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J. Bacteriol. 2004, 186(5):1398. DOI:
10.1128/JB.186.5.1398-1408.2004.

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Relevance of Peptide Uptake Systems to the Physiology and Virulence of *Streptococcus agalactiae*

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Received 12 September 2003/Accepted 21 November 2003

Streptococcus agalactiae is a major cause of invasive infections in human newborns. To satisfy its growth requirements, *S. agalactiae* takes up 9 of the 20 proteinogenic amino acids from the environment. Defined *S. agalactiae* mutants in one or several of four putative peptide permease systems were constructed and tested for peptide uptake, growth in various media, and expression of virulence traits. Oligopeptide uptake by *S. agalactiae* was shown to be mediated by the ABC transporter OppA1-F, which possesses two substrate-binding proteins (OppA1 and OppA2) with overlapping substrate specificities. Dipeptides were found to be taken up in parallel by the oligopeptide permease OppA1-F, by the dipeptide ABC transporter DppA-E, and by the dipeptide symporter DpsA. Reverse transcription-PCR analysis revealed a polycistronic organization of the genes *oppA1-F* and *dppA-E* and a monocistronic organization of *dpsA* in *S. agalactiae*. The results of quantitative real-time PCR revealed a medium-dependent expression of the operons *dppA-E* and *oppA1-F* in *S. agalactiae*. Growth of *S. agalactiae* in human amniotic fluid was shown to require an intact *dpsA* gene, indicating an important role of DpsA during the infection of the amniotic cavity by *S. agalactiae*. Deletion of the *oppB* gene reduced the adherence of *S. agalactiae* to epithelial cells by 26%, impaired its adherence to fibrinogen and fibronectin by 42 and 33%, respectively, and caused a 35% reduction in expression of the *fbsA* gene, which encodes a fibrinogen-binding protein in *S. agalactiae*. These data indicate that the oligopeptide permease is involved in modulating virulence traits and virulence gene expression in *S. agalactiae*.

Streptococcus agalactiae has a limited capacity to synthesize amino acids and must acquire from the environment 9 of the 20 proteinogenic amino acids for growth (38). The amino acid requirements of *S. agalactiae* can be satisfied by the uptake of peptides, which are cleaved in the cytoplasm to their respective amino acids (69, 70). In microorganisms, two different peptide uptake systems have been described (23, 41). The most common peptide transporters are binding-protein-dependent permeases, consisting of multiple components, belonging to the ATP-binding cassette (ABC) transporter superfamily (24). The process of peptide transport by ABC transporters involves the extracytoplasmic binding of the substrate by a substrate-binding protein, transfer of the substrate to two membrane-integrated permeases for translocation across the cytoplasmic membrane, and ATP hydrolysis by one or two proteins located on the cytoplasmic side of the membrane (24). In numerous bacteria, the uptake of di- and oligopeptides is mediated by these ABC transporters (41). In contrast, some microorganisms take up di- and/or tripeptides by single proteins with similarity to eukaryotic peptide transport proteins of the PRT family (62). These bacterial permeases use the proton motive force across the cytoplasmic membrane for the uptake of peptides (23, 37). They exhibit a broad substrate specificity, but size recognition is restricted to di- and tripeptides only (16). Examples of this kind of peptide symporter are the tripeptide permeases from *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (17, 60), and the di- and tripeptide permeases (DtpT) of *Lactococcus lactis* and *Lactobacillus helveticus* (23, 37).

In *L. lactis*, whose peptide uptake systems have been the focus of intense research, di- and oligopeptides are taken up by two distinct ABC transporters, termed DppA-E and OppA-F, respectively (52, 64). Di- and tripeptide uptake is also mediated in *L. lactis* by the proton-driven permease DtpT (23). In contrast, in *Streptococcus pyogenes*, dipeptides are taken up exclusively by the ABC transporter system DppA-E (44).

In addition to nutrient uptake, peptide permeases of several organisms are involved in sensing pertinent environmental signals in the form of peptides, culminating in diverse responses such as sporulation (51), chemotaxis (1, 32), conjugation (29), development of competence (4, 5, 26), or expression of virulence determinants (11, 34, 44, 45). Signature-tagged mutagenesis identified the putative dipeptide ABC transporter DppA-E as a requirement for growth and survival of *S. agalactiae* in a rat sepsis model (28), suggesting an important role of peptides as nutrients or signaling molecules for the virulence of these bacteria.

S. agalactiae is the predominant cause of septicemia, pneumonia, and meningitis in neonates and poses a significant threat to parturient women (8, 54). A common cause of neonatal infection is the colonization of the human rectovaginal tract with *S. agalactiae*, ascending spread of the bacteria into the amniotic fluid, and aspiration of contaminated amniotic fluid by the infant (8). Although amniotic fluid contains only low amounts of free amino acids (35), it supports growth of *S. agalactiae* to high concentrations (14, 66), indicating that *S. agalactiae* satisfies its amino acid requirements in amniotic fluid by the uptake of peptides.

Recently, the genome sequences of the two *S. agalactiae* strains NEM316 and 2603 V/R were published, thereby allowing a genome-wide search for putative peptide permease genes in *S. agalactiae*.

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TABLE 1. Primers used for targeted deletion or disruption of *S. agalactiae* genes encoding putative peptide uptake systems

Target gene	NEM316 gene designation	Type of inactivation	Primers (5'→3') ^a	Cloning sites
<i>oppA1</i>	<i>gbs0144</i>	Deletion	CCGCGGATCCGTC AAGGAAGCAATAGCAGC CCCATCCACTAAACTTAAACAGCAACTGAAGCAAGTGAAGG TGTTTAAGTTTGTAGTGGATGGGACTCTACTTCCGTAGTGG TGGCACAAGCTTATCATACGTGATACTGGCTG	<i>Bam</i> HI <i>Hind</i> III
<i>oppA2</i>	<i>gbs0966</i>	Disruption	TGGCACAAGCTTTGAGTCAAAGAACTCTGG CCGCGGATCCGGACTTGTCAATTTAAAGG	<i>Hind</i> III <i>Bam</i> HI
<i>oppB</i>	<i>gbs0145</i>	Deletion	CCGCGGATCCAGATGCTGATGTTCTCTGC CCCATCCACTAAACTTAAACAGCAAGAACTGGCAATTACC TGTTTAAGTTTGTAGTGGGCTATCTTGATAACAGACG TGGCACAAGCTTGAATACCTACGATGATGCGC	<i>Bam</i> HI <i>Hind</i> III
<i>dppA</i>	<i>gbs0187</i>	Deletion	GGGGGTACCTCGAGACAGCAATGGCGTGTATC CCCATCCACTAAACTTAAACACGCCAGAAACAAGCAAC TGTTTAAGTTTGTAGTGGGACTTGGGATGAATCTGC CCGCGGATCCCTCAATCAAGCAATTTCCC	<i>Kpn</i> I <i>Bam</i> HI
<i>dppB</i>	<i>gbs0188</i>	Deletion	TGGCACAAGCTTTCAAGGCTAGTAACTGGG CCCATCCACTAAACTTAAACAGAGTCGAGGAAACCATTCC TGTTTAAGTTTGTAGTGGGCTGATACCCGCTACTTCC CCGCGGATCCCTTGTAGCACCTTTCCCAAC	<i>Hind</i> III <i>Bam</i> HI
<i>dpsA</i>	<i>gbs1444</i>	Deletion	CCGCGGATCCCTACGAGCAAGTACACAGTC CCCATCCACTAAACTTAAACACGAAAATAGCACGCATACC TGTTTAAGTTTGTAGTGGGCTCACCAGTTGGATTATCAG TGGCACAAGCTTCATTGGTACACAGATGG	<i>Bam</i> HI <i>Hind</i> III
<i>gbs1577</i>	<i>gbs1577</i>	Disruption	GAGCGGGTACCCTTACGCTTGTGGGGAG CCGCGGATCCCTTTTACAACCCATTGAC	<i>Kpn</i> I <i>Bam</i> HI

^a Restriction sites are underlined, and regions used for crossover PCR are in boