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Identification and Molecular Analysis of PcsB, a Protein Required for Cell Wall Separation of Group B Streptococcus

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Group B streptococcus (GBS) is the leading cause of bacterial sepsis and meningitis in neonates. N-terminal sequencing of major proteins in the culture supernatant of a clinical isolate of GBS identified a protein of about 50 kDa which could be detected in all of 27 clinical isolates tested. The corresponding gene, designated *pcsB*, was isolated from a GBS cosmid library and subsequently sequenced. The deduced PcsB polypeptide consists of 447 amino acid residues (M_r, 46,754), carries a potential N-terminal signal peptide sequence of 25 amino acids, and shows significant similarity to open reading frames of unknown function from different organisms and to the murein hydrolase P45 from *Listeria monocytogenes*. Northern blot analysis revealed a monocistronic transcriptional organization for *pcsB* in GBS. Insertional inactivation of *pcsB* in the genome of GBS resulted in mutant strain Sep1 exhibiting a drastically reduced growth rate compared to the parental GBS strain and showing an increased susceptibility to osmotic pressure and to various antibiotics. Electron microscopic analysis of GBS mutant Sep1 revealed growth in clumps, cell separation in several planes, and multiple division septa within single cells. These data suggest a pivotal role of PcsB for cell division and antibiotic tolerance of GBS.

Group B streptococcus (GBS), also known as *Streptococcus agalactiae*, is part of the normal human flora colonizing the respiratory, gastrointestinal, and urogenital tracts. It is also the leading cause of bacterial sepsis and meningitis in neonates in the United States and western Europe, and it is a major cause of endocarditis and fever in parturient women (2). In the last decade, the incidence of GBS infections has increased especially in the elderly and in immunocompromised persons (58), but despite its clinical importance, GBS is only poorly understood on the molecular level.

Bacterial cell division is a complex interplay of topological processes, biosynthetic reactions, and cleavage mechanisms. Septum formation is initiated by the cooperative action of division inhibitors, topological specificity factors, and proteins constituting the cell division apparatus both found in gram-negative and gram-positive bacteria (reviewed in reference 5, 31, and 42). In globular cells, like streptococci, the division septum can be arranged in several orientations, resulting in the generation of two daughter cells of the same size and shape. The choice of the division plane determines the three-dimensional organization of the multicellular arrays of progeny cells that are formed. In GBS, the plane of division is approximately the same in each division cycle, resulting in the formation of frequently long chains (20). The division septum that is formed between the daughter cells is composed of newly synthesized membrane and peptidoglycan components. Peptidoglycan biosynthesis is accomplished by the insertion of peptide-carrying disaccharide units into the existing murein sacculus by transglycosylation and transpeptidation (34). Cell division is terminated by the action of murein hydrolases that split the peptidoglycan septum, thereby resulting in the release of the daughter cells (reviewed in references 23, 45, and 46). Murein hydrolases have been shown to participate in a number of important biological processes during cell growth and division, including daughter cell separation, cell wall growth, peptidoglycan recycling, and turnover (1, 24, 45). In addition, these enzymes have been shown to contribute to the pathogenicity of bacteria and are required for the bacterial susceptibility to antibiotics (24). Those murein hydrolases that lead to the destruction of the cell wall and cause cell lysis are known as autolysins.

This study describes the characterization of a protein, termed PcsB, which is required for ordered cell wall separation of GBS. Insertional inactivation of the *pcsB* gene exhibited a significant influence on cell septum formation and on the susceptibility of GBS to different antibiotics.

MATERIALS AND METHODS

Bacterial strains and culture conditions. GBS strain 6313 is a serotype III clinical isolate obtained from an infected neonate and has been described previously (55). GBS mutant Sep1 is a *pcsB*::pG$^+$host6 derivative of GBS 6313 carrying a disrupted *pcsB* gene. The GBS strains belonging to different serotypes are clinical isolates and have been described elsewhere (6). Strains from *Enterococcus faecium, Lactococcus lactis, Staphylococcus aureus*, and *Bacillus subtilis* were kindly provided by A. Podbielski (University of Rostock). *Escherichia coli* DH5α (19) was used for the construction of a GBS pTEX5236 cosmid gene library and served as host for the recombinant pG$^+$host6 plasmid. *E. coli* BL21(DE3) (9) harbored the recombinant plasmid pET28 and was used for the production of PcsB fusion protein.

GBS, *E. faecium*, and *S. aureus* were cultivated at 37°C in Todd-Hewitt yeast (THY) broth consisting of Todd-Hewitt broth (Oxoid) supplemented with 1% of yeast extract. Cultivation of the GBS mutant Sep1 was performed at 37°C in THY medium containing D-sorbitol (500 mM) and erythromycin (5 μg/ml). *L. lactis* was grown in M17 broth (Oxoid) at 30°C, while *B. subtilis* and *E. coli*

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were grown at 37°C in Luria broth (LB). Recombinant *E. coli* clones carrying cosmid pTEX5236 and plasmid pET28a were selected in the presence of chloramphenicol (15 μg/ml) and kanamycin (50 μg/ml), respectively.

Plasmids and cosmids used for cloning purposes. A pTEX5236 (51) cosmid gene library from GBS 6313 was constructed essentially as described by Xu et al. (60). Briefly, 500 μg of chromosomal DNA was digested with 0.1 U of *Sau*3A for 30 min at 37°C. Fragments were size separated in a sodium chloride salt gradient by ultracentrifugation. A fraction containing fragments of ≥20 kb was ligated with the *Bam*HI-digested cosmid pTEX5236, packaged by using the Gigapack III Gold packaging extract kit (Stratagene), and used to infect *E. coli* DH5α. Recombinant clones were screened on LB medium containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 50 μg/ml); 1,160 individual white colonies from the primary selection plate were picked and stored in 96-well microtiter dishes in LB-tetracycline-glycerol solution.

