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Influence of proteins Bsp and FemH on cell shape and peptidoglycan composition in group B streptococcus

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Group B streptococcus (GBS) is surrounded by a capsule. However, little is known about peptidoglycan metabolism in these bacteria. In the present study, a 65 kDa protein was isolated from the culture supernatant of GBS and N-terminally sequenced, permitting isolation of the corresponding gene, termed *bsp*. The *bsp* gene was located close to another gene, designated *femH*, and reverse transcription-PCR revealed a bicistronic transcriptional organization for both genes. The Bsp protein was detected in the culture supernatant from 31 tested clinical isolates of GBS, suggesting a wide distribution of Bsp in these bacteria. Overexpression of *bsp* resulted in lens-shaped GBS cells, indicating a role for *bsp* in controlling cell morphology. Insertional disruption of *femH* resulted in a reduction of the L-alanine content of the peptidoglycan, suggesting that *femH* is involved in the incorporation of L-alanine residues in the interpeptide chain of the peptidoglycan of GBS.

Keywords: *Streptococcus agalactiae*, murein hydrolase, *fem*-like genes

INTRODUCTION

Group B streptococcus (GBS), also named *Streptococcus agalactiae*, is a frequent colonizer of the human respiratory, gastrointestinal and urogenital tracts of humans and several mammals. GBS is also the major cause of bacterial sepsis and meningitis in human newborn infants, and poses a significant threat to parturient women (Baker & Edwards, 1995). As the incidence of GBS infections has increased significantly during the last decade, particularly in immunocompromised persons (Waite *et al*., 1996), considerable research has been focused on the identification of putative virulence factors from GBS. A variety of studies have addressed the capsule of GBS (Rubens *et al*., 1991; Wessels *et al*., 1992; Kogan *et al*., 1996). In contrast, the function of most extracellular proteins and the nature of peptidoglycan metabolism in these bacteria are only poorly understood. In common with many other Gram-positive bacteria, the peptidoglycan stem peptide of GBS consists of the pentapeptide L-Ala-D-iGln-L-Lys-D-Ala-D-Ala (iGln, isoglutamine). However, in GBS, different peptidoglycan strands are cross-linked by short interpeptide bridges of L-Ala-L-Ala or L-Ala-L-Ser dipeptides, connecting the L-lysine of one stem peptide to the D-alanine in position 4 of a neighbouring subunit (Schleifer & Kandler, 1972).

Since the interpeptide bridge is a species-specific feature, it has been the focus of intense research in other pathogens. In *Staphylococcus aureus*, the interpeptide bridge consists of five glycine residues which are synthesized by the sequential addition of glycyl residues in the presence of the proteins FmhB, FemA and FemB, respectively. FmhB was shown to be required for the first step of interpeptide synthesis by attaching the first glycine to the ε-amino group of a lysine residue in the stem peptide (Rohrer *et al*., 1999; Tschierske *et al*., 1999), FemA directs the incorporation of the second and the third glycine, while FemB is required for the addition of the fourth and fifth glycines (Stranden *et al*., 1997).

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Abbreviation: GBS, group B streptococcus.

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In *Streptococcus pneumoniae*, the peptidoglycan stem peptides can be either directly linked to each other or cross-linked by an interpeptide bridge carrying L-Ala-L-Ala or L-Ser-L-Ala dipeptides (Garcia-Bustos *et al*., 1987). Recently, the *fem*-like genes *murM* and *murN* were identified in *Strep. pneumoniae* and shown to be required for the formation of the interpeptide bridge (Filipe & Tomasz, 2000; Weber *et al*., 2000). Insertional mutagenesis revealed that *murM* is involved in the incorporation of the first amino acid, while *murN* is required for the addition of the second amino acid of the interpeptide bridge (Filipe *et al*., 2000).

As the interpeptide bridge has a species-specific amino acid composition, it represents the target of specific bacteriolytic enzymes that cleave the interpeptide bridge and cause lysis of the target cell. *Staphylococcus simulans* biovar *staphylolyticus* and *Staphylococcus capitis*, respectively, secrete the glycyl-glycine endopeptidases lysostaphin and Ale-1, which recognize and cleave the pentaglycine interpeptide chain in the peptidoglycan of *Staph. aureus*, resulting in the lysis of this organism (Sugai *et al*., 1997a; Thumm & Götz, 1997). The former strains protect their own cell walls from cleavage by the incorporation of serine molecules at positions 3 and 5 within the interpeptide chain (Ehlert *et al*., 2000). The immunity factors that mediate the incorporation of serine into the interpeptide chain reveal high similarity to Fem-like proteins (Sugai *et al*., 1997b; Thumm & Götz, 1997). Similar bacteriolytic enzymes and immunity factors from different streptococcal species have also been described (Beatson *et al*., 1998; Simmonds *et al*., 1997; Beukes & Hastings, 2001).

The present study describes the identification and characterization of the genes *bsp* and *femH*, which appear to play a role in cell morphogenesis and peptidoglycan metabolism in GBS. By insertional mutagenesis, the *bsp* gene was deleted and the *femH* gene was disrupted in the genome of GBS. The mutant strains were characterized for their growth behaviour, β-lactam susceptibility and cell wall composition. Furthermore, the *bsp* gene was overexpressed in GBS, and the shape of the resultant strain was compared with that of the parental strain. The results obtained reveal that overexpression of *bsp* causes altered cell morphology, while the disruption of *femH* changes the amino acid composition of the peptidoglycan of GBS.

