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The puzzle of *zmpB* and extensive chain formation, autolysis defect and non-translocation of choline-binding proteins in *Streptococcus pneumoniae*

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Summary

Choline-binding proteins (CBPs) from *Streptococcus pneumoniae* are involved in several important processes. Inactivation of *zmpB*, a gene that encodes a surface-located putative zinc metalloprotease, in a *S. pneumoniae* serotype 4 strain was recently reported to reveal a composite phenotype, including extensive chain formation, lysis defect and transformation deficiency. This phenotype was associated with the lack of surface expression of several CBPs, including the major autolysin LytA. LytA, normally 36 kDa in size, was reported to form an SDS-resistant 80 kDa complex with CinA. ZmpB was therefore proposed to control translocation of CBPs to the surface, possibly through the proteolytic release of CBPs (and RecA) from CinA. Based on the use of 12 independent *mariner* insertions in the *zmpB* gene of the well-characterized R6 laboratory strain, we could not confirm several of these observations. Our *zmpB* mutants: (i) did not form chains; (ii) lysed normally in the presence of deoxycholate, which indicates the presence of a functional autolysin; (iii) transformed at normal frequency; and (iv) contained bona fide CinA and LytA species. Polymorphism of ZmpB between R6 and the serotype 4 isolate could not account for the discrepancy, as inactivation of *zmpB* (through replacement by transposon-inactivated *zmpB* R6 alleles) in the latter strain did not affect separation of daughter cells and autolysis. The conflicting observations could be explained by our finding that the reportedly serotype 4 *zmpB* 'mutant' differed from its *S. pneumoniae* parent in lacking capsule and in exhibiting characteristic traits of the *Streptococcus viridans* group, including resistance to optochin.

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Introduction

The Gram-positive bacterium *Streptococcus pneumoniae*, a human commensal and a pathogen, contains choline residues incorporated into the cell wall teichoic acids and in the membrane-bound lipoteichoic acid (Tomasz, 1967). Choline residues serve to anchor a family of surface-exposed proteins that share a specialized choline-binding domain (Sánchez-Puelles *et al*., 1990), the choline-binding proteins (CBPs; for a review, see García *et al*., 1998). Choline residues bound to teichoic acid have also been shown to be absolutely required for the activity of LytA (Holtje and Tomasz, 1975). LytA is a 36 kDa *N*-acetylmuramoyl-L-alanine amidase, the major autolytic enzyme of *S. pneumoniae* (for a review, see Tomasz, 1984; García *et al*., 1986) and the first identified CBP.

During a search for surface-exposed proteins of *S. pneumoniae* involved in adhesion, Novak *et al*. (2000) identified a putative zinc metalloprotease, ZmpB. A global loss of cell surface expression of the CBPs (CbpA, CbpE, CbpF and CbpJ) was observed in a ZmpB-deficient mutant. Immunoelectron microscopy failed to detect any intracellular or extracellular CbpE, CbpF and CbpJ. However, the majority of CbpA was found trapped in the cytoplasm of the *zmpB* mutant. LytA was also detected mostly in the cytoplasm. This phenomenon was tentatively attributed to alteration in the translocation of CBPs to the surface. In addition, no 36 kDa bona fide LytA band could be detected by Western blot. Two immunoreactive species at 80 kDa and 60 kDa were detected instead (Novak *et al*., 2000). The 80 kDa species was concluded to correspond to a 'complex' between LytA and CinA, a protein induced when cells become competent for genetic transformation (Masure *et al*., 1998; Mortier-Barrière *et al*., 1998), because (i) immunoelectron microscopy suggested that the two proteins co-localized in the cytoplasm;

Table 1. Bacterial strains, plasmids and oligonucleotide primers used in this study.