Plasmid pUC18 (57) was used for subcloning of the *pcsB* gene after partial digestion of a *pcsB*-carrying pTEX5236 cosmid with *Sau*3a and cloning of 2- to 4-kb fragments into the *Bam*HI site of pUC18. Plasmid pET28a (Novagen) was used for expression of the His-tagged PcsB fusion protein. A truncated *pcsB* gene lacking its signal peptide-encoding sequence was amplified by PCR using the primers 5′CGCGGATCCGATGACTTGACTCGAA and 5′TGGCACAAGCTTTCCAATCGTCTGAGACAC) (the *Bam*HI and *Hind*III restriction sites used for cloning are underlined). After digestion of the *pcsB* PCR product and of plasmid pET28 with *Bam*HI and *Hind*III, the *pcsB* gene was ligated into pET28 and transformed into *E. coli* BL21.

Construction of the GBS *pcsB* mutant Sep1. The thermosensitive plasmid pG$^+$host6 (Appligene) was used for targeted disruption of *pcsB* in GBS 6313 to construct mutant Sep1. An internal *pcsB* fragment was amplified by PCR using the primers 5′CGCGGATCCGATGACTTTGACTCGAA and 5′TGGCACAAGCTTTCCAATCGTCTGAGACAC) (the *Bam*HI and *Hind*III restriction sites used for cloning are underlined). The resulting PCR product and plasmid pG$^+$host6 were digested with *Bam*HI and *Hind*III, ligated, and transformed into *E. coli* DH5α. Transformation of the plasmid into GBS was performed as described by Ricci et al. (40). Integration of the plasmid into the chromosome of GBS 6313 was performed by a temperature shift to 37°C essentially as described by Maguin et al. (32), with the modification that all growth media contained D-sorbitol (500 mM). Successful disruption of *pcsB* was confirmed by Southern blotting with *Pst*I-digested chromosomal DNA of GBS 6313 and of GBS Sep1 by using a digoxigenin-labeled *pcsB* fragment obtained with the primers 5′TCTTCAACACCTAGAGCG and 5′TCCAATCGTCTGAGACAC.

RNA preparation and Northern blot analysis. Total RNA from GBS 6313 was prepared after growth to an optical density of 0.4. Cells were disrupted mechanically using glass beads in a Ribolyser (Hybaid), and RNA was purified with a RNeasy kit (Qiagen). Northern blot analysis using a digoxigenin-labeled PCR product of *pcsB* obtained with the primers 5′GATGACTTTGACTCGAA and 5′GCTTGCTTGTTAGCTGC was performed using a Dig labeling and detection kit (Roche Molecular Biochemicals) as instructed by the manufacturer, with subsequent detection by enhanced chemiluminescence (ECL).

General DNA techniques. Chromosomal GBS DNA was isolated as described by Pospiech and Neumann (39). 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. (43).

Electron microscopy and antibiotic testing. Transmission electron microscopy and scanning electron microscopy were performed as described by Valentin-Weigand et al. (54, 55). MICs of penicillin G, cefotaxime, imipenem, meropenem, ceftazidime, vancomycin, ofloxacin, ciprofloxacin, gentamicin, netilmicin, and co-trimoxazole were determined on sheep blood agar plates using antibiotic-containing E-test strips (AB Biodisk) as instructed by the manufacturer.

N-terminal sequencing of proteins and peptides. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), subsequently transfered onto a polyvinylidene difluoride 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 of internal PcsB peptides using endoproteinase Lys-C (Promega) and separation of the peptides were performed as described by Maiorino et al. (33).

Preparation of PcsB fusion protein and generation of anti-PcsB antibodies. PcsB fusion protein was synthesized in recombinant *E. coli* BL21 by the addition of 1 mM IPTG after the culture had reached an optical density of 1.0. The cells were disrupted using a French press cell, and the PcsB fusion protein was solubilized with 6 M guanidine hydrochloride because it formed inclusion bodies in *E. coli*. The fusion protein was purified by cobalt affinity chromatography in the presence of 8 M urea as instructed by Clontech. The purified PcsB fusion protein was dialyzed against 100 mM phosphate buffer (pH 7.4), resulting in the precipitation of high amounts of PcsB. After centrifugation of the precipitate, the PcsB protein in the supernatant was used for the enzymatic digestion of GBS cell walls. For the generation of anti-PcsB antibodies, affinity-purified PcsB fusion protein was size separated by SDS-PAGE and blotted onto nitrocellulose; after staining with Ponceau S, the PcsB-containing band was cut out. After the nitrocellulose membrane was dissolved in dimethyl sulfoxide, 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 for detection of PcsB. Western blotting was performed essentially as described by Talay et al. (50), using a 1:500 dilution of the anti-PcsB antiserum, a 1:15,000 dilution of goat anti-mouse Fab fragments (Dianova), and subsequent detection using an Amersham/Pharmacia ECL kit. A 1:500 dilution of mouse preimmune serum was used for testing nonimmune binding of antibodies to proteins in culture supernatants of the different bacterial species.

Determination of lytic activity by SDS-PAGE. Peptidoglycan lytic activity was analyzed in a zymogram assay essentially as described by Foster (11). Culture supernatant of GBS was concentrated by precipitation with saturated $(NH_4)_2SO_4$ and subsequent dialysis with 10 mM Tris-HCl (pH 7.6). The concentration of total proteins present in each preparation was determined by the Bradford assay (Bio-Rad) according to the manufacturer's directions. A 15-μg aliquot of each preparation was loaded onto an SDS–10% polyacrylamide gel containing 0.4% lyophilized GBS cells. Following electrophoretic separation of the loaded proteins, the murein hydrolases were allowed to hydrolyze the embedded bacterial cells by incubation of the gel in 1% Triton X-100–25 mM Tris-HCl (pH 7.6) buffer overnight at 37°C. After this incubation, the gel was stained using a 1% methylene blue–0.01 M KOH solution and destained in distilled water. Following destaining, the gel was photographed over a light box.