METHODS

Bacterial strains and culture conditions. GBS strain 6313 is a serotype III clinical isolate obtained from an infected neonate (Valentin-Weigand *et al*., 1996). GBS strain SMB is a *bsp* deletion mutant of strain 6313, and strain FBH is a *femH*::pG$^+$host6 derivate of GBS 6313, carrying an insertionally disrupted *femH* gene in the chromosome. The GBS strains belonging to different serotypes are clinical isolates and have been described elsewhere (Chhatwal *et al*., 1984). *Escherichia coli* DH5α (Hanahan, 1985) served as host for the pTEX5236 cosmid gene library and the recombinant pG$^+$host6 plasmids. *E. coli* BL21(DE3) (Dubendorff & Studier, 1991) harboured the recombinant pET28 plasmid and was used for production of the hexahistidyl-tagged Bsp fusion protein. GBS was cultivated at 37 °C in Todd–Hewitt yeast (THY) broth containing 1% yeast extract. GBS strains carrying recombinant pG$^+$host6 or pAT28 derivatives were selected in the presence of erythromycin (5 μg ml^{-1}) and spectinomycin (200 μg ml^{-1}), respectively. *E. coli* was grown at 37 °C in Luria broth (LB), and clones carrying cosmid pTEX5236, plasmid pET28a or plasmid pAT28 were selected in the presence of chloramphenicol (15 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or spectinomycin (100 μg ml^{-1}). For visual inspection of the mean chain length, the GBS strains were cultivated overnight in THY liquid medium and examined by light microscopy.

Plasmids and cosmids used for cloning purposes. A pTEX5236-based (Teng *et al*., 1998) cosmid gene library from GBS was used for the isolation of the *bsp*-encoding region (Reinscheid *et al*., 2001). Plasmid pUC18 (Vieira & Messing, 1982) was used for subcloning of the *bsp* gene after partial digestion of a *bsp*-carrying pTEX5236 cosmid with *Sau*3AI. Plasmid pET28a (Novagen) was used for the expression of the hexahistidyl-tagged Bsp fusion protein, which was constructed as follows. A truncated *bsp* gene, devoid of its signal-peptide-encoding sequence, was amplified by PCR using the primers 5′-CGCGGATCCGATCAAACTACATCGG-3′ and 5′-TGGCACAAGCTTCAATATAGCGACGAA-3′. The *Bam*HI and *Hin*dIII restriction sites used for cloning are underlined. After digestion of the *bsp* PCR product and of plasmid pET28a with *Bam*HI and *Hin*dIII, the *bsp* gene was ligated into pET28 and transformed into *E. coli* BL21. Plasmid pAT28 (Trieu-Cuot *et al*., 1990) was used for the overexpression of *bsp* in GBS. For this purpose, the *bsp* gene was amplified from the genome of GBS by PCR using the primers 5′-GCTAGAATTCGGAACGATGAATTCAACCC-3′ and 5′-CGTGACTCTAGAGACGTAAATCTCCACTG-3′. After digestion of the PCR product and of plasmid pAT28 with *Eco*RI and *Xba*I, the *bsp* gene was ligated into pAT28, resulting in plasmid pAT*bsp*. Transformation of recombinant plasmids in GBS was performed as described by Ricci *et al*. (1994).

Construction of GBS deletion and insertion mutants. As the *bsp* and *femH* genes are transcriptionally linked, the *bsp* gene was deleted in the chromosome to rule out a polar effect on the expression of the downstream-located *femH* gene. For the deletion of the *bsp* gene, the thermosensitive plasmid pG$^+$host6 (Appligene) was used. Two *bsp* flanking fragments were amplified by PCR using the primer pairs bsp_del1 (5′-CGCGGATCCAAGCAGAAGGTGTAGAGC-3′) and bsp_del2 (5′-*CCCATCCACTAAACTTAAACA*CGTAGAGAGTAAGA-TTGC-3′) as well as bsp_del3 (5′-*TGTTTAAGTTTAGTGGATGGG*TAGTAGATGGTCATCAGTGG-3′) and bsp_del4 (5′-TGGCACAAGCTTTTGGCATAGCCTTGCAATGC-3′). Complementary DNA sequences in primers bsp_del2 and bsp_del3 are shown in italics, and the *Bam*HI and *Hin*dIII restriction sites in primers bsp_del1 and bsp_del4 are shown underlined. The *bsp* flanking PCR products were mixed in equal amounts with each other and subjected to crossover PCR by using primers bsp_del1 and bsp_del4. The resulting PCR product consisted of the *bsp* flanking regions on a single DNA fragment. The crossover PCR product and plasmid pG$^+$host6 were digested with *Bam*HI and *Hin*dIII, ligated, and transformed into *E. coli* DH5α. The resulting plasmid, pG$^+$*bsp*, was transformed into GBS 6313, and transformants were selected by growth on erythromycin agar at 30 °C. Cells in which pG$^+$*bsp* had integrated into the GBS chromosome were obtained by growth of the transformants at 37 °C with erythromycin selection, as described elsewhere

(Maguin *et al*., 1996). Four such integrant strains were serially passaged for 3 days in liquid medium at 30 °C without erythromycin selection, to facilitate the excision of plasmid pG$^+$*bsp*, producing the desired *bsp* deletion in the chromosome. Dilutions of the serially passaged cultures were plated onto agar plates, and single colonies were tested for erythromycin sensitivity to identify pG$^+$*bsp* excisants. Chromosomal DNA of GBS 6313 and of 24 erythromycin-sensitive GBS excisants was tested by Southern blotting after *Hin*dIII digestion, using a digoxigenin-labelled *bsp* flanking fragment obtained with primers bsp_del1 and bsp_del2.

For targeted disruption of *femH*, an internal *femH* fragment, ranging from bp 214 to bp 684 of the structural *femH* gene, was amplified by PCR using primers 5′-CGCGGATCCAATTCAGCCTTGCCTTC-3′ and 5′-TGGCACAAGCTTCAGGACCAGTTCATTC-3′. The *Bam*HI and *Hin*dIII restriction sites used for cloning are underlined. The resulting PCR product and plasmid pG$^+$host6 were digested with *Bam*HI and *Hin*dIII, and the PCR product was subsequently ligated into pG$^+$host6 and transformed into *E. coli* DH5α, resulting in plasmid pG$^+$*femH*. After transformation of pG$^+$*femH* into GBS 6313, integration of the plasmid into the chromosome of GBS 6313 was performed by means of a temperature shift to 37 °C as described elsewhere (Maguin *et al*., 1996). Successful disruption of *femH* was confirmed by Southern blot analysis with *Cla*I-digested chromosomal DNA from GBS and a digoxigenin-labelled *femH* probe obtained by PCR with primers 5′-TTATGCCAGTCACTGGTGG-3′ and 5′-AGAGCGTTGCCTATGATAC-3′. The *femH* mutation was stably maintained by growing the *femH*-inactived GBS strain at 37 °C in the presence of 5 μg erythromcyin ml^{-1}.

RNA preparation and RT-PCR analysis. Total RNA from 250 ml mid-exponential-phase GBS 6313 culture was prepared by using the RNeasy Midi extraction kit (Qiagen) and was treated for 30 min with 150 U RNase-free DNase (Promega). For analysis of the transcriptional organization of *bsp* and *femH* in GBS, 1 μg RNA samples were used for RT-PCR with primers 3′ *bsp* (5′-TAGTAGATGGTCATCAGTGG-3′) and 5′ *femH* (5′-GGTCCTGAATCAATTTTC-3′). For comparative expression analysis of *bsp* in GBS strains 6313(pAT28) and GBS 6313(pAT*bsp*), RNA samples from the two strains containing 5, 1, 0·5, 0, 1, 0·05 or 0·01 ng RNA were tested by RT-PCR for the presence of a *bsp*-specific transcript, using primers 5′-GAGACAAGTGCGTCAAGTG-3′ and 5′-TGAGTTGGACTCGCTACC-3′. RT-PCR was performed using the OneStep RT-PCR kit (Qiagen) according to the instructions of the manufacturer.

General DNA techniques. Chromosomal GBS DNA was isolated according to Pospiech & Neumann (1995). Conventional techniques for DNA manipulation, such as restriction enzyme digests, PCR, ligation, transformation by electroporation and Southern blotting, were performed as described by Sambrook *et al*. (1989).

Electron microscopy and antibiotic testing. Scanning electron microscopy of mid-exponential-phase cultures was performed as described previously (Reinscheid *et al*., 2001). Determination of the MICs for penicillin G, oxacillin, cefotaxim, imipenem and vancomycin was performed on sheep-blood agar plates by using antibiotic-containing E-test strips (AB Biodisk) according to the manufacturer's instructions. Penicillin-induced lysis was measured as described by Fontana *et al*. (1990). Briefly, a bacterial overnight culture was diluted in fresh THY medium to an OD_{600} of 0·2. Penicillin G was added to give concentrations of 0·032 μg ml^{-1}, 0·064 μg ml^{-1} and 0·128 μg ml^{-1}, respectively. Cultures were incubated with shaking at 37 °C, and 1 ml aliquots were removed every 30 min to determine the OD_{600}.

N-terminal sequencing of proteins and peptides. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane (Amersham/Pharmacia), and visualized with amido black. N-terminal amino acid sequencing was performed on excised bands, using an Applied Biosystems 447A pulsed-liquid protein sequencer. Generation and separation of internal Bsp peptides using endoproteinase Lys-C (Promega) was performed as described by Maiorino *et al*. (1996). Briefly, a gel slice containing Bsp was washed twice with 100 μl 100 mM sodium bicarbonate and 50 % acetonitrile. Cysteine residues were reduced for 30 min at 55 °C with 45 mM dithiothreitol in 50 μl 8 M urea and 0·4 M ammonium bicarbonate and alkylated for 30 min by the addition of 5 μl 100 mM iodoacetamide; this was followed by a washing step with H_2O. Digestion of Bsp was performed at 37 °C for 20 h in a total volume of 50 μl containing 100 mM ammonium bicarbonate, 10 % acetonitrile, 1 % Triton X-100 and 0·5 μg endoproteinase Lys-C (sequencing grade; Promega). The solution was collected, vacuum-dried and subsequently dissolved in 20 μl 20 % acetonitrile. Samples (10 μl each) were analysed by HPLC (Applied Biosystems) on an Aquapore OD-300 RP-18 column at 37 °C and at a flow rate of 40 μl min^{-1}. Solvent A was 0·06 % trifluoroacetic acid in water and solvent B 0·05 % was trifluoroacetic acid in 80 % acetonitrile. After sample injection, a linear gradient was started to reach 45 % of solvent B in 75 min. Peaks were detected at 214 nm and collected manually in 0·5 ml micro-centrifuge vials.

Preparation of Bsp fusion protein and generation of anti-Bsp antibodies. Bsp fusion protein was synthesized in recombinant *E. coli* BL21 by the addition of 1 mM IPTG after the culture had reached an OD_{600} of 1·0. The cells were disrupted using a French pressure cell, and purification of the fusion protein was performed according to the instructions of Clontech by using cobalt affinity chromatography. For the generation of anti-Bsp antibodies, affinity-purified Bsp fusion protein was size-separated by SDS-PAGE, blotted onto nitrocellulose and, after staining with Ponceau S, the Bsp-containing band was excised. After the nitrocellulose membrane had been dissolved in DMSO, the solution was used for the immunization of mice. Immunization consisted of two intramuscular applications of the purified protein within 2 weeks. Serum was collected 4 weeks after immunization.

Western blot analysis and quantification of cell-surface hydrophobicity. Western blotting was performed essentially as described previously (Reinscheid *et al*., 2001), using a 1:500 dilution of the anti-Bsp antiserum, a 1:15000 dilution of goat anti-mouse-Fab fragments (Dianova), and subsequent detection by chemiluminescence using the ECL kit (Amersham/Pharmacia). Culture supernatant and cell wall proteins from GBS were isolated as described elsewhere (Kling *et al*., 1999). Cell-surface hydrophobicity was determined in an aqueous/hexadecane emulsion by quantification of the amount of bacteria in the aqueous phase as described by Rosenberg *et al*. (1981).

