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-ιGln-L-Lys-D-Ala-D-Ala (ιGln, 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 glycy1 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-liked 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 bacteriocytic enzymes that cleave the interpeptide bridge and cause lysis of the target cell. *Staphylococcus simulans* biovar staphylohyticus and *Staphylococcus capitis*, respectively, secrete the glycol-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 & Görtz, 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 (Ehler 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 & Görtz, 1997). Similar bacteriocytic enzymes and immunity factors from different streptococcal species have also been described (Beatson et al., 1998; Simmonds et al., 1997; Beuks & 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*<sup>+</sup>*host6* derivative of GBS 6313, carrying an insertional 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*<sup>+</sup>*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*<sup>+</sup>*host6 or pAT28 derivatives were selected in the presence of erythromycin (7 µg ml<sup>-1</sup>) and spectinomycin (200 µg ml<sup>-1</sup>), 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<sup>-1</sup>), kanamycin (50 µg ml<sup>-1</sup>) or spectinomycin (100 µg ml<sup>-1</sup>). For visual inspection of the mean 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) 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 *Sal*<sup>III</sup>. Plasmid pET28a (Novagen) was used for the expression of the bacteriocytic-tagged enzyme. 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 (Ehler 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).

**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 thermostable plasmid pG*<sup>+</sup>*host6 (Appligene) was used. Two *bsp* flanking fragments were amplified by PCR using the primers *5′*-GGATCCATCGGATCCATTCA-GCC-3′ and *5′*-TGGCCTGATGCTTCAATATAGCTGAGAA-3′. The BamH<sup>I</sup> and HindIII restriction sites used for cloning are underlined. After digestion of the *bsp* PCR product and of plasmid pET28a with BamH<sup>I</sup> and HindIII, 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′*-GCCGATCCGGAAGGATCTCTG-3′ and *5′*-CGGCTGATGCTTCAATATAGCTGAGAA-3′. After digestion of the PCR product and of plasmid pAT28 with EcoRI and XbaI, 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).

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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 bps excisants. Chromosomal DNA of GBS 6313 and of 24 erythromycin-sensitive GBS excisants was tested by Southern blotting after HindIII digestion, using a digoxigenin-labelled bsp flanking fragment obtained with primers bsp-de1 and bsp-de2.

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'-CGCGGATCCAGTTACCGGTGCCGCTTGCATCTAC-3' and 5'-GAGCGGATTCTCTTCTC-3'. The BamHI and HindIII restriction sites used for cloning are underlined. The resulting PCR product and plasmid pG host6 were digested with BamHI and HindIII, 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 ClaI-digested chromosomal DNA from GBS and a digoxigenin-labelled femH probe obtained by PCR with primers 5'-TTATGCCAGCTACGTGGG-3' and 5'-AGACGGTGCTTACATGAC-3'. The femH mutation was stably maintained by growing the femH-inactive GBS strain at 37 °C in the presence of 5 µg erythromycin ml-1.

RNA preparation and RT-PCR analysis. Total RNA from 250 ml mid-exponential-phase GBS 6313 culture was prepared by using the RNaseasy Midi extraction kit (Qiagen) and was treated for 30 min with 150 µ 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'-TAGTAGATGTCATGCG-G-3') and 5'-femH (5'-GGTCTCCTAATCACATTTTTTCC-3'). For comparative expression analysis of bsp in GBS strains 6313(pAT28) and GBS 6313(pATbosp), RNA samples from the two strains containing 5, 1, 0.5, 1, 0.05 or 0.01 µg RNA were tested by RT-PCR for the presence of a bsp-specific transcript, using primers 5'-GAGACAGTGGTGCCTCAAGTG-3' and 5'-TGAGTTGGACCTCGTACCATC-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 OD600 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 OD600.

N-terminal sequencing of proteins and peptides. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane (Amersham/Pharmacia), and visualized with an anti-mouse antibody. N-terminal amino acid sequencing was performed on excised bands, using an Applied Biosystems 477A pulsed-liquid protein sequencer. Generation and separation of internal Bsp peptides using endopeptidase 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 H2O. 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 endopeptidase Lys-C (sequencing grade; Promega). The solution was collected, rinsed, rinsed 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 % 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 OD600 of 1.0. The cells were disrupted using a French press machine, 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 DMF, 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. Peptidoglycanolytic 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 OD600 of 0.2.
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 (Hölzel 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_{2}O (containing 20 mM thioglycolic acid as antioxidant) at 110 °C for 16 h. Amino acids, derivatized to their N-trifluoroacetyl-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 (SKHIGDLGTV-TN1AVLEG) of the 90 kDa polypeptide revealed significant similarity to the chaperone Hsp70 from *Lactococcus lactis*, while the N-terminus (EPDSDV-WAAR) of the 60 kDa protein did not show any similarity to the N-terminal regions of peptidoglycans available in public-domain databases. Interestingly, the N-terminus (DQTTSVQVNN) of the 65 kDa protein (P65) revealed 60% identity to the N-terminal region (DSNNSVSQVNN) 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.

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**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 endoprotease Lys-C and N-terminal sequencing of five peptides yielded the sequences Bsp1 (TGVYNIIGSTEVK), Bsp2 (DQTTSVQVNN), Bsp3 (VASPTQFTLDK), Bsp4 (TLPEQGNYYS) and Bsp5 (VSSPVEFNFQK), respectively. Two degenerate primers (5’-GTCWARGTNAYAAYCARAC-3’ and 5’-CCDATDATRTTTRTANA-CNCC-3’) were synthesized, according to the N-terminus and the internal sequence Bsp1, and used to amplify a bsp internal fragment of about 1 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 60-17 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 (AAGGAAG) 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
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 Staphylococcus biovar staphylolyticus, and 22.2% similarity to the glycyl-glycine endopeptidase Alc-1 from Staphylococcus capitis. The deduced FemH protein shows 62.3% similarity to the MurN protein from Streptococcus pneumoniae, 58.6% similarity to the zoon immunity factor (Zif) from Streptococcus equi subsp. zooepidermicus, and 36.4% similarity to the FemA protein from Staphylococcus 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 bacteriocytic endopeptidases lysostaphin and Alc-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, Staphylococcus aureus and streptococci of the serological groups A, B, C, G and L, indicating that Bsp does not represent a bacteriocytic 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, impenem, 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 (OD₀/₀ 0·34). However, the amount of the bsp mutant SMB in the aqueous phase was reduced (OD₀/₀ 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 pATbsp. RT-PCR analysis revealed that plasmid-mediated expression of bsp increased the amount of bsp-specific transcript in GBS 6313(pATbsp) 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(pATbsp), 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(pATbsp) (b). Cells of GBS 6313(pAT28) are spherical while those of GBS 6313(pATbsp) 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(pATbsp) are lens-shaped. Similar differences were also observed when comparing cells from strain SMB(pAT28) with those from SMB pATbsp (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-
isoglutamine as the peptidoglycan of GBS exclusively as the

distinguish the relative proportions of the stereoisomers

amino acids by electron-impact MS allowed us to

analytical methods showed a 14% lower amount of

molar ratios of serine, lysine and isoglutamine. How-

amino acid ratios within the peptidoglycan from GBS

purity of the murein preparations. A comparison of the

amino acids alanine, serine, isoglutamine and lysine

both performed in duplicate, are summarized in Fig. 5.

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 ± 50.

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. How-

ever, for the peptidoglycan of GBS mutant FBH, both

analytical methods showed a 14 % lower amount of D-
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

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 in-

tracellular 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 biosynthe-
sis, 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

gen genes for the bacteriolytic endopeptidases lysostaphin,

Ale-1, millericin B and zoocin A, respectively, are

clustered with genes encoding Fem-like resistance pro-

tiens 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 super-

natant 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

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natant revealed no bacteriolytic activity against a variety

of different Gram-positive bacterial species. In addition,
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 (Hölter & 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 the L-alanine residues into the interpeptide bridge of the peptidoglycan. Similarly, the disruption of femAB and murMN in Staphylococcus 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; Strand 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 susceptible 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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REFERENCES


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