Enzymatic digestion of GBS cell walls. Cell walls were prepared essentially as described by Thumm and Götz (52). GBS was grown in 500 ml of THY medium to exponential phase, centrifuged, and washed two times with distilled water. The pellet was resuspended in 5 ml of distilled water and boiled for 30 min to inactivate autolysins. After cooling to 4°C, the cells were centrifuged and washed two times with distilled water. The pellet was resuspended in 2 volumes of distilled water, and 700-μl portions were transferred to new microcentrifuge tubes, each containing 250 μg of glass beads (150 to 212 μm). The cells were mechanically disrupted in a Ribolyzer (Hybaid), and the glass beads were removed by centrifugation at 2,000 × *g* with subsequent decanting of the supernatant. The pellet was washed two times with distilled water and lyophilized. Then 0.5-mg aliquots of lyophilized GBS cell walls were resuspended in 1 ml of 100 mM phosphate buffer, pH 7.4, followed by the addition of 100 μl of phosphate buffer containing no protein (negative control), 5 μg of mutanolysin (positive control), or 50 μg of purified PcsB fusion protein. The samples were incubated at 37°C, and the turbidity was measured at 30-min intervals at 600 nm. Protein concentrations were determined by the Bradford assay (Bio-Rad) according to the manufacturer's directions.

Nucleotide sequence accession number. The nucleotide sequence of the *pcsB* region from *S. agalactiae* was submitted to the EMBL nucleotide sequence database and assigned accession no. AJ277292.

RESULTS

N-terminal sequencing of major proteins in the culture supernatant of GBS. Concentrated culture supernatant of the GBS serotype III strain 6313 was subjected to SDS-PAGE and subsequently stained with Coomassie brilliant blue (Fig. 1A). Three major protein bands with sizes of 70, 55, and 50 kDa were identified in the culture supernatant of GBS 6313. The N terminus of the 70-kDa polypeptide (ETINPETSLTMATA) showed significant similarity to the N terminus of a protein of unknown function from group A streptococcus, named immunogenic secreted protein (35). The N termini of the 55-kDa protein (PPDAIVPSNDDFA) and the 50-kDa protein (DDF DSKIAATDSVINTLSGQQ), however, showed no similarity to any protein in the database.

SDS-PAGE analysis of 27 different clinical isolates of GBS belonging to six different serotypes revealed a major protein band of around 50 kDa in all strains tested (not shown), sug-

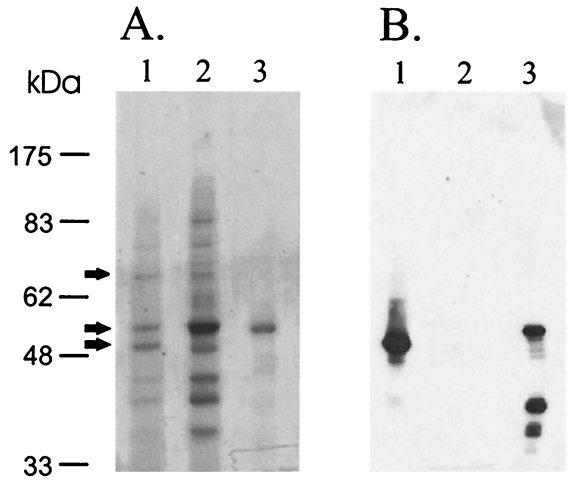

FIG. 1. SDS-PAGE analysis of culture supernatant from GBS strain 6313 (lanes 1) and GBS strain Sep1 (lanes 2), and SDS-PAGE of purified PcsB fusion protein (lanes 3). Following SDS-PAGE, proteins were either stained with Coomassie blue (A) or transferred onto a nitrocellulose membrane and subjected to Western blotting using anti-PcsB polyclonal antibodies (B). Protein bands subjected to N-terminal sequencing are marked by arrows, and positions of molecular weight standards are shown at the left.

gesting that this protein, termed P50, could be common among virulent GBS strains. This prompted us to initiate studies to isolate the corresponding *p50* gene and to analyze the biological function of P50 for GBS.

Isolation and characterization of the *p50* gene. After proteolytic digestion of P50 with endoproteinase Lys-C, the N termini of the two peptides Pep2 (GQVGALESQQSELEA QNAQ) and Pep3 (GNLTNYINTILNSK) were obtained. The structural data were used to synthesize three degenerate primers for the amplification of *p50* internal sequences by PCR. Primer 1 (GATGATTTYGAYWSNAARATH) was derived from the N terminus of P50, while primers 2 (YTGNGCRTT YTGNGCYTC) and 3 (NATDGTRTTDATRTARTTNGT) correspond to the N-terminal peptide sequences of Pep2 and -3, respectively. PCR using primers 1 and 2 and primers 1 and 3 amplified two different fragments of 180 and 280 bp, respectively, from the chromosome of GBS 6313. Sequencing of both fragments revealed sequence identity between the 180- and 280-bp fragments, showing that the two primer sets had amplified the same chromosomal region of GBS. The 280-bp fragment was subsequently used as a digoxigenin-labeled probe to isolate the *p50* gene from a GBS cosmid library in *E. coli*. Three of 378 cosmid clones hybridized to the 280-bp fragment. One of the cosmids was partially digested, and fragments ranging in size between 2 to 4 kb were ligated in the *E. coli* plasmid pUC18. The insert of one clone carrying the putative *p50* gene on a 2,437-bp fragment was subsequently sequenced. Computer analysis of the obtained sequence revealed one complete open reading frame (ORF1) extending from bp 627 to 1967 and one incomplete ORF (ORF2) extending from bp 2094 to the end of the sequenced 2,437-bp fragment (Fig. 2A). Downstream of ORF1, at positions 1991 to 2002, a region of dyad symmetry followed by several T residues similar to rho-independent transcription terminators (41) could be identified, indicating transcriptional termination immediately downstream of ORF1. The deduced polypeptide of ORF1 has a size of 447 amino acids with a molecular mass of 46,754 Da, corresponding in average to the size of the P50 protein. Further inspection of the deduced amino acid sequence of ORF1 revealed at the N terminus a typical signal sequence of 25 amino acids with a putative cleavage site between Ala-25 and Asp-26. However, no C-terminal cell wall anchor motif (LPXTG) could be detected, indicating that this protein is translocated across the cytoplasmic membrane but not covalently bound to the cell wall. Immediately downstream of the predicted signal cleavage site, a stretch of amino acids matched exactly the previously sequenced N terminus of the P50 protein. In addition, the two internal peptide sequences of P50 were identical to portions of the deduced amino acid sequence of the *p50* gene. ORF1, therefore, represents the *p50* gene. Since subsequent functional analysis revealed that P50 is a protein for cell separation of GBS, the *p50* gene was termed *pcsB*. Analysis of the deduced amino acid sequence of ORF2 showed that it encodes a polypeptide with high similarity to phosphoribosyl pyrosphosphate synthetases which are involved in the synthesis of ribonucleoside monophosphates. ORF2 was therefore designated *prs*.