Determination of murein hydrolase activity. Peptidoglycan-lytic activity was analysed by zymography and by using a turbidity assay as described previously (Reinscheid *et al*., 2001). The bacteriolytic activity of Bsp fusion protein was tested according to Simmonds *et al*. (1997). Cell autolysis was performed essentially as described by Qin *et al*. (1998). Briefly, samples from exponential and stationary growth phase were washed three times with 100 mM phosphate buffer (pH 7·4) and the suspension was subsequently adjusted to an OD_{600} of

0·3. The suspension was then incubated at 37 °C, and the OD_{600} was measured at 30 min intervals for 6 h.

Preparation and separation of muropeptides. After growth of GBS at 37 °C, peptidoglycan was prepared as described by Hakenbeck *et al.* (1998). Digestion of lyophilized peptidoglycan with *Streptomyces globisporus* mutanolysin (25 μg ml^{-1}) and reduction of the muropeptides with borohydride were performed as described by Weber *et al.* (2000). Muropeptides were separated by reversed-phase HPLC as previously described (Hakenbeck *et al.*, 1998). The analysis of the muropeptide profiles, including the preparation of peptidoglycan, was done in duplicate.

Amino acid analysis of peptidoglycan. Purified peptidoglycan was hydrolysed in 6 M HCl at 166 °C for 1 h. Samples were subsequently subjected to amino acid analysis (Biotronic LC 5000) after drying. Amino acid enantiomers were quantified by the technique of enantiomer labelling (Frank *et al.*, 1978) after separation by GC and detection by electron-impact MS as previously reported (Höltzel *et al.*, 2001). The total amount of an amino acid was calculated by adding the amounts of the two enantiomers of the amino acid. Lyophilized peptidoglycan (1 mg) was hydrolysed in 6 M DCl/D_2O (containing 20 mM thioglycolic acid as antioxidant) at 110 °C for 16 h. Amino acids, derivatized to their *N*-trifluoracetyl-*O*-ethyl esters, were analysed; the data collected were processed on a model 5973 gas chromatograph coupled on-line with a model 6890 electron-impact mass spectrometer using the manufacturer's protocols and software (Hewlett Packard). Separation was achieved on a 250 μm × 25 m column of fused silica modified with 30 % 2,6-dipentyl-3-butyrylcyclodextrin in PS255 with a film thickness of 0·13 mm and a 250 μm × 25 m Chirasil-L-Val capillary using appropriate temperature gradients.

Both procedures for amino acid analysis, including the preparation of peptidoglycan, were done in duplicate.

RESULTS

N-terminal sequencing of proteins in the culture supernatant of GBS

SDS-PAGE of the concentrated culture supernatant of GBS strain 6313 previously identified three major secreted proteins (Reinscheid *et al.*, 2001). However, by loading higher quantities of concentrated culture supernatant onto the gel, three further protein bands with sizes of 90, 65 and 60 kDa could be visualized (data not shown); these bands were isolated and subjected to N-terminal sequencing. The N-terminus (SKIIGIDLGTTNSAVAVLEG) of the 90 kDa polypeptide revealed significant similarity to the chaperone Hsp70 from *Lactococcus lactis*, while the N-terminus (EPDSVWAAR) of the 60 kDa protein did not show any similarity to the N-terminal regions of polypeptides available in public-domain databases. Interestingly, the N-terminus (DQTTSVQVNN) of the 65 kDa protein (P65) revealed 60 % identity to the N-terminal region (DSNNSVSVNN) of an M-like protein from *Streptococcus pyogenes* M65. Since M-like proteins are important virulence factors in *Strep. pyogenes*, we initiated studies to isolate the P65 gene and to identify and characterize the function of the P65 protein in GBS. As P65 represents a group B streptococcal secreted protein, it was designated Bsp.

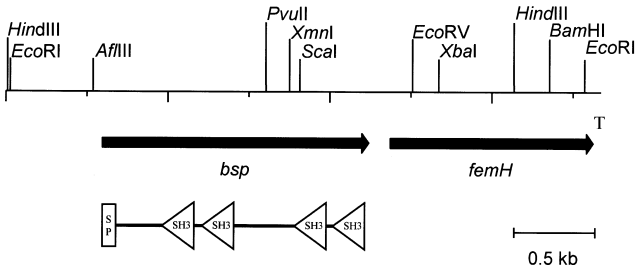

Fig. 1. Restriction map of the *bsp*- and *femH*-encoding region in GBS, and a diagrammatic representation of structural features and domains of the deduced Bsp protein. Open arrows indicate the positions of the *bsp* and *femH* genes, and 'T' represents the proposed transcriptional terminator downstream of the *femH* gene. SP, putative signal peptide; SH3, putative cell-wall-binding SH3 domain.