Strain/plasmid/primer	Relevant characteristics	Source/reference
Streptococcus strain		
ET-*zmpB*	JNR.7/87, but *zmpB*::pJDC9; Ery^R Not *S. pneumoniae* according to our observations	E. Tuomanen/Novak *et al.* (2000)
S. pneumoniae strains		
FP25	JNR.7/87, but non-capsulated, Δ*cps*::*kan*; Kan^R	This study
FP251	FP25, but *zmpB*::*spc* insertion a (by transformation with R736 chromosomal DNA); Spc^R, Kan^R	This study
FP252	FP25, but *zmpB*::*spc* insertion d (by transformation with R737 chromosomal DNA); Spc^R, Kan^R	This study
FP253	FP25, but *str41*; Sm^R, Kan^R	This study
JNR.7/87	Serotype 4 clinical isolate; TIGR sequence	E. Tuomanen/Bricker and Camilli (1999)
M31	M11 derivative, Δ*lytA*	Sánchez-Puelles *et al.* (1986)
R800	R6 derivative	Lefèvre *et al.* (1979)
R304	R800, but *str41*, *rif23*, *nov1*; Sm^R, Rif^R, Nov^R	Mortier-Barrière *et al.* (1998)
R704	R800, but *comA*::*ermAM*; Ery^R	B. Grossiord
R736	R704, but *zmpB*::*spc* insertion a; Spc^R, Ery^R	This study
R737	R704, but *zmpB*::*spc* insertion d; Spc^R, Ery^R	This study
R738	R800, but *zmpB*::*spc* insertion a (by transformation with R736 chromosomal DNA); Spc^R	This study
R739	R800, but *zmpB*::*spc* insertion d (by transformation with R737 chromosomal DNA); Spc^R	This study
R742	R704, but *zmpB*::*spc* insertion l; Spc^R, Ery^R	This study
R745	R704, but *zmpB*::*spc* insertion h; Spc^R, Ery^R	This study
R761	R800, but *zmpB*::*spc* insertion l (by transformation with R742 chromosomal DNA); Spc^R	This study
R762	R800, but *zmpB*::*spc* insertion h (by transformation with R745 chromosomal DNA); Spc^R	This study
Plasmid		
pR412	ColE1, Ap^R, Spc^R; carries a 1146 bp minitransposon containing the inverted repeats (IRs) of the *Himar1* transposon flanking the *spc* Spc^R gene ('*mariner* cassette')	Martin *et al.* (2000)
Primer	Sequence; gene; position within the deposited sequence indicated; accession number	
BG23	GAGGCGCCAAGTTTACG; *recA*; 606–590; Z17307	
BM15	CTTCATGCAGTTGCACAAGCGCA; *recA*; 293–315; Z17307	
MP127	CCGGGGACTTATCAGCCAACC; *mariner* cassette universal primer; internal to terminal IRs; outward orientation	
MP128	TACTAGCGACGCCATCTATGTG; *mariner* cassette universal primer; adjacent to IRL, outward orientation	
zmpBUP	AGTCTTACCCAAGCTGGTCC; *hk09*; 2120–2139; SPAJ6398	
zmpBDO	CCCCCAAAGTCCTAAAATCA; *pabS* (downstream of *zmpB*; see Fig. 1); TIGR	
7693	CAACAGCTTCACGTTCCAAT; *zmpB*; 4831–4812; AF221126; type 4 specific	
8594	CGACCTGAAAGTGTGCCTGT; *zmpB*; 5732–5713; AF221126; type 4 specific	
OF889	GGCCCGGGAACTATTCAC; 16S rDNA; 1393–1375; TIGR	
OF890	TCAAAKGAATTGACGGGGGC; 16S rDNA; 916–935; TIGR	

(ii) addition of the molecular weight of CinA and LytA results in a molecular weight (81 kDa) potentially corresponding to that of the putative complex; and (iii) Western blot analysis using anti-CinA antibody detected an 80 kDa species instead of the 45 kDa CinA band in the parent strain. Detection of the 'complex' on SDS−10% polyacrylamide gels (Novak *et al.*, 2000) suggested covalent linkage of the proteins. Although *cinA* and *lytA* are part of an operon expressed at the onset of competence for genetic transformation (Mortier-Barrière *et al.*, 1998), they are separated by two genes, *recA* and *dinF*. Therefore, the putative CinA–LytA complex could not be produced by a simple frameshift.

LytA is responsible for the sensitivity of *S. pneumoniae* to deoxycholate (DOC), which is considered as a species characteristic trait. DOC, an allosteric activator of LytA, triggers autolysis. In line with the postulated defect in the translocation of CBPs, including LytA, the *zmpB* mutant did not undergo autolysis. Resistance to autolysis was observed (i) in the stationary phase of growth; (ii) after the addition of a 10-fold excess of DOC; or (iii) when treated with up to 10× the minimum inhibitory concentration of penicillin (Novak *et al.*, 2000). The *zmpB* mutant was also reported to grow in chains of 2000–3000 cells, whereas *S. pneumoniae* normally grows mainly as diplococci (hence its former name *Diplococcus*). This could be explained by the loss of the LytB CBP, a murein hydrolase essential for the separation of daughter cells (García *et al.*, 1999).

Finally, based on a previous report that CinA formed a

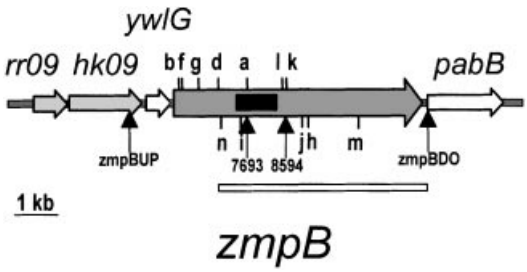

Fig. 1. Location of *mariner* cassette insertions in the *zmpB* gene of *S. pneumoniae*. Cassette location and orientation was first established by PCR and confirmed by sequencing one of the two chromosome–cassette junctions. Letters (a–n) identify independent *spc* insertions. b, f, g and d insertions occurred, respectively, after positions 108, 183, 536 and 1008 with respect to the start of *zmpB* (accession no. AF221126). The *zmpB* region that differs in sequence between the serotype 4 (strain JNR.7/87) and the serotype 19F (strain G54) isolates (see Fig. 5) is indicated by an open rectangle below the map. Letters above and below the map correspond to cassette orientation co-transcribed and antitranscribed with respect to *zmpB* respectively. The location of primers used to amplify the *zmpB* region (zmpUP and zmpBDO) and of the type 4-specific primers (7693 and 8594) is indicated by arrows below the map. The black rectangle within *zmpB* represents the fragment used for insertion–duplication mutagenesis of *zmpB* by Novak *et al.* (2000). *rr09* and *hk09* (Lange *et al.*, 1999), also named *488rr* and *488hk* (Throup *et al.*, 2000) or *zmpR* and *zmpS* (Novak *et al.*, 2000), encode the response regulator and the histidine kinase of a two-component regulatory system; no evidence for a functional relationship to *zmpB* was reported (Novak *et al.*, 2000). *ywlG* is homologous to a *Bacillus subtilis orf* with unknown function, and *pabB* probably encodes subunit A of *para*-aminobenzoate synthase.