The *pcsB* gene is transcribed monocistronically. To test if *pcsB* forms a transcriptional unit with the proximal *prs* gene, the transcriptional organization of *pcsB* was characterized by Northern blot analysis. Total RNA from GBS strain 6313 isolated at mid-logarithmic growth phase was probed with a 450-bp digoxigenin-labeled *pcsB* DNA fragment, giving a single band at 1.5 kb (Fig. 2B). Since the *pcsB* structural gene has a size of 1.3 kb, this result indicates that *pcsB* is transcribed monocistronically in GBS.

PcsB is similar to proteins from different gram-positive bacteria. Database analysis of the PcsB protein revealed 41.8% similarity to Usp45 from *L. lactis* (56), 34% similarity to P54 from *E. faecium* (13), 28.3% similarity to P45 from *Listeria monocytogenes* (44), and 27.9% similarity to the deduced amino acid sequence of an ORF, named YvcE, in the genome of *B. subtilis* (29). Among the proteins that exhibit homology to PcsB, a biological function is only known for the *L. monocytogenes* protein P45, which has been shown to exhibit murein hydrolase activity (44). The C-terminal portion of P45 reveals significant similarity to the C terminus of the Iap protein from *L. monocytogenes*, also representing a murein hydrolase (59).

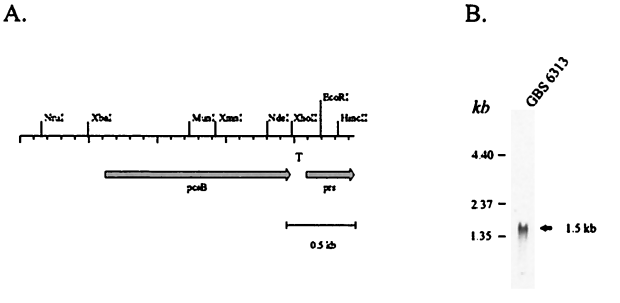

FIG. 2. (A) Restriction map and transcriptional organization of the *pcsB*-carrying region from GBS, showing positions of the two ORFs (open arrows) and the proposed transcriptional terminator of the *pcsB* gene (T). Northern blot analysis using a *pcsB*-specific probe revealed a single band of 1.5 kb for the *pcsB* transcript (B).

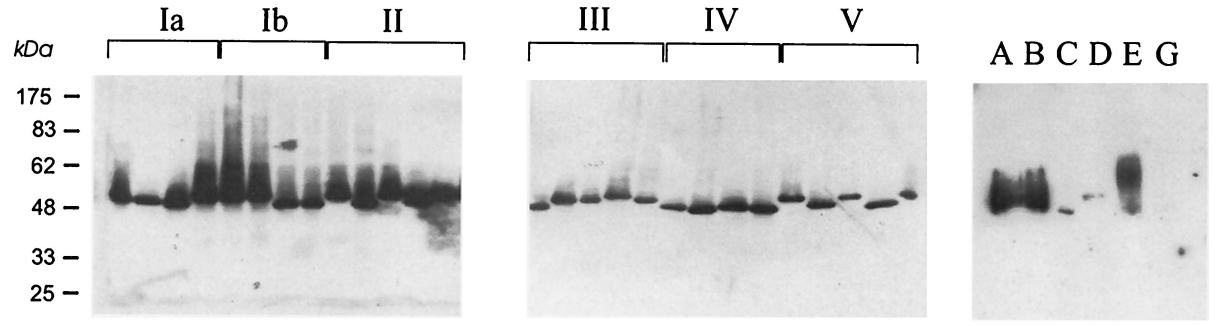

FIG. 3. Western blot analysis of culture supernatants from different bacterial species and from GBS strains belonging to six different serotypes, using anti-PcsB serum. Aliguots (15 μg) of total protein isolated from the supernatants were tested by using mouse polyclonal anti-PcsB antibodies. Lanes: Ia to V, GBS serotypes; A, GBS 6313; B, *E. faecium*; C, *L. lactis*; D, *S. aureus*; E, *B. subtilis*; G, *E. coli*.

Iap and P45 both have at their C termini a stretch of amino acids (FDCSG) carrying a conserved cysteine residue which is discussed to be required for catalytic activity (59). Interestingly, this stretch of amino acids can also be found in P54 from *E. faecium* (44) and YcvE from *B. subtilis*. Although PcsB from GBS and Usp45 from *L. lactis* do not possess this conserved region, they share a conserved cysteine residue at a location similar to that of the cysteine-carrying motif in P45 from *L. monocytogenes*.