Isolation and characterization of the *bsp* gene

Endoproteolytic digestion of Bsp with endoproteinase Lys-C and N-terminal sequencing of five peptides yielded the sequences Bsp1 (TGVYNIIGSTEVK), Bsp2 (DQTTSVQVNN), Bsp3 (VASPTQFTLDK), Bsp4 (TLPEQGNYVYS) and Bsp5 (VSSPVEFNFQK), respectively. Two degenerate primers (5′-GTWCARGTNAAYAAYCARAC-3′ and 5′-CCDATDATRTTRTANACNCC-3′) were synthesized, according to the N-terminus and the internal sequence Bsp1, and used to amplify a *bsp* internal fragment of about 1·0 kb from the chromosome of GBS. The *bsp*-specific PCR product was used as a digoxigenin-labelled probe to isolate the entire *bsp* gene from a GBS cosmid library in *E. coli*. Subcloning of the *bsp*-encoding region in pUC18 resulted in the identification of a 3·7 kb insert which was finally sequenced. As shown in Fig. 1, analysis of the *bsp*-encoding region identified two ORFs extending from bp 590 to bp 2230 (ORF1) and from bp 2374 to bp 3609 (ORF2). ORF1 is preceded by a typical ribosome-binding site (AAGGAAG) and encodes a polypeptide of 544 aa with a predicted molecular mass of 60·417 Da. As the six peptide sequences obtained by N-terminal sequencing of Bsp and Bsp-derived peptides exactly matched the deduced polypeptide of ORF1, it was concluded that ORF1 represents the *bsp* gene. The deduced Bsp protein carries, at its N-terminus, a typical signal peptide sequence (Nielsen *et al.*, 1997) of 42 aa, and possesses, at its C-terminus, a cell wall anchor motif (Schneewind *et al.*, 1993) (LPKTG), suggesting that Bsp is transported across the cytoplasmic membrane and is covalently attached to the cell wall of GBS. Interestingly, analysis of Bsp with the SMART program (http://smart.embl-heidelberg.de/smart) identified four SH3 domains, which are suggested to be involved in the binding of proteins to the bacterial cell wall (Ponting *et al.*, 1999).

ORF2 starts 144 bp downstream of the *bsp* gene, is preceded by a ribosome-binding site (AAGAAAGG) and is transcribed in the same direction as the *bsp* gene. Since the deduced polypeptide revealed significant homology to Fem-like proteins (see below), ORF2 was

designated *femH*. The *femH* gene is followed by a putative rho-independent terminator structure, suggesting transcriptional termination downstream of the *femH* gene. To test the possibility of *bsp* and *femH* co-transcription, RT-PCR was used to amplify from total RNA of GBS the junction region between 3′ *bsp* and 5′ *femH*. By using RT-PCR, a DNA fragment of 432 bp was amplified, of which the DNA sequence was identical to the targeted *bsp–femH* region (data not shown). No PCR product was obtained without the addition of reverse transcriptase. These results indicate that *bsp* and *femH* have a bicistronic organization in GBS.

Homology search of Bsp and FemH

Database analysis of the deduced Bsp protein revealed 42·6 % similarity to an unknown ORF in the incomplete genome sequence of *Streptococcus mutans* (http://www.genome.ou.edu), 25·2 % similarity to the glycyl-glycine endopeptidase lysostaphin from *Staph. simulans* biovar *staphylolyticus*, and 22·2 % similarity to the glycyl-glycine endopeptidase Ale-1 from *Staph. capitis*. The deduced FemH protein shows 62·3 % similarity to the MurN protein from *Strep. pneumoniae*, 58·6 % similarity to the zoocin immunity factor (Zif) from *Streptococcus equi* subsp. *zooepidermicus*, and 36·4 % similarity to the FemA protein from *Staph. aureus*. Since MurN, Zif and FemA are known to be involved in the formation of the interpeptide bridge during peptidoglycan biosynthesis, the database analysis indicates that FemH is involved in the synthesis of the interpeptide bridge of the peptidoglycan in GBS, whereas Bsp appears to be a putative peptidoglycan-cleaving endopeptidase.

Functional analysis and serological detection of the Bsp protein

Because of the sequence similarity of Bsp to the bacteriolytic endopeptidases lysostaphin and Ale-1, a hexahistidyl-tagged recombinant Bsp protein and Bsp-containing culture supernatant of GBS were tested in agar diffusion tests for bacteriocidal activity against different bacterial species. However, no such activity could be observed against *Lactococcus lactis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Staph. aureus* and streptococci of the serological groups A, B, C, G and L, indicating that Bsp does not represent a bacteriolytic enzyme from GBS. In addition, neither the Bsp fusion protein nor Bsp in the culture supernatant of GBS revealed autolytic activity against cell walls of GBS (data not shown).

To investigate the distribution of Bsp in different GBS serotypes, purified Bsp fusion protein was used for the production of antibodies against Bsp. Culture supernatants of 31 clinical isolates of GBS, belonging to six serological groups, were subsequently tested for the presence of the Bsp protein (Fig. 2). In the culture supernatant of every GBS strain, the anti-Bsp antibodies detected a single protein of 65 kDa, indicating a wide distribution of Bsp in GBS and a high degree of conservation of the size of the Bsp protein.

Construction of a *bsp* deletion mutant and a *femH* insertion mutant of GBS

To analyse the importance of *bsp* and *femH* for GBS, *bsp* was deleted and *femH* insertionally inactivated in the genome of GBS 6313, resulting in the *bsp* mutant SMB and in the *femH* mutant FBH. The successful deletion of *bsp* and the disruption of *femH* in the two mutant strains was confirmed in both by Southern blot analysis (data not shown). In Western blotting experiments, culture supernatant of GBS SMB lacked a Bsp-specific band, while culture supernatants from the GBS strains 6313 and FBH revealed identical amounts of Bsp (data not shown). GBS mutants SMB and FBH exhibited growth rates and final optical densities similar to those of their parental strain, 6313, indicating that *bsp* and *femH* are not essential for GBS under these growth conditions. Since Bsp and FemH are suggested to be involved in the cell wall metabolism of GBS, the autolysis rate of the two mutant strains was compared with GBS 6313. However, no difference in autolysis could be observed between the strains (data not shown). Subsequently, the sensitivity of the GBS mutants SMB and FBH to different β-lactam antibiotics and vancomycin was determined. The MICs of penicillin G, oxacillin, imipenem, cefotaxim and vancomycin for the GBS mutants SMB and FBH and the GBS parental strain were identical at 0·064 μg ml^{-1}, 0·38 μg ml^{-1}, 0·064 μg ml^{-1}, 0·032 μg ml^{-1} and 0·5 μg ml^{-1}, respectively. In addition, the lysis rates of the three strains in the presence of 0·032 μg ml^{-1}, 0·064 μg ml^{-1} and 0·128 μg ml^{-1} penicillin G were identical (data not shown). These data indicate that *bsp* and *femH* do not influence the sensitivity of GBS to antibiotics that act on the cell walls of these bacteria.

