cell division proteins were FtsQ/DivIB, FtsB/DivIC, PBP2x, FtsK, FtsL and ZapA, consistent with the same behaviour shown by the *E. coli* counterparts (Di Lallo *et al.*, 2003; P. Ghelardini, unpublished results; see below).

As shown in Table 2, a total of 29 hetero-interactions were detected among *S. pneumoniae* proteins and of these seven, FtsA–FtsK, FtsA–FtsL, FtsZ–FtsW, FtsZ–FtsQ/DivIB, FtsZ–FtsL, FtsK–FtsW, FtsL–FtsI/PBP2x, have not been reported previously in *E. coli* (Di Lallo *et al.*, 2003; Karimova *et al.*, 2005), although a positive FtsZ–FtsW interaction was found in *Mycobacterium tuberculosis* (Datta *et al.*, 2002). Interestingly, it has been suggested recently that binding of FtsZ to the C-tail of FtsW may modulate its interactions with FtsI, regulating septal peptidoglycan biogenesis (Datta

et al., 2006). It would be interesting to determine if this is also the case for *S. pneumoniae*, where surprisingly no interaction between FtsW and any of the PBPs tested was detected.

Two other hetero-interactions, FtsZ–FtsB(DivIC) and FtsW–FtsB(DivIC), were identified and seemed to be specific of *S. pneumoniae* (see below).

Among the PBPs, PBP2B was found to interact strongly with FtsZ and ZapA, further supporting the presence of this PBP at the mid-cell, as reported recently (Zapun *et al.*, 2008; D. Fadda & O. Massidda, unpublished results).

Co-immunoprecipitation of interacting protein pairs identified by THA

To confirm the results of the THA, co-immunoprecipitation was used. Because of the large number of interactions detected between the proteins pairs examined, only some of them were tested with this additional method. In particular, we chose to confirm those interactions that were shown only in *S. pneumoniae* and not detected previously in *E. coli*, namely: FtsA–FtsK, FtsA–FtsL, FtsZ–FtsW, FtsZ–FtsQ/DivIB, FtsZ–FtsL, FtsK–FtsW, FtsL–PBP2x, FtsZ–FtsB/DivIC and FtsW–FtsB/DivIC.

Since specific antibodies were not available for all these studied proteins, the assays were performed on the division

proteins fused with tags, i.e. the GFP and/or GST for which commercial antibodies are available and the specificity of the assay has already been demonstrated (D'Ulisse *et al.*, 2007; Fadda *et al.*, 2007).

The results, presented in Fig. 1, show that, as expected from the THA, an interaction was observed for each of the eight pairs of proteins tested. On the other hand, control experiments with non-interacting protein pairs, like FtsL–FtsB, FtsK–PBP2x and FtsW–FtsA, did not co-immunoprecipitate (data not shown).

An interaction network genetically conserved in *S. pneumoniae* and *E. coli*

E. coli is the only micro-organism for which the whole interaction web of the divisome proteins has been described (Di Lallo *et al.*, 2003; Karimova *et al.*, 2005; D'Ulisse *et al.*, 2007), and therefore the only possible reference with which to compare the data from *S. pneumoniae* described above. As noted, these two species share at least nine division proteins and to compare the two interaction webs, we used the data

already published on *E. coli* (Di Lallo *et al.*, 2003) integrated with the data on new interactions detected for *E. coli* ZapA and FtsB proteins with themselves and with the other *E. coli* division partners. The results, reported in Table 3, show that ZapA interacted with itself and with all the other cell division proteins tested except FtsI, while FtsB interacted with itself, ZapA, FtsQ, FtsL and FtsI.

In addition, given the large number of interactions shown by *S. pneumoniae* FtsL (FtsL_{SPN}), the interaction profile of the FtsL *E. coli* (FtsL_{EC}) counterpart was re-evaluated. While we found consistent results confirming the hetero-interactions already reported (Di Lallo *et al.*, 2003), the ability of FtsL_{EC} to self-interact was also detected, given the 22 % residual β -galactosidase activity, thus correcting the value reported previously (Di Lallo *et al.*, 2003).