PcsB and PcsB-related proteins are widely distributed in GBS and other gram-positive bacteria. The *pcsB* gene was cloned, in frame, without its signal peptide-encoding sequence into the pET28 expression system, placing a hexahistidine affinity tag at the N terminus of the resultant fusion protein. The recombinant plasmid was subsequently transformed into *E. coli* BL21(DE3). By Co^{2+} affinity chromatography, an apparently pure preparation of the PcsB fusion protein was obtained as shown by SDS-PAGE and subsequent Coomassie blue staining (Fig. 1A, lane 3). Polyclonal antibodies against PcsB were obtained after immunization of mice with the purified PcsB fusion protein. To test for the distribution of the PcsB protein in different GBS serotypes and in other bacterial species, culture supernatants of *E. faecium*, *L. lactis*, *S. aureus*, *B. subtilis*, and *E. coli*, and of 21 GBS strains belonging to six different serotypes, were tested for the presence of the PcsB protein (Fig. 3). Bands reacting with the anti-PcsB serum could be detected in the culture supernatant of every GBS strain tested, indicating that PcsB is common to GBS. However, the detected proteins ranged in size between 50 and 55 kDa, suggesting size variation of PcsB among different GBS strains. Interestingly, the culture supernatant of *E. faecium* and *B. subtilis* contained proteins of 50 and 62 kDa, respectively, that interacted strongly with the anti-PcsB antibodies. In addition, the culture supernatant of *L. lactis* showed weak cross-reactivity of the antibodies with a 50-kDa protein. Although the culture supernatant of *S. aureus* contained a protein of 53 kDa that interacted with the anti-PcsB antibodies, experiments with preimmune serum showed a nonimmune interaction of this protein with immunoglobulins (not shown), suggesting that this protein is the immunoglubulin-binding protein A from *S. aureus*. In the culture supernatant of *E. coli*, no protein could be identified that interacted with the anti-PcsB antibodies. These data suggest a wide distribution of PcsB-related proteins in different gram-positive bacteria and support the results of the database analysis that identified PcsB-homologous polypeptides in *E. faecium*, *L. lactis*, and *B. subtilis*.

Construction of the GBS *pcsB* mutant strain Sep1. To analyze the function of PcsB for GBS, we attempted to disrupt the *pcsB* in the genome of GBS 6313 by insertional inactivation using a 458-bp internal *pcsB* fragment cloned into the thermosensitive shuttle vector pG^+host6. However, despite the isolation of several putative *pcsB* mutants, subsequent Southern blot experiments revealed an unaltered organization of the *pcsB* gene in these strains. We therefore speculated on an essential function of PcsB for GBS under the chosen experimental conditions. Since PcsB is located extracellularly and shows limited similarity to the autolysin P45 from *L. monocytogenes* (44), we assumed that the disruption of *pcsB* might result in destabilization of the GBS cell wall with subsequent osmotic lysis. Therefore, insertional inactivation was performed in the presence of 500 mM D-sorbitol, which is not fermented by GBS but increases the osmotic strength of the medium (20). This approach resulted in the isolation of a *pcsB* mutant strain, which was termed Sep1. The successful inactivation of *pcsB* in strain Sep1 was confirmed by Southern blot analysis (not shown). Western blotting using polyclonal anti-PcsB antiserum showed a strong band in the culture supernatant of GBS 6313 but no band in the culture supernatant of its isogenic mutant Sep1 (Fig. 1B). These data confirm the successful inactivation of the *pcsB* gene in GBS strain Sep1. Interestingly, a dramatically higher amount of a 55-kDa protein was detected in the culture supernatant of GBS Sep1 than in that of GBS 6313 (Fig. 1A). This indicates the attempt of strain Sep1 to compensate for the *pcsB* deficiency by overproduction of the 55-kDa protein, which might serve similar functions as PcsB.

Growth characteristics and antibiotic susceptibility of GBS mutant Sep1. While the GBS parent grew well on THY agar and blood agar plates as well as in THY broth, the Sep1 mutant exhibited poor growth both on THY agar and blood agar plates and no growth in THY broth. The two strains grew to about the same optical density in sorbitol-supplemented THY broth; however, mutant Sep1 showed a doubling time of 150 min, compared to 30 min for the GBS parent. A GBS control mutant carrying a chromosomal pG^+host6 insertion in the *femH* gene exhibited a growth rate similar to that of the GBS parent (not shown). These results show that disruption of *pcsB* in GBS results in a dramatic reduction of growth rate.

In *S. aureus*, *Streptococcus pneumoniae*, and *Streptococcus*

TABLE 1. MICs of various antibiotics for growth of GBS 6313 and GBS mutant Sep1

Strain	MIC (μg/ml)										
	β-lactams					Vancomycin	Quinolones		Aminoglycosides		Sulphonamide + trimethoprim (cotrimoxazole)
	Penicillin G	Carbapenems		Cephalosporins							
		Imipenem	Meropenem	Cefotaxime	Ceftazidime		Ofloxacin	Ciprofloxacin	Gentamicin	Netilmicin	
GBS 6313	0.064	0.064	0.023	0.032	0.75	0.50	16	4.0	382	>256	>32
GBS Sep1	0.012	0.006	0.003	0.006	0.094	0.38	2.0	0.50	8.0	1.5	0.094

pyogenes, a defect in cell wall biosynthesis and turnover results in an altered susceptibility to β-lactam antibiotics (3, 14, 18, 53). Therefore, the GBS parent and its isogenic mutant Sep1 were tested for sensitivity to different classes of antibiotics. As shown in Table 1, mutant strain Sep1 was 5 to 10 times more susceptible than the GBS parental strain to all β-lactam antibiotics tested. However, the two strains differed only slightly in sensitivity to vancomycin, known to complex with the D-alanyl-D-alanine subunits of the peptidoglycan polymer (38). Strikingly, the GBS parent and the Sep1 mutant differed significantly in susceptibility to different classes of antibiotics known to act intracellularly. The Sep1 strain was eight times more sensitive than the GBS parent to different quinolones, and it showed 45- to 170-fold-increased susceptibility to different aminoglycoside antibiotics. Toward cotrimoxazole, a sulfonamide-trimethoprim combination, mutant strain Sep1 exhibited 340-fold-higher sensitivity than the parental GBS strain.