The surface hydrophobicity of GBS mutants SMB and FBH was compared to that of GBS 6313. The assay was performed in an aqueous/hydrocarbon emulsion by photometric quantification of the bacteria in the aqueous phase. The amount of bacteria in the aqueous phase was comparable for the *femH* mutant FBH and the parental GBS strain (OD_{600} 0·34). However, the amount of the *bsp* mutant SMB in the aqueous phase was reduced (OD_{600} 0·225), indicating that the presence of Bsp decreases the cell-surface hydrophobicity of GBS.

Bsp affects the cell shape of GBS

To analyse the effect of *bsp* overexpression on the chain length and morphology of GBS, strains 6313 and SMB were transformed with the *E. coli/Streptococcus* shuttle vector pAT28 or the *bsp*-carrying plasmid pAT*bsp*. RT-PCR analysis revealed that plasmid-mediated expression of *bsp* increased the amount of *bsp*-specific transcript in GBS 6313(pAT*bsp*) about fivefold compared to GBS 6313(pAT28) (data not shown). In Western blotting experiments, overexpression of *bsp* resulted in threefold higher amounts of Bsp in the culture supernatant of GBS 6313(pAT*bsp*), while Bsp was not detected in cell wall preparations of either of the GBS strains (data not shown). The 6313- and SMB-derived strains were

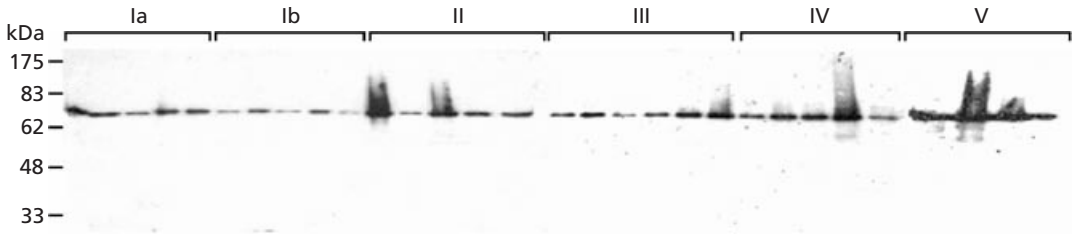

Fig. 2. Western blot analysis of culture supernatants from different GBS strains belonging to serotypes Ia, Ib, II, III, IV and V, respectively, for the presence of the Bsp protein. The strains represent different clinical isolates that had been isolated from infected neonates. Total protein (15 μg) isolated from culture supernatant of the respective strains was tested by using mouse polyclonal anti-Bsp antibodies.

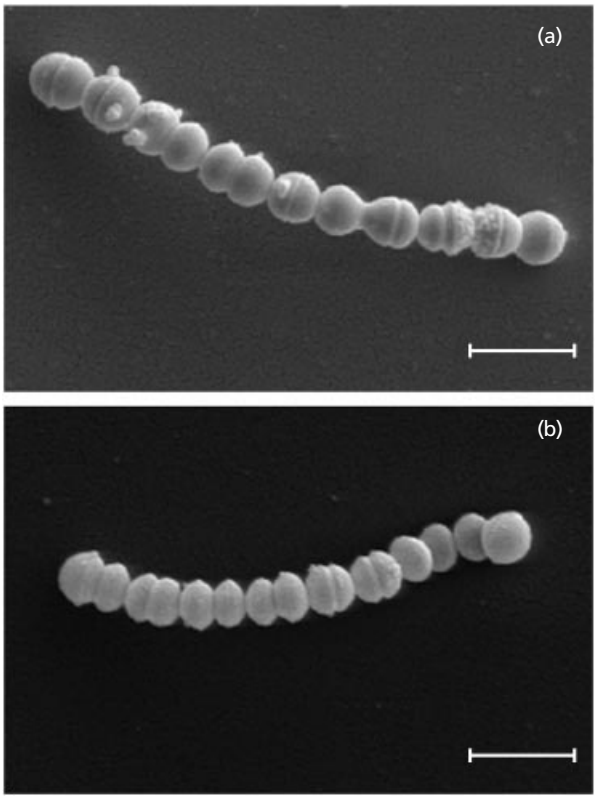

Fig. 3. Scanning electron micrographs of GBS 6313(pAT28) (a) and its *bsp*-overexpressing derivate, GBS 6313(pAT*bsp*) (b). Cells of GBS 6313(pAT28) are spherical while those of GBS 6313(pAT*bsp*) are lens-shaped. Bars, 2 μm.

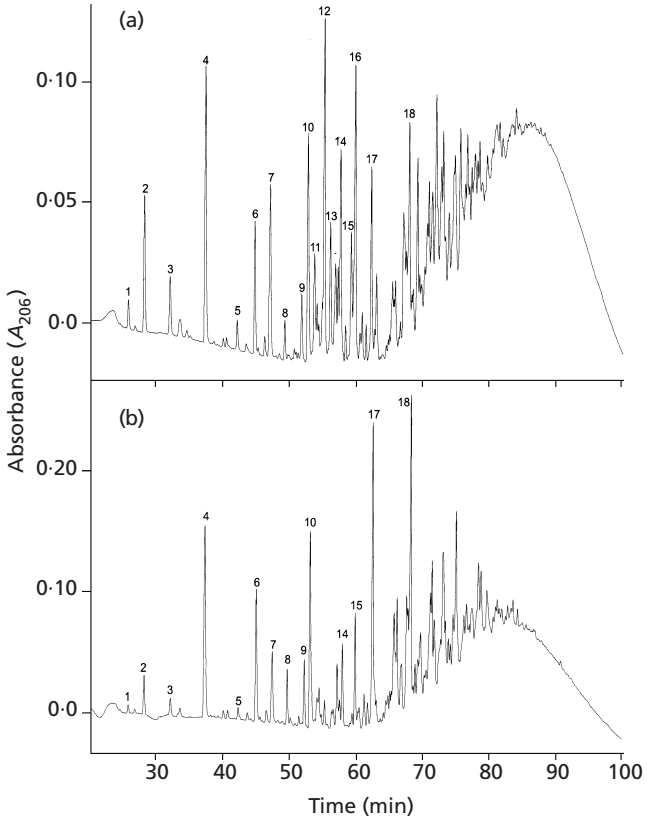

Fig. 4. HPLC analysis of mutanolysin-digested cell walls of the GBS strains 6313 and FBH. Isolated muropeptides were subjected to reversed-phase HPLC as described in Methods. (a) GBS 6313; (b) GBS FBH. Peaks are numbered sequentially.