As shown in Fig. 2, in both organisms it is possible to identify a complex interaction network, in which each protein interacts with at least one, but more often with several partners. Although it was clear that in *S. pneumoniae* the interactions are more numerous than

Fig. 1. Co-immunoprecipitation to determine the specificity of *S. pneumoniae* cell division protein interactions. Immunoprecipitation of GFP-FtsA–GST-FtsK (lanes 1–2) and GFP-FtsA–GST-FtsL (lanes 3–4), GST-FtsW–GFP-FtsZ (lanes 5–6), GST-FtsL–GFP-FtsZ (lanes 7–8), GST-DivIC–GFP-FtsZ (lane 9–10), GST-FtsK–GFP-FtsW (lanes 11–12), GST-DivIC–GFP-FtsW (lanes 13–14), and GFP-PBP2x–GST-FtsL (lanes 15–16) complexes is shown. Protein extracts were precipitated with anti-GST (lanes 1, 3, 6, 8, 10, 12, 14 and 15) or anti-GFP (lanes 2, 4, 5, 7, 9, 11, 13 and 16) antibodies and detected with anti-GFP or anti-GST antibodies, respectively. In the case of the interaction between DivIB and FtsZ, the extract was precipitated with anti-GFP antibodies (directed to GFP_DivIB) and the co-immunoprecipitate detected with polyclonal anti-FtsZ antibodies (lane 17). Lanes M contain molecular mass markers. Immunoprecipitation results for non-interacting protein pairs are not shown.

Table 3. Interactions between the *E. coli* FtsB and ZapA proteins with themselves and with the other division proteins

The interaction of ZapA with FtsA and FtsZ have been described previously (Di Lallo *et al.*, 2003). The results represent the means of at least five independent determinations, in which standard deviation did not exceed ± 4 . Values of less than 50 % (in bold) indicate an interaction between the two proteins under investigation, as reported in Methods.

	FtsA	FtsZ	FtsK	FtsQ	FtsB	FtsL	FtsW	FtsI	ZapA
FtsB	85	90	67	36	21	27	63	30	
ZapA	25	19	8	29	5	6	8	85	2

those in *E. coli*, 71 % vs 64 %, respectively, a closer look revealed a high similarity. Indeed, a large part of the interaction pattern between these two organisms seemed to be conserved. Out of 45 protein pairs tested for each bacterium, 30 show the same behaviour: 23 interacted and seven did not. Notably, all eight homo-interactions were conserved in both organisms. On the other hand, nine interactions appeared specific to *S. pneumoniae* (Fig. 2a), and six to *E. coli* (Fig. 2b).

Concerning the protein pairs FtsA–FtsQ/DivIB, FtsA–FtsW, FtsA–FtsB/DivIC, FtsZ–FtsI (PBP2x/PBP3), ZapA/FtsI, FtsK–FtsB/DivIC, FtsW–FtsW, no interactions were detected in either organism.

Unexpectedly, the *S. pneumoniae* FtsW did not interact with PBP2x, or any other PBP tested. However, it should

be noted that *ftsW* is absent from the *dcw* cluster of many Gram-positive organisms, in particularly cocci (Massidda *et al.*, 1998). Furthermore, it has been reported recently that in *Staphylococcus aureus* the recruitment of PBP2 to the division site is dependent on its substrate (Pinho & Errington, 2005). If this is also the case for *S. pneumoniae* PBPs, it could explain the lack of this interaction, although this hypothesis needs to be confirmed.

Heterologous interactions between *E. coli* and *S. pneumoniae* division proteins

Heterologous interactions between cell division proteins were first reported by Wang *et al.* (1997) using a yeast THA. These authors showed that both FtsZ and FtsA of *B. subtilis* were able to interact with FtsZ of *E. coli*, despite the phylogenetic distance between the two species.

The common pattern of interactions, conserved between two organisms as phylogenetically distant as *E. coli* and *S. pneumoniae*, suggests that, in this case, the interactions between these proteins (or their domains/structures) could have been maintained over the course of evolution, being fundamental to the division process. It was therefore very interesting to see whether the single members of each pair could cross-interact with the respective orthologous protein, i.e. if a selected *S. pneumoniae* protein would interact with its interactive *E. coli* counterpart and vice versa. To test this possibility, cross-interactions among heterologous proteins were tested with the THA for all those proteins that showed a conserved interaction profile in the species-specific assay.

Fig. 2. Species-specific interaction profile among the nine cell division proteins common to *S. pneumoniae* and *E. coli*. (a) Interaction web among nine selected *S. pneumoniae* division proteins, FtsZ, FtsA, ZapA, FtsK, FtsQ/DivIB, FtsB/DivIC, FtsL, FtsW and FtsI/PBP2x, described in Table 2. (b) Interaction web among the nine *E. coli* cell division proteins, FtsZ, FtsA, FtsK, FtsQ/DivIB, FtsB/DivIC, FtsL, FtsN, FtsW and FtsI/PBP3 as described by Di Lallo *et al.* (2003), supplemented with the results reported in Table 3. Homodimerization is represented by circular arrows. The unbroken arrows indicate conserved interactions, while the dashed arrows indicate species-specific interactions. The double arrows indicate that heterodimerization was studied bi-directionally, with both possible constructions of fusion proteins: 434–X/P22–Y and 434–Y/P22–X.