Microscopic analysis of GBS mutant Sep1. Light microscopic inspection of the GBS parent and the Sep1 mutant revealed growth in irregular clumps for the Sep1 mutant, compared to regular chains for the GBS parent (Fig. 4). Scanning electron microscopy showed a severely disturbed morphology for the Sep1 mutant (Fig. 5). Importantly, no uniform cell forms were observed for this strain. While some cells were slice shaped or kidney shaped (Fig. 5A1 and A2), others had an irregular giant size (Fig. 5B) or exhibited cell division in different planes (Fig. 5C). This result prompted us to analyze the Sep1 mutant in more detail by transmission electron microscopy. As depicted in Fig. 6A, the GBS parent shows single septum formation in one plane, with subsequent cell wall separation between daughter cells. During exponential growth, new septum formation is initiated before splitting of the septum of the daughter cells occurs. Transmission electron microscopy of the Sep1 mutant revealed severalfold ingrowth of septa in different planes without subsequent cell wall separation (Fig. 6B). Unequal septum formation of Sep1 not only resulted in the formation of giant cells (Fig. 6C) but also produced slice-shaped and kidney-shaped bacteria (Fig. 6D). Taken together, these results indicate that PcsB is required for cell division of GBS.

Test of PcsB for murein hydrolase activity. Cell division is terminated by the action of murein hydrolases that cleave bonds in the peptidoglycan polymer. To analyze if PcsB represents a murein hydrolase of GBS, concentrated culture supernatants of the GBS parent and the Sep1 mutant as well as purified PcsB fusion protein were tested in zymograms using embedded GBS cells as substrate. However, the culture supernatants of the GBS parent and the mutant strain Sep1 revealed no difference in their zymographic profiles, nor did the PcsB fusion protein show lytic activity (not shown). To avoid the denaturing conditions of SDS-PAGE, the purified PcsB protein was tested for autolytic activity using GBS cell walls in a photometric assay. Here again, the PcsB fusion protein was not able to hydrolyze GBS cell walls (not shown), indicating the absence of murein hydrolase activity of PcsB under the conditions tested.

DISCUSSION

Cell division of a bacterium requires a sophisticated concert of topological mechanisms, cell wall biosynthesis, and restricted cell wall degradation. Division is initiated by the ingrowth of a septum, usually located equidistant from the two cell poles. Selection of the midcell site for septum formation and the assembly of several proteins for the formation of a cell division apparatus have been the focus of intense research in gram-negative and gram-positive bacteria (reviewed in references 5, 31, and 42).

Homologs to *E. coli* proteins known to be involved in the initiation of septum formation and in the assembly of the cell division apparatus have been identified in a variety of bacterial species, including streptococci (42). Since the amino acid sequence of PcsB reveals no similarity to the above-mentioned proteins, it can be speculated that PcsB either is a novel com-

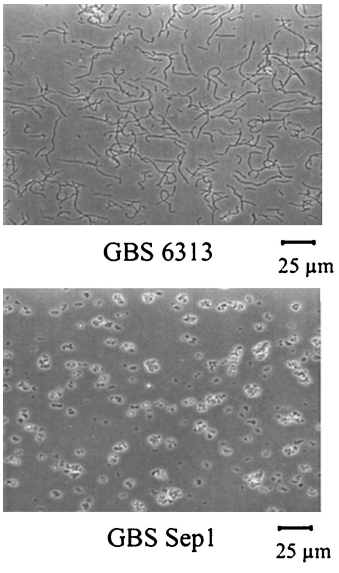

FIG. 4. Light microscopic analysis of GBS 6313 and the GBS mutant Sep1. While strain 6313 shows typical growth in chains, the Sep1 mutant grows in clumps.