complex with RecA that was translocated to the membrane upon induction of competence (Masure *et al.*, 1998), Novak *et al.* (2000) examined the cellular distribution of RecA. They could not observe trafficking of RecA to the membrane in the *zmpB* mutant, which they attributed to the trapping of CinA in the CinA–LytA complex (see above). In addition, the *zmpB* mutant was observed to exhibit a 99% reduction in genetic transformation efficiency, which was tentatively attributed to an alteration in RecA trafficking.

Collectively, these observations led Novak *et al.* (2000) to propose that ZmpB is important for the CinA-dependent transport of RecA, LytA and other CBPs to the cell membrane, possibly required for the proteolytic release of CBPs and RecA from CinA.

We have studied the role of CinA and failed to confirm the existence of the specific interaction between CinA and RecA reported by Masure *et al.* (1998) (Mortier-Barrière, 1999; I. Mortier-Barrière, M. Prudhomme, L. Fontaine, B. Martin and J.-P. Claverys, in preparation). We therefore set out to examine the reported formation of a complex between CinA and LytA in a *zmpB* mutant. A straightforward test would be the absence of the putative 80 kDa CinA–LytA complex from a ZmpB-deficient strain after the introduction of a *cinA* null mutation. We therefore generated a series of *zmpB* mutants by *in vitro mariner* mutagenesis (Akerley *et al.*, 1998). Our results failed to confirm that the translocation of CBPs to the cell surface is altered in *zmpB* mutants of *S. pneumoniae* and provided no evidence for the existence of a ZmpB protease-dependent regulatory mechanism governing the translocation of CinA and the CBPs or the hypothesis that CinA is involved in the transport of LytA to the cell membrane.

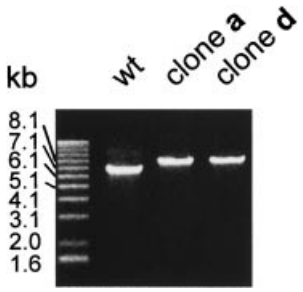

Fig. 2. Inactivation of *zmpB* by cassette insertion. The *zmpB* region was PCR amplified from chromosomal DNA of the wild type and of two *zmpB* mutants (insertions a and d; strain R738 and R739 respectively; Table 1) with the zmpBUP–zmpBDO primer pair. The predicted size of PCR fragments is 6845 bp for the wild type and 7993 bp for *mariner* cassette insertion mutants. Similar patterns were obtained for 12 independent *zmpB* insertion mutants (data not shown).

Results

In vitro mariner *mutagenesis of the* zmpB *region*

A 6845 bp fragment was amplified from the wild-type *S. pneumoniae* strain R800 by polymerase chain reaction (PCR) with the zmpBUP–zmpBDO primer pair (Table 1), designed from the *S. pneumoniae* type 4 sequence (http://www.tigr.org). This fragment was mutagenized *in vitro* using purified *Himar1* transposase and plasmid pR412 (Table 1) as a source of the spectinomycin resistance (Spc^R) *mariner* minitransposon; transposition products were used as donors in the transformation of an *S. pneumoniae* recipient strain (*Experimental procedures*). Twelve independent Spc^R transformants were isolated. For each carefully subcloned putative insertion mutant, the orientation and the site of insertion of the *spc* cassette in the chromosome were deduced from the presence or absence and the size of PCR fragments generated with the cassette-specific primer MP128 (Table 1) combined with zmpBUP or zmpBDO. Seven insertions corresponded to the co-transcribed orientation of the cassette with respect to *zmpB* (Fig. 1). Among 12 insertions in *zmpB*, seven led to more severe truncations of ZmpB than in the insertion–duplication mutant used by Novak *et al.* (2000), and two generated similarly sized truncated proteins (insertions l *and* k; Fig. 1). For eight insertions,

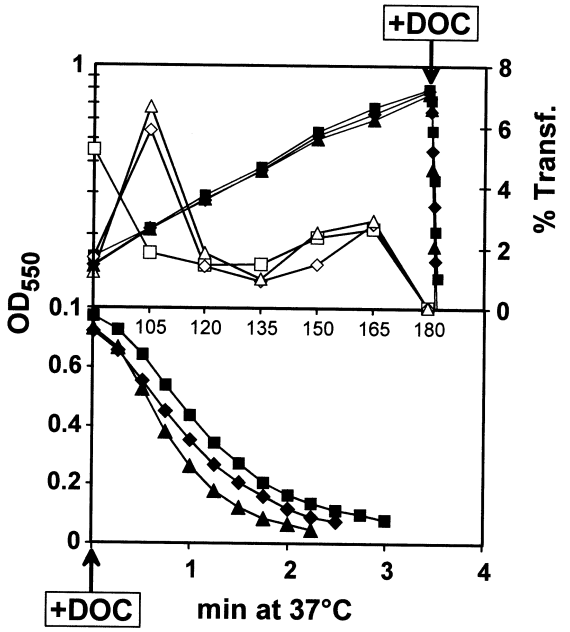

Fig. 3. Kinetics of transformation and of DOC-triggered autolysis. Top. Transformation (open symbols) was assayed during exponential growth in C+Y medium for *zmpB* mutant strains R738 (insertion a, squares) and R739 (insertion d, diamonds) and the wild type (triangles). OD_{550} (filled symbols) was measured in parallel. DOC (0.05%) was added (arrow) to cultures at the end of the exponential phase of growth. Bottom. Kinetics of lysis (filled symbols) are shown on an expanded time scale.

cassette–*S. pneumoniae* DNA junctions were characterized by sequencing PCR products with MP128 as primer (*Experimental procedures*; Fig. 1 and see below).