The results, presented in Fig. 3, indicate that most of the shared common interactions detected previously, on the basis of the comparison of the two interaction webs, were also conserved in cross-interaction experiments. The cross-interactions, illustrated in the figures, concern the protein pairs FtsZ_{EC}–FtsZ_{SPN}, FtsA_{EC}–FtsZ_{SPN}, FtsQ_{EC}–DivIB_{SPN}, FtsQ_{EC}–FtsL_{SPN}, FtsL_{EC}–FtsL_{SPN}, FtsL_{EC}–FtsW_{SPN}, FtsK_{EC}–FtsZ_{SPN}, FtsK_{EC}–DivIB_{SPN}, FtsK_{EC}–FtsL_{SPN} and vice versa, with the exception of the pairs FtsK–FtsZ and FtsK–FtsQ. In these cases we observed an interaction only unidirectionally between FtsK_{EC}–FtsZ_{SPN} and FtsK_{EC}–DivIB_{SPN}; in the opposite orientation, i.e. FtsK_{SPN}–FtsZ_{EC} and FtsK_{SPN}–FtsQ_{EC}, no interactions were detected. Finally, five cross-interactions were observed between ZapA_{EC} and the streptococcal proteins FtsK_{SPN}, DivIC_{SPN}, DivIB_{SPN}, FtsL_{SPN} and FtsW_{SPN}. In this case, however, analysis of the reverse interactions was not performed.

DISCUSSION

In *S. pneumoniae* the cell division gene organization differs significantly from that of other Eubacteria although the division proteins are phylogenetically conserved (Massidda *et al.*, 1998). In this organism, at least 12 proteins are known or believed to be involved in the process and, among them, nine, FtsZ, FtsA, ZapA, FtsK, FtsQ/DivIB, FtsB/DivIC, FtsL, FtsW and FtsI/PBP2x, are common with both other Gram-positive and Gram-negative bacteria.

A function has been attributed to only a few division proteins. This identification of function is difficult because often the inactivation of the respective genes is not lethal in conditions of overexpression of other division proteins. A common property of all these proteins is their colocalization at the division site. In the last few years,

substantial progress has been made in elucidating several aspects of cell division in various bacterial models, such as identification of genes, and structure and activity of the proteins involved in the process, their cytological localization, Z-ring formation, etc. All this knowledge cannot however give us information on the assembly of the division proteins or the regulation and the structure of the divisome.

Studies on division proteins' interactions in various bacterial species, such as *E. coli*, *B. subtilis*, *S. pneumoniae* and *M. tuberculosis* (Tormo *et al.*, 1986; Wang *et al.*, 1997; Ma & Margolin, 1999; Haney *et al.*, 2001; Datta *et al.*, 2002, 2006; Haeusser *et al.*, 2004; Lara *et al.*, 2005; Fadda *et al.*, 2007) indicate that they engage in many interactions between each other. These results, however, refer to sporadic tests of a few interactions performed in the species reported above, whereas the only whole interaction web currently available is that of *E. coli* (Di Lallo *et al.*, 2003; Karimova *et al.*, 2005; D'Ulisse *et al.*, 2007).

It is now generally accepted that knowledge on interactions conserved in other organisms, or 'interologs' (Walhout *et al.*, 2000) ought to represent useful information that will allow the formulation and testing of biological hypotheses (Walhout *et al.*, 2000; Wojcik & Schachter, 2001). On this basis, in this work we identified the interaction web amongst 11 division proteins of *S. pneumoniae* in their 66 possible pairwise combinations and compared the results obtained with those for *E. coli*.

The results showed the existence of a close net constituted of at least 37 homo- and/or heterodimeric protein associations in which each protein generally interacts with more partners, up to 10 for FtsL and FtsZ, whereas the mean number of partners is two or three. PBP1A is the only case described in our results in which the protein interacts with a single partner, i.e. FtsL.

Fig. 3. Cross-interaction between selected cell division protein pairs from *S. pneumoniae* and *E. coli*. The unbroken arrows indicate the presence of an interaction between species, while dotted arrows that are crossed out indicate the absence of interaction. In most cases, we observed interaction with both the 434–X_{EC}/P22–Y_{SPN} and the 434–X_{SPN}/P22–Y_{EC} pairs. Only the interaction between FtsK_{EC}–FtsZ_{SPN} and FtsK_{EC}–DivIB_{SPN} were observed unidirectionally. Cross-interactions between ZapA and the other division proteins were tested only with the 434–ZapA_{EC} and P22–Y *S. pneumoniae* partners.

The comparison of this network with the one already established for *E. coli* (Di Lallo *et al.*, 2003) showed, for the nine common proteins, that among these 45 possible pairs of proteins, 30 show an analogous pattern in both micro-organisms: 23 interact and seven do not. The similarity of these two interactomes is not surprising since various 'comparative proteomics' strategies applied to the search for pairs of potential orthologues of known interacting protein partners in *Saccharomyces cerevisiae* have been performed to identify potentially conserved interactions, or 'interologs' (Walhout *et al.*, 2000), in *Caenorhabditis elegans*. At least 16% of the protein interactions in these networks are indeed conserved.