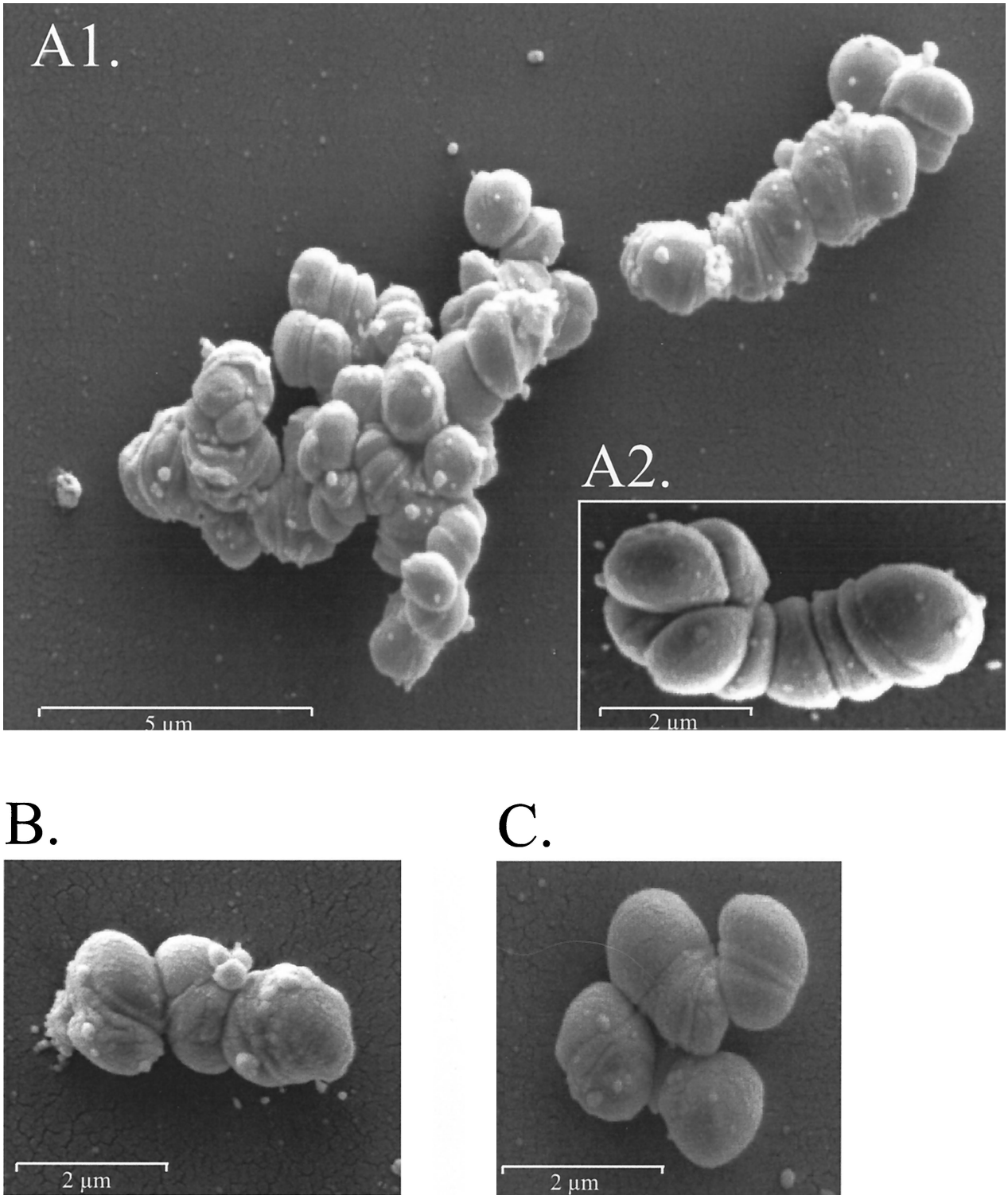

FIG. 5. Scanning electron analysis of GBS mutant strain Sep1. Cells of GBS Sep1 are irregular and slice shaped (A), exhibit a giant size (B), and show division in different planes (C).

ponent required for placement of the septum at the midpoint of the cell or plays a crucial role in the splitting of the septum. *E. coli* mutants impaired in localizing the midpoint of the cell are characterized by the generation of small spherical minicells (12), while those with defects in formation of the cell division apparatus grow uniformly as long, nonseptated filaments (21, 22). However, cultures of the GBS mutant Sep1, carrying a disrupted *pcsB* gene, revealed the presence of huge, small, as well as slice-shaped cells, a phenotype not matching the uniform phenotypes of the above-mentioned *E. coli* mutants.

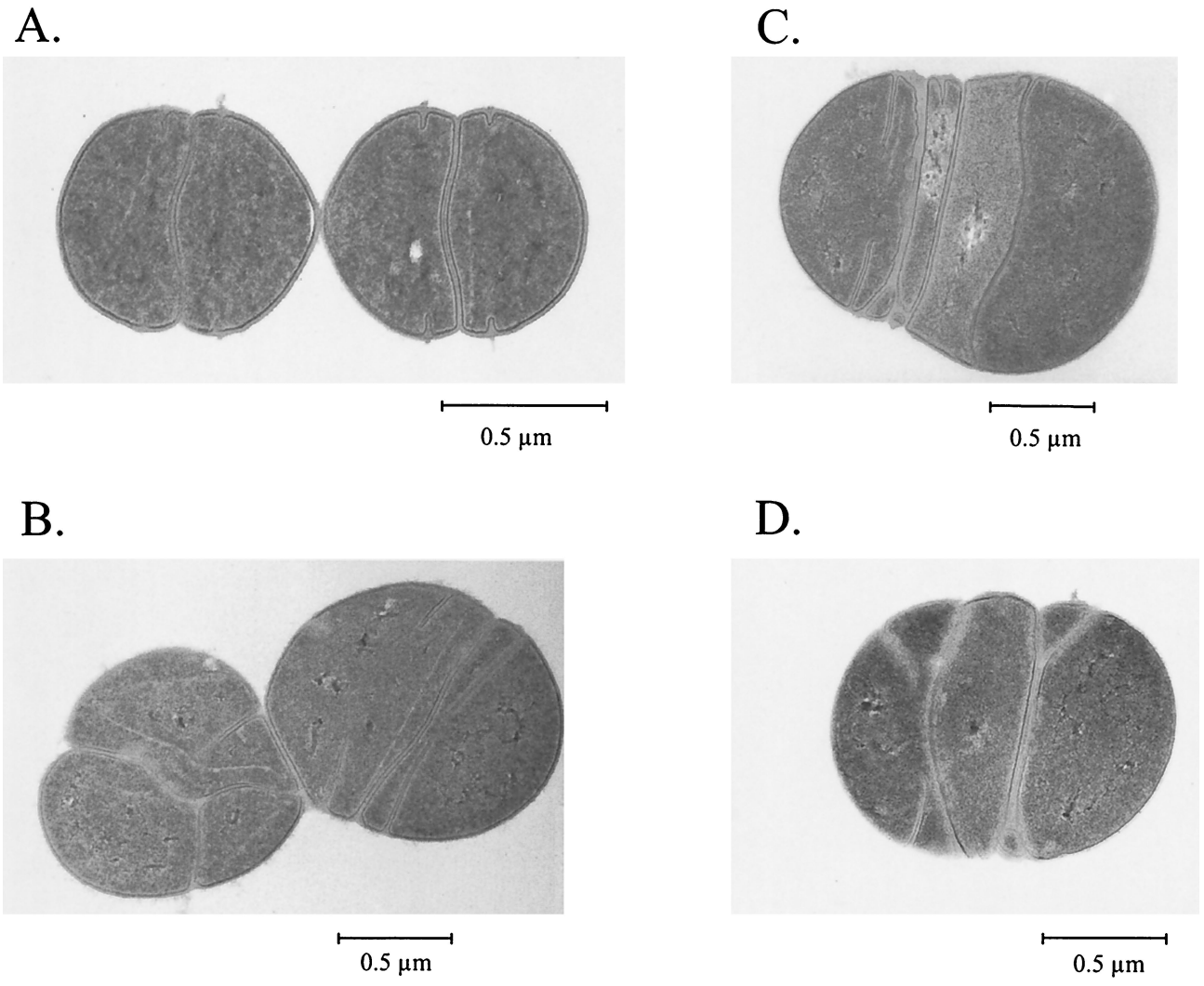

FIG. 6. Transmission electron analysis of GBS 6313 (A) and its isogenic mutant Sep1 (B to D). GBS 6313 shows septum formation in equal planes (A), while mutant Sep1 exhibits septum formation in different planes (B and D) and multifold septum formation (C).

Since GBS mutant Sep1 continued to form new septa between still unseparated cells, it can be suggested that Sep1 is impaired in septum separation instead of the placement of the septum.

In *E. coli*, *B. subtilis*, and *S. pyogenes*, mutations in penicillin-binding proteins (PBPs) have been shown to affect septum separation and to alter the shape of the bacteria (7, 8, 18, 47). PBPs, collectively exhibiting covalent binding to β-lactam antibiotics, represent transpeptidases, endopeptidases, and carboxypeptidases, respectively, that are involved in peptidoglycan cross-linking and metabolism of eubacteria (15, 48). In several GBS strains, six different PBPs, with molecular masses of 86, 85, 80, 76, 70, and 42 kDa, have been identified (10, 25). PcsB from GBS, migrating during SDS-PAGE at 50 kDa, does not correspond to the size of either of the PBPs from GBS. In addition, the amino acid sequence of PcsB does not possess any of the highly conserved motifs of PBPs (16), suggesting that PcsB does not represent a PBP from GBS.

A plausible explanation for the phenotype of mutant Sep1 would be a lack of murein hydrolase activity at its septal sites. Murein hydrolases cleave bonds in the bacterial peptidoglycan, thereby allowing enlargement of the cell wall during growth (23, 45). Furthermore, they are required for splitting of the septum during cell division (46). Interestingly, PcsB reveals similarity to the *L. monocytogenes* protein P45, which was shown to possess murein hydrolase activity (44). The alignment of PcsB with proteins exhitibing similarity to PcsB revealed in the aligned proteins at approximately the same position a conserved cysteine residue, speculated to be required for murein hydrolase activity (44, 59). Since the PcsB protein revealed autolytic activity neither in zymograms nor in a photometric assay, it can be speculated that PcsB does not represent a murein hydrolase from GBS. However, our failure to detect murein hydrolase activity of PcsB could also be explained by an irreversible denaturation of the fusion protein during the purification procedure. Since the PcsB fusion protein formed inclusion bodies in *E. coli*, it had to be purified in the presence of urea, possibly resulting in a loss of enzymatic activity. PcsB might also require additional factors for activity that are missing in the purified protein preparation. Furthermore, not all murein hydrolases are potential autolytic enzymes (46), sugg-

gesting that PcsB might play a pivotal role in murein hydrolysis without being able to function as an autolysin.

Inhibition of murein synthesis by β-lactam antibiotics is suggested to result in the lysis of the cell by an uncontrolled action of murein hydrolases (23). For *E. coli*, enterococci, and pneumococci, it has been demonstrated that a decrease in murein hydrolase activity is correlated with a lower susceptibility to penicillin G and that an increase in murein hydrolase activity results in enhanced penicillin G-induced lysis (17, 49, 53). Penicillin G tolerance of GBS has also been suggested to be correlated with a defect in the autolytic system (27). In contrast, GBS mutant Sep1, speculated to be impaired in murein hydrolase activity, reveals an increased susceptibility to β-lactam antibiotics. Although the basis for this phenotype remains unclear, GBS mutant Sep1 is also significantly more sensitive to different classes of antibiotics that function intracellularly, i.e., quinolones, aminoglycosides, and a sulfonamide-trimethroprim combination. Since in gram-positive organisms the lipid bilayer of the cytoplasmic membrane represents the major permeability barrier for antibiotics, it could be argued that the cytoplasmic membrane of mutant Sep1 exhibits a higher permeability for different classes of antibiotics. However, the permeability of the cytoplasmic membrane cannot be changed significantly, because this would result in improper functioning of membrane proteins, subsequently leading to cell death (37). It can therefore be speculated that the increased susceptibility of mutant Sep1 to antibiotics acting intracellularly originates in defects in its cell wall rather than within its cytoplasmic membrane. In fact, the uptake of aminoglycosides by enterococci has been demonstrated to be dependent on the composition of the cell wall (36). In these bacteria, a combination of a cell wall-active β-lactam antibiotic with an aminoglycoside results in a synergistic bactericidal activity; i.e., the cell wall-active drug enhances the ability of the aminoglycoside to reach the 16S subunit of the ribosome (26, 30). Taken together, our data indicate that a defect in the cell wall metabolism of mutant Sep1 results in an increased uptake of different kinds of antibiotics.

The drastically increased susceptibility of GBS strain Sep1 to different classes of antibiotics offers an exciting perspective for the treatment of GBS infections. Although GBS is uniformly susceptible to penicillin G, significant higher doses of this antibiotic are required for the treatment of a GBS infection compared to a *S. pyogenes* infection (2). In addition, 5 to 17% clinical isolates of GBS show increased *in vitro* tolerance against penicillin G; that is, the antibiotic is less bactericidal against these strains (4, 28). The importance of PcsB for the growth of GBS and for the sensitivity of GBS to different classes of antibiotics makes this protein an exciting subject for further research to elucidate its biochemical function and role in cell separation of GBS.

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