subsequently subjected to a light-microscopic inspection, and the mean number of cells within 50 arbitrarily chosen chains was determined. No difference in the chain length could be observed between the different strains. Scanning electron microscopy revealed no differences in the cell morphology of GBS strain 6313 (pAT28) and its *bsp* mutant, SMB(pAT28). However, as shown in Fig. 3, cells of GBS 6313(pAT28) exhibit a spherical shape, while those of GBS 6313(pAT*bsp*) are lens-shaped. Similar differences were also observed when comparing cells from strain SMB(pAT28) with those from SMB pAT*bsp* (data not shown).

FemH affects the muropeptide profile and amino acid composition of peptidoglycan

To examine the role of *femH* in cell wall biosynthesis in GBS, peptidoglycan of the GBS strains 6313 and FBH was isolated, digested with muramidase, and the muro-

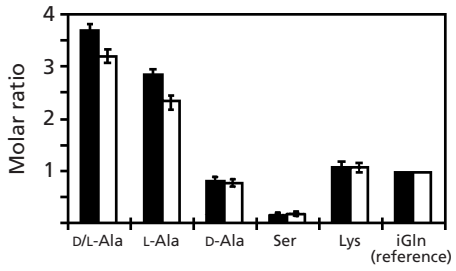

Fig. 5. Total amino acid analysis of purified peptidoglycan from GBS strains 6313 (■) and FBH (□). The amount of each amino acid is expressed as a molar ratio relative to iGln, for which the amount was set as 1·0. The amount of D,L-Ala, Ser, Lys and iGln was determined both in an amino acid analyser and by GC, followed by electron-impact MS. Each experimental approach was performed in duplicate. The amount of the D- and L-stereoisomers of alanine was determined in duplicate by GC, followed by electron-impact MS. Data represent means ± SD.

peptides analysed by reversed-phase HPLC (Fig. 4). The disruption of *femH* in mutant FBH resulted in the virtual absence of four muropeptides (Fig. 4, peaks 11, 12, 13 and 16) and an increased amount of two muropeptides (Fig. 4, peaks 17 and 18), indicating significant differences in the peptidoglycan of GBS mutant FBH compared to GBS 6313. To analyse these changes in more detail, the peptidoglycan of GBS strains 6313 and FBH was subjected to total amino acid analysis by two experimental approaches: amino acid racemates were quantified both in an amino acid analyser and by GC followed by electron-impact MS. The latter method was also used to quantify the enantiomers of each amino acid. The results of the two experimental approaches, each performed in duplicate, are summarized in Fig. 5. Both analytical procedures exclusively identified the amino acids alanine, serine, isoglutamine and lysine within the peptidoglycan of GBS, indicating a high purity of the murein preparations. A comparison of the amino acid ratios within the peptidoglycan from GBS 6313 and mutant FBH revealed no differences in their molar ratios of serine, lysine and isoglutamine. However, for the peptidoglycan of GBS mutant FBH, both analytical methods showed a 14% lower amount of DL-alanine compared to that in GBS 6313. Quantification of amino acids by electron-impact MS allowed us to distinguish the relative proportions of the stereoisomers of each amino acid. Serine and lysine were present in the peptidoglycan of GBS exclusively as the L-enantiomer, isoglutamine as the D-isomer, and alanine as both D- and L-enantiomers. A detailed analysis of the data obtained by electron-impact MS revealed no difference in the molar ratio of D-alanine within the peptidoglycan of GBS 6313 and mutant FBH (Fig. 5). However, the amount of L-alanine within the peptidoglycan of GBS mutant FBH was reduced by 16%. Taking into account the presence of one L-alanine in the peptidoglycan stem peptide of the two strains, the L-alanine content of the interpeptide chain of GBS mutant FBH is reduced by 25% compared to that of GBS parental strain 6313. These data indicate that GBS mutant FBH carries shorter L-alanine-containing interpeptide chains than GBS 6313.

DISCUSSION

Although the bacterial murein sacculus appears as a static structure that protects the cell against its intracellular pressure, it is a highly flexible meshwork that allows bacterial growth and separation during cell division (Höltje, 1998). This requires a permanent turnover of peptidoglycan, i.e. concomitant biosynthesis, cleavage and recycling. Despite the functional coupling of cell wall biosynthesis and degradation, a genetic linkage of enzymes involved in peptidoglycan biosynthesis and degradation is found only in a few bacteria, i.e. *Staph. simulans* biovar *staphylolyticus*, *Staph. capitis*, *Streptococcus milleri* and *Streptococcus equi* subsp. *zooepidermicus*. In these organisms, the genes for the bacteriolytic endopeptidases lysostaphin, Ale-1, millericin B and zoocin A, respectively, are clustered with genes encoding Fem-like resistance proteins which modify the interpeptide bridge of the peptidoglycan, thereby protecting these strains against their own bacteriolytic enzymes (Beatson *et al.*, 1998; Sugai *et al.*, 1997b; Thumm & Götz, 1997; Beukes & Hastings 2001). In the present study, the *bsp* gene, whose product reveals similarity to the bacteriolytic enzyme lysostaphin, was found to be clustered and co-transcribed with the *femH* gene, encoding a Fem-like protein. This genetic organization and the apparent similarity of Bsp and FemH to bacteriolytic enzymes and immunity factors, respectively, might indicate that Bsp is a bacteriolytic enzyme and FemH a protein conferring protection against Bsp. However, functional analysis of a Bsp fusion protein and Bsp-containing culture supernatant revealed no bacteriolytic activity against a variety of different Gram-positive bacterial species. In addition, insertional mutagenesis of *femH* revealed no effect on the viability of GBS, which is in contrast to the Fem-like immunity factors that are essential for the viability of those strains that produce a bacteriolytic enzyme. Taken together, these data suggest that *bsp* and *femH* do not encode a bacteriolytic enzyme and an immunity factor, respectively, from GBS.