The structure of each insertion was controlled by PCR amplification of the *zmpB* chromosomal region with the zmpBUP–zmpBDO primer pair for each mutant. A fragment exhibiting the size expected after insertion of the *mariner* cassette was obtained in all cases (Fig. 2; data not shown). Disappearance of the wild-type fragment in *mariner* insertion mutants (Fig. 2) indicated that no rearrangement had occurred and ruled out possible artifacts such as the presence of an intact copy of the *zmpB* gene in the mutants through duplication of the region.

Cell morphology

Surprisingly, microscopic examination of the cell morphology of this series of *zmpB* mutants revealed that they all grew in THY or in C+Y medium mainly as diplococci (data not shown). No extensive chain formation of the sort described by Novak *et al*. (2000) was observed. This suggested that at least one CBP, LytB, which is required for separation of daughter cells (García *et al*., 1999), was not affected by disruption of the *zmpB* gene.

DOC-triggered autolysis of zmpB *mutants*

It has been reported that a *zmpB* mutant is resistant to a 10-fold excess of DOC and that lysis of the mutant required 45 min treatment with 25 mg ml^{-1} lysozyme in the presence of 0.1 g ml^{-1} glycine (Novak *et al*., 2000). We therefore investigated a possible autolysis defect by treating *zmpB* mutants with DOC. All *zmpB* mutants were readily lysed in the presence of 1× DOC (0.05%; data not shown). Examination of the kinetics of DOC-triggered autolysis of four randomly chosen *zmpB* mutants indicated that complete lysis occurred within less than 2 min after the addition of 1× DOC, revealing no significant difference from the wild-type parent (Fig. 3; data not shown). These results strongly suggest that a second CBP, LytA, the protein responsible for autolysis, remained fully functional in *zmpB* mutant context.

Spontaneous development of competence in zmpB *mutants*

Novak *et al*. (2000) also reported that the trafficking of RecA (and CinA) to the membrane, previously reported to

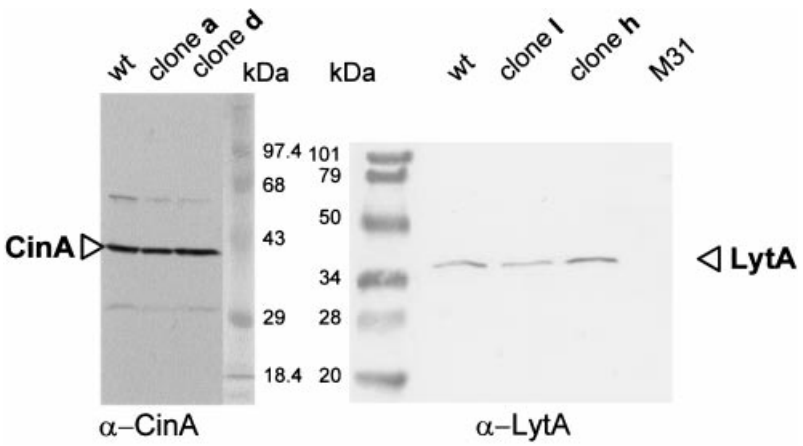

Fig. 4. Western blot analysis of CinA and LytA in *zmpB* mutants. Lysates of the wild type (strain R800) and of derivatives carrying *zmpB*::*spc* insertion a (strain R738), d (strain R739), l (strain R761) or h (strain R762) were subjected to SDS–PAGE (12%) and probed with polyclonal mouse antibodies directed against CinA (α-CinA; lanes 1–3) or LytA (α-LytA; lanes 6–9). Lanes (from left to right): 1, wild type (R800); 2, $zmpB^-$ insertion a; 3, $zmpB^-$ insertion d; 4 and 5, prestained molecular size markers; 6, wild type; 7, $zmpB^-$ insertion l; 8, $zmpB^-$ insertion h; 9, M31 (Δ*lytA* strain).

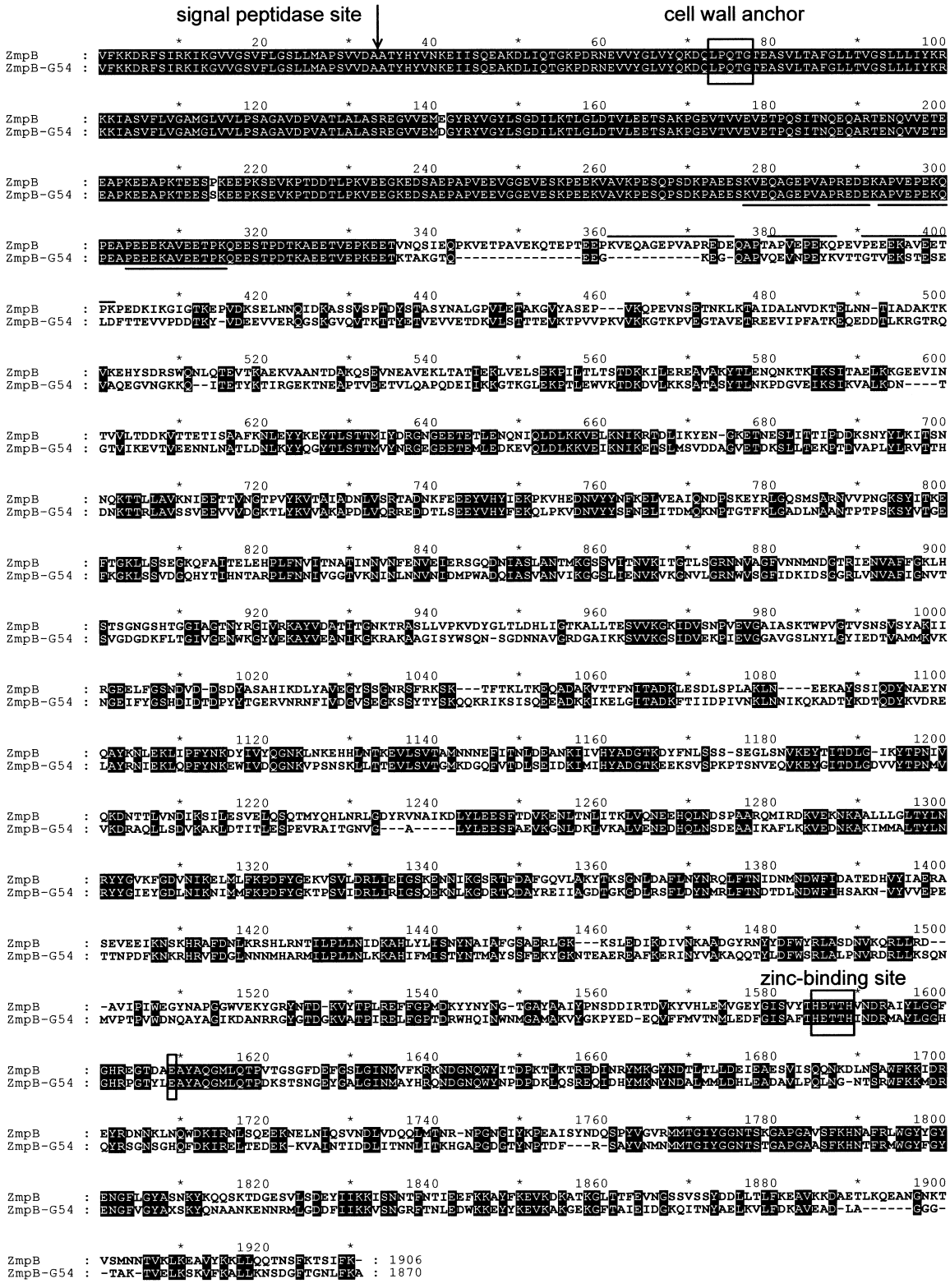

Fig. 5. Comparison of type 4 and G54 ZmpB sequences. Gaps indicated by dashes were introduced by the CLUSTALW program. Identical amino acids are in white letters on a black background. The proposed signal peptidase cleavage site (positions 33–34), which conforms to the (−3, −1) rule, the putative N-terminal cell wall anchor motif (LPNTG; positions 73–77) and the putative C-terminal zinc-binding sequence (HETTH; positions 1562–1566 in ZmpB type 4 and 1540–1544 in ZmpB-G54, followed by an E residue 20 amino acids downstream) are indicated. The region between residues 277 and 315 (underlined) is almost exactly repeated in ZmpB type 4 (amino acids 361–402), but not in ZmpB-G54.

occur in competent cells of *S. pneumoniae* (Masure *et al*., 1998), was not observed in their *zmpB* mutant. In line with this observation, they reported a 99% reduction in the efficiency of genetic transformation of the *zmpB* mutant compared with the parent strain. The addition of competence stimulating peptide (CSP; Håvarstein *et al*., 1995) did not improve transformation efficiency.

Although we could not confirm a differential cellular localization of RecA (and CinA) between competent and non-competent wild-type cells (Mortier-Barrière, 1999; I. Mortier-Barrière, M. Prudhomme, L. Fontaine, B. Martin and J.-P. Claverys, in preparation), spontaneous competence of the *zmpB* mutants was assayed under standard conditions in C+Y medium (*Experimental procedures*). Our *zmpB* mutants displayed normal competence profiles and efficiencies of genetic transformation similar to those of the parental strain (Fig. 3, top). These observations indicated that the RecA protein remained fully functional in *zmpB* mutants. They also strongly suggested that translocation of a third CBP, CBP3, which was recently shown to be required for transformation (Rimini *et al*., 2000), was not affected by disruption of the *zmpB* gene.