Our data raise some considerations: (i) the maintenance (conservation) of analogous interactomes between two phylogenetically distant species could be indicative of the biological significance of each interaction between the protein and its partners; (ii) the presence of interaction overlaps in the two networks suggests the existence of a common core of divisome proteins; (iii) if this interpretation is correct we should expect to find cross-interactions among *S. pneumoniae* and *E. coli* division proteins.

This hypothesis is supported both by the results shown in Fig. 3 and by the observation of Wang *et al.* (1997) of heterologous interactions between division proteins (FtsZ and FtsA) of *B. subtilis* and *E. coli*. Notably, the cross-interactions described here were observed between proteins of two bacterial species with a discrete sequence homology. The percentage of identical amino acid residues determined by Lipman–Pearson protein alignment between *S. pneumoniae* and *E. coli* proteins FtsA, FtsZ, DivIB and PBP2x (FtsI) is 33.0, 54.7, 17.1 and 24.1%, respectively (Massidda *et al.*, 1998).

The cross-interaction ability described in this paper may reflect a functional inter-exchangability from one organism to another. This is the case for *S. pneumoniae* DivIB protein, which phenotypically suppresses the defects of an *E. coli* FtsQ null mutant (D'Ulisse *et al.*, 2007). Heterologous complementation among division functions of phylogenetically distant bacteria have also been described by other authors (Ma *et al.*, 1997; Gaikwad *et al.*, 2000; Momynaliev *et al.*, 2002; Dziadek *et al.*, 2003; Osawa & Erickson, 2006). We are presently performing other complementation assays to establish whether other *S. pneumoniae* division proteins can substitute for their *E. coli* orthologues.

It is generally admitted that various levels of prudence need to be considered when analysing the results obtained by THA. This last notion comes from the application of the *Saccharomyces cerevisiae* THA, the first and most widely used tool in protein–protein interaction. This assay often highlights false positive or negative interactions. False positives are due to the overexpression of the reporter protein fusions, which could result in magnifying potentially weak interactions whereas the false negatives could be due, for example, to low expression levels and/or instability of the molecules tested. Presently, we have no data that

allow evaluation of the limit of the THA used in this work. However, although the number of interactions tested with the phage THA is very low compared with that obtained with the yeast THA, some data suggest that our system excludes the false (or indirect) interactions: formally, if a protein X interacts with both A and B, false positives could be due to indirect interaction between A and B mediated by X in a complex such as A–X–B. Besides the examples reported in previous papers (Di Lallo *et al.*, 2003; D'Ulisse *et al.*, 2007), the absence of interaction between ZipA and FtsA in *E. coli* suggests that the bridge eventually formed with FtsZ, which interacts with both proteins, is not revealed. Analogously, the assay does not reveal interactions between the *E. coli* proteins FtsZ–FtsW and FtsZ–FtsB although all of them also interact with ZapA. In addition, the interactions described in this paper were observed in a heterologous system, *S. pneumoniae* proteins tested in *E. coli*. The formation of false positives should need an *E. coli* 'bridge' protein able to interact with both the *S. pneumoniae* partners. Other kinds of false positives are, however, possible, such as the one due to the overexpression of proteins magnifying potentially weak interactions and leading to false signals. In these cases, levels of residual β -galactosidase synthesis close to 50% could represent weak or transient interactions amplified by protein overexpression. In any case, the *S. pneumoniae* species-specific interactions have already been confirmed by co-immunoprecipitation, similarly to previous reports (D'Ulisse *et al.*, 2007; Fadda *et al.*, 2007). Finally, as far as false negatives are concerned, this aspect can be related to the expression level and/or stability of the proteins under investigation. Generally, it is easily solved by observing whether there are two molecules, X and Y, that do not interact with each other, but each of which is able to interact with other partners; thus the lack of interaction between them is real. This condition was verified for 29 protein pairs that did not show any interaction between them.

In conclusion, the comparative analysis of the division protein interactomes reported in this work allows the identification of protein–protein interaction representatives of a minimal common divisome in Bacteria. For this reason, these results may be helpful for both a deeper comprehension of some aspects related to bacterial divisome formation and the elucidation of the cell division process in the Gram-positive coccus *S. pneumoniae*, where some genetic tools are as yet underused. Finally, given the importance of the cell division proteins as primary targets for the development of new broad-spectrum antibacterial inhibitors, the knowledge of the conserved protein–protein interactions, between phylogenetically distant species, should reinforce their attractiveness in this respect.

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