Bsp from GBS has limited similarity to the murein hydrolases lysostaphin and Ale-1, respectively, and possesses four SH3 domains which are suggested to mediate binding to the bacterial cell wall (Ponting *et al.*, 1999). As both lysostaphin and Ale-1 contain SH3 domains (Ponting *et al.*, 1999), it can be speculated that Bsp, like these murein hydrolases, is capable of recognizing, and binding to, the bacterial cell wall. However, in our studies, the Bsp protein could not be detected in cell wall preparations of GBS, indicating that Bsp is not tightly linked to the cell wall. Interestingly, overexpression of *bsp* resulted in a lens-shaped morphology for GBS. Although GBS cells are typically spherical, a shape which is generally believed to be the most simple cellular morphology, recent findings with different streptococci, including GBS, have clearly shown the importance of

extracellular proteins for the cell morphology of these bacteria (Reinscheid *et al*., 2001; Chia *et al*., 2001; Mattos-Graner *et al*., 2001). It is therefore tempting to speculate that Bsp plays a role in controlling the cell shape of GBS. Although the *bsp* deletion mutant SMB did not reveal an altered cell morphology, its *bsp* deficiency might be compensated for by alternative mechanisms. Similarly, different components involved in the peptidoglycan metabolism of *E. coli* have been shown to be highly redundant (Höltje & Heidrich, 2001).

Disruption of *femH* in GBS resulted in a significant decrease in the amount of L-alanine in the cell wall of the GBS mutant FBH. As the interpeptide chain of the peptidoglycan of GBS is composed of L-alanyl-L-alanine and L-alanyl-L-serine dipeptides, FemH of GBS is conceivably involved in the incorporation of L-alanine residues into the interpeptide bridge of the peptidoglycan. Similarly, the disruption of *femAB* and *murMN* in *Staph. aureus* and *Strep. pneumoniae*, respectively, result in a significant reduction of the amount of glycine and alanine, respectively, in the interpeptide chains of the resultant mutants (Filipe *et al*., 2000; Stranden *et al*., 1997). The deduced protein sequence of *femH* reveals striking similarity to MurN from *Strep. pneumoniae*, which is required for the addition of the second L-alanine into the interpeptide chain of the bacteria (Filipe *et al*., 2000). Because of this sequence similarity, it is tempting to speculate that FemH from GBS incorporates L-alanine into position 2 of the interpeptide chain. However, the *femH* mutant FBH revealed no L-Ser reduction and a reduction of only 0·5 L-Ala per interpeptide chain, while, in the case of the *murN* mutant of *Strep. pneumoniae*, one L-Ala per interpeptide chain is lost (Filipe *et al*., 2000). This discrepancy might be explained by incomplete inactivation of *femH* in GBS mutant FBH. Although the *femH* gene was insertionally inactivated by a fragment, corresponding in size and location to the one used for disrupting *murN* in *Strep. pneumoniae*, the possibility that the truncated FemH protein in GBS retained some catalytic activity cannot be ruled out. Alternatively, the disruption of *femH* might be partially compensated for by another *fem*-like gene from GBS. Similarly, the Fem-like lysostaphin immunity factor Lif was shown to complement a FemB deficiency in *Staph. aureus* (Tschierske *et al*., 1997).

Although GBS remains uniformly susceptible to β-lactam antibiotics, the treatment of GBS infections requires 4–10-fold higher doses of β-lactam antibiotics compared to *Strep. pyogenes* infections (Fernandez *et al*., 1998). Penicillin resistance can result from the acquisition of specific penicillin-binding proteins that, in the presence of β-lactam antibiotics, take over the function of the cells' own suceptible penicillin-binding proteins in cell wall biosynthesis (de Jonge & Tomasz, 1993). Interestingly, the peptidoglycan interpeptide bridges of β-lactam-resistant strains of *Staph. aureus* and *Strep. pneumoniae* are essential for the resistance of these strains against methicillin and penicillin, respectively. Thus, the disruption of the *femAB* genes in highly methicillin-resistant *Staph. aureus* strains results in virtually a complete loss of methicillin resistance (Stranden *et al*., 1997). Similarly, the inactivation of the *murMN* genes in pencillin-resistant *Strep. pneumoniae* strains causes a complete loss of the penicillin resistance of these strains (Filipe & Tomasz, 2000). Since FemH from GBS exhibits significant similarity to MurN and to FemA from *Strep. pneumoniae* and *Staph. aureus*, respectively, FemH might be required for the intrinsic reduced β-lactam susceptibility of GBS. However, the disruption of *femH* in GBS did not increase the susceptibility of GBS to different β-lactam antibiotics, revealing that *femH* is not involved in the lower β-lactam sensitivity of GBS. It is interesting to note that in *Strep. pneumoniae* strains, which are intrinsically sensitive to β-lactam antibiotics, the disruption of *murMN* does not further increase their susceptibility to β-lactams (Filipe & Tomasz, 2000). Therefore, it would be interesting to analyse the effect of disrupting *femH* in β-lactam-resistant GBS strains (Kim, 1985). Finally, it is tempting to speculate that FemH might be used as a target in future studies of β-lactam-resistant strains of GBS.

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