Analysis of CinA and LytA in zmpB *mutants*

The finding that our *zmpB* mutants displayed normal cell morphology, autolysis and genetic transformation efficiency, three properties relying on the presence of at least three functional CBPs, LytB, LytA and CBP3, strongly suggested that secretion or translocation of CBPs was unaffected in the mutants. Nevertheless, we carried out a Western blot analysis of LytA. In contrast to Novak *et al*. (2000), who reported the absence of a band displaying the apparent molecular mass expected for LytA (36 kDa), we readily detected this protein with anti-LytA antibodies in cell extracts of four of the *zmpB* mutants chosen at random (Fig. 4; data not shown).

As a last control, we decided to investigate the CinA protein. According to Novak *et al*. (2000), no 'free' CinA species could be detected in extracts from a *zmpB* mutant on SDS gels. Using standard methods (see *Experimental procedures*), we detected the expected 45 kDa CinA species in extracts of competent cells of both parental and *zmpB* mutants using anti-CinA antibodies (Fig. 4; data not shown), with no hint of an 80 kDa CinA–LytA complex similar to that described by Novak *et al*. (2000).

Collectively, these data demonstrate that the secretion and translocation of the CBPs is unaltered in our *zmpb* mutants of *S. pneumoniae*, in contrast to the report of Novak *et al*. (2000) based on their *zmpb* mutant.

Polymorphism of the zmpB *gene in* S. pneumoniae

Only four out of eight sequenced *mariner* cassette–*zmpB* junctions could be mapped at the nucleotide level using the published *zmpB* type 4 sequence (insertions b, f, g and d; Fig. 1; data not shown). Other insertions (a, l, h and m; Fig. 1) could be mapped only with respect to the sequence of the *zmpB* gene of strain G54 (EMBL accession no. AL449926), a 19F capsular-type clinical isolate (Rimini *et al*., 2000; data not shown). This observation suggested the existence of a polymorphism between the *zmpB* gene of the type 4 clinical isolate and of R6, and that R6 and strain G54 share similar *zmpB* alleles. Comparison of the sequences of ZmpB type 4 [1906 amino acids, instead of 1882 amino acids as indicated previously by Novak *et al*. (2000), because of an incorrect assignment of the start] and of ZmpB-G54 (1870 amino acids) revealed that the proteins are 99% identical over the 335 first amino acids and 42% identical in the polymorphic region (Fig. 5). Although no significant identity is detected at the DNA level in the polymorphic region, except for five small (from 37 to 218 nucleotides long) segments with an average identity of 78%, sequence identity resumes 20 nucleotides upstream of the *pabB* start codon (Fig. 1). Polymorphism of surface-exposed proteins as observed for ZmpB is not unprecedented in *S. pneumoniae*, as sequence variability has been reported in several instances (e.g. PspA and PspC/CbpA/SpsA; Brooks-Walter *et al*., 1999; Hollingshead *et al*., 2000). We observed the existence of a peptide of 33 amino acids with typical features of a prokaryotic export signal sequence at the N-terminus of ZmpB (Fig. 5), confirmed the presence of a cell wall anchor domain and of a zinc-binding motif in the N-terminal and in the C-terminal region, respectively (Fig. 5), but failed to detect the previously reported '10 nearly identical tandem repeats of a 25-mer of unknown function' in either protein.

After communication of our observations, E. Tuomanen suggested that, although located at the same chromosomal position between *ywlG* and *pabB* (Fig. 1), *zmpB*-R6 and *zmpB* type 4 could constitute two different genes, with different functions. This seemed rather unlikely in view of the overall conservation of the ZmpB proteins (Fig. 5). In addition, taking into account the central role proposed for ZmpB in the trafficking of CBPs (Novak *et al*., 2000) and the importance of CBPs for *S. pneumoniae*, one would expect that the function is conserved in the species. Nevertheless, to resolve the apparently conflicting data, we inactivated the *zmpB* gene in the type 4 strain. Substitution of *zmpB* type 4 by an inactivated copy of *zmpB*-R6 was readily obtained in two independent experiments by transformation of strain FP25, a non-capsulated derivative of the serotype 4 isolate (Table 1), with chromosomal DNA from strains R736 (*zmpB*::*spc* insertion a) and R737 (*zmpB*::*spc* insertion d). The structure of two independent transformants in each experiment was controlled by PCR amplification of the *zmpB* chromosomal region with the zmpBUP–zmpBDO primer

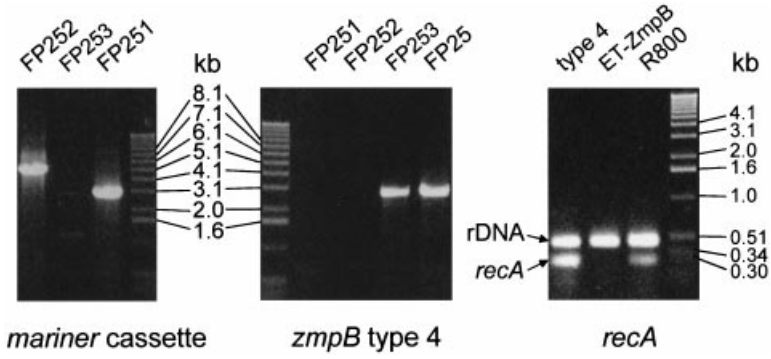

Fig. 6. Inactivation/substitution of *zmpB* type 4 (left and centre) and control PCR of the ET-*zmpB* mutant (right). Analysis of transformants of strain FP25 (non-capsulated derivative of the serotype 4 strain; Table 1) harbouring the *zmpB*::*spc* insertion a (strain FP251), the *zmpB*::*spc* insertion d (strain FP252) or the *str41* Sm^R chromosomal allele (strain FP253).Left ('*mariner* cassette'). Presence and location of the *mariner* cassette. Primer pairs and expected PCR products are as follows: zmpBUP-MP128, 2700 bp for FP251 and no product for FP253; zmpBDO-MP127, 4798 bp for FP252.Centre ('*zmpB* type 4'). Disappearance of a *zmpB* type 4-specific fragment in the transformants. Predicted size of PCR fragment with the zmpBUP-7693 primer pair: 2729 bp for FP25 and FP253; no product expected for FP251 and FP252.Right ('*recA*'). Control PCR using a *recA*-specific primer pair (BM15-BG23; Table 1) and a universal primer pair specific for 16S rDNA (OF890–OF889; Table 1) carried out on the serotype 4 parental strain and the ET-*zmpB* mutant received from E. Tuomanen, and on strain R800 (R6 derivative). Expected PCR products: *recA*, 313 bp (arrow *recA*); rDNA, 477 bp (arrow rDNA). Amplification of the *recA*-specific fragment from the ET-*zmpB* mutant also failed using the *recA* primer pair in an individual reaction, ruling out possible PCR competition artifacts (data not shown).

pair. A fragment exhibiting the size predicted after substitution of the *zmpB* type 4 gene by the cassette-containing *zmpB*-R6 gene was obtained in all cases (data not shown, but see Fig. 2). Disappearance of the wild-type fragment in the transformants indicated that no rearrangement had occurred and ruled out possible artifacts such as the presence of an intact copy of the *zmpB* type 4 gene in the transformants through duplication of the region (data not shown, but see Fig. 2). The presence and location of the *mariner* cassette in the transformants was confirmed by PCR using the cassette-specific primers MP127 or MP128 (Table 1) combined with zmpBDO or zmpBUP (Fig. 6). In addition, the disappearance of *zmpB* type 4-specific fragments in the transformants was monitored by PCR using primer zmpBUP in combination with primers 7693 (Fig. 6A) or 8594 (data not shown); the latter two primers are type 4 specific (Table 1 and Fig. 1).

None of the two *zmpB* mutants of the type 4 strain generated this way displayed extensive chain formation of the sort described by Novak *et al*. (2000); they both grew mainly as diplococci (data not shown). In addition, examination of the kinetics of DOC-triggered autolysis revealed no significant difference from the wild-type parent (data not shown). Thus, the polymorphism of the *zmpB* gene between R6 and the serotype 4 isolate cannot account for the conflicting observations.

Resolving the conflicting observations

Upon receipt of freshly subcloned and controlled strains (the Ery^R ZmpB mutant and its serotype 4 parent) kindly sent by E. Tuomanen, we first confirmed the two phenotypes previously attributed to the *zmpB* mutant (Novak *et al*., 2000), long-chain formation and increased resistance to DOC (data not shown). Then, in an effort to account for the conflicting observations, we further analysed the *zmpB* mutant isolated by Novak *et al*. (2000) ('ET-*zmpB* mutant' hereafter). Anonymous duplicate samples of the two strains were passed to the Laboratoire de Microbiologie de l'Hôpital Purpan (Toulouse, France), a service in charge of the identification of bacterial species infecting hospital patients. Although the duplicates of the parental serotype 4 strain were readily recognized as *S. pneumoniae*, those corresponding to the ET-*zmpB* mutant were not. They differed from their 'parent' in lacking capsule (negative latex agglutination test), in displaying an alkaline phosphatase-positive reaction (rapid ID 32 Strep system, bioMérieux; Freney *et al*., 1992) and in resistance to optochin. On the basis of these results, the ET-*zmpB* mutant was classified as *Streptococcus viridans* (possibly *Streptococcus oralis*) or, more likely, might belong to the group of atypical oral streptococci (Whatmore *et al*., 2000).

In complete agreement with this conclusion, PCR using a primer pair specific for the *recA* gene (Fig. 6) yielded a fragment for strain R800 and for the serotype 4 wild-type strain, but failed to amplify any fragment from the ET-*zmpB* mutant. The presence of template in the latter case was demonstrated by a control PCR using a universal primer pair specific for 16S rDNA (Fig. 6).

Discussion

Based on the use of 12 independent *mariner* insertions in

the *zmpB* gene of the well-characterized R6 laboratory strain, we observed that the mutants (i) do not show extensive chain formation, which indicates the presence of a functional LytB CBP; (ii) undergo normal DOC-triggered autolysis, which relies on the presence of a functional LytA CBP; (iii) transform at a normal frequency, which requires the presence of at least a functional CBP3; and (iv) contain bona fide CinA and LytA species. In addition, polymorphism of the *zmpB* gene between strain R6 (R800) and the serotype 4 strain used by Novak *et al*. (2000) could not account for the discrepancy, as we observed that inactivation of *zmpB* in the latter does not affect the separation of daughter cells and autolysis. Thus, our findings do not confirm that the secretion and translocation of CBPs to the cell surface is altered after inactivation of the *zmpB* gene of *S. pneumoniae*.

A possible explanation for the discrepancy was suggested to us by the striking similarity between the pleiotropic phenotype reported for the *zmpB* mutant (Novak *et al*., 2000) and that previously attributed to PsaD mutants (Novak *et al*., 1998). The PsaD phenotype included (i) reduced sensitivity to the lytic and killing effects of penicillin; (ii) growth in chains of 200–300 cells; (iii) autolysis defect and loss of sensitivity to low concentrations of DOC; (iv) absence of LytA; (v) almost complete loss of CBPs; (vi) loss of transformability. The only difference is in the Mn^{2+} requirement for growth in a chemically defined medium displayed by PsaD mutant cells. As with the ZmpB mutant phenotype, the phenotypic traits of Psa mutants could not be confirmed by other laboratories (Claverys *et al*., 1999). These observations suggest that the phenotypes might not result from the introduced mutations but from low-level contamination of pneumococcal laboratory stocks by a distinct but transformable streptococcal species. This species would be related closely enough to *S. pneumoniae* so as to accept *psa* or *zmpB* knock-out plasmids, but would also display inherently the phenotypes described – extensive chain formation, absence of autolysis and global modification of CBPs. This hypothesis is consistent with the observation of an unexplained increase in the size of RecA in some of the mutants (Novak *et al*., 1998), whereas DNA divergence could explain the apparent low transformability of the mutants. The emergence of low-level contaminants could readily be accounted for by selection for penicillin tolerance in one case (Novak *et al*., 1998). In the other case (Novak *et al*., 2000), it could be relevant that the *S. pneumoniae* serotype 4 isolate used is notoriously difficult to transform (Bricker and Camilli, 1999).

In the case of the *zmpB* mutant used by Novak *et al*. (2000), the contaminant hypothesis was supported by our finding that this strain does not belong to the *S. pneumoniae* species. The '*zmpB* mutant' differed from its *S. pneumoniae* serotype 4 parent in displaying resistance to optochin, although sensitivity to optochin is a characteristic trait of the species, and in lacking a capsule. The strain was tentatively classified as belonging to the *S. viridans* group. The contamination of *S. pneumoniae* cultures by related species is not unprecedented, as this occurred at an early stage of the TIGR *S. pneumoniae* genome sequence project (Adams and Venter, 1996).

Collectively, our findings do not confirm that the translocation of CBPs to the cell surface is altered in *zmpB* mutants of *S. pneumoniae*. They provide no evidence for the existence of the ZmpB protease-dependent regulatory mechanism governing the translocation of CinA and the CBPs of *S. pneumoniae* postulated by Novak *et al*. (2000). They also do not support the hypothesis that CinA is involved in the transport of LytA to the cell membrane. The functions of ZmpB and of CinA in *S. pneumoniae* remain to be established.

Experimental procedures

Bacterial strains, growth conditions and competence

The bacterial strains and plasmids used are listed in Table 1. Competence profiles, which assess spontaneous transformability, were generated from cultures grown in C+Y medium as described previously (Alloing *et al*., 1996). In short, stocks of bacteria grown in CAT medium to an OD_{550} of 0.4 were diluted 20-fold in C+Y medium and incubated at 37°C. Samples were withdrawn at 15 min intervals, from 90 min to 180 min after inoculation, and incubated with R304 donor DNA for 20 min. For CSP-induced competence, precompetent cells prepared as described previously (Martin *et al*., 1995) but in THY (30 g l^{-1} Todd–Hewitt, 5 g l^{-1} yeast extract; Difco) instead of CTM 1 were incubated with synthetic CSP (25 ng ml^{-1}; CSP2 for strain FP25) in CTM 2 (Martin *et al*., 1995) at 37°C for 10–15 min before the addition of DNA.

Cells were incubated at 30°C during DNA uptake. Transformants were determined by plating in 10 ml of CAT agar, followed by challenge with a 10 ml overlay containing the appropriate antibiotic after phenotypic expression for 120 min at 37°C. Antibiotic concentrations used for the selection of transformants were 0.05 μg ml^{-1} for erythromycin (Ery), 250 μg ml^{-1} for kanamycin (Kan) and 200 μg ml^{-1} for spectinomycin (Spc) and streptomycin (Sm).

In vitro mariner *mutagenesis*

Transposition reactions were performed using purified *Himar1* transposase as described previously (Lampe *et al*., 1996). The target for transposition of the *spc* minitransposon carried by plasmid pR412 (Table 1) was a *zmpB* PCR product. Gaps in transposition products were repaired as described previously (Akerley *et al*., 1998), and repaired transposition products were used as donors in transformation.

PCR reactions and characterization of mariner *cassette–*S. pneumoniae *DNA junctions*

PCR was performed using hot *Tub* DNA polymerase (Amersham), primers (1 μM) and 30 cycles of amplification (30 s denaturation at 94°C, 30 s annealing at 55°C and 7–12 min extension at 72°C). For DNA sequencing, the *zmpb* chromosomal region was amplified with the zmpBUP–zmpBDO primer pair. PCR products were purified with the Qiaquick DNA cleanup system (Qiagen) and sequenced with the ThermoSequenase cycle sequencing kit (USB) using the cassette-specific primer MP128.

Proteins

Preparation of cell extracts was as described previously (Martin *et al*., 1995). Samples were subjected to 12% SDS–PAGE, followed by immunoblot transfer with anti-CinA or anti-LytA antibodies as described previously (Martin *et al*., 1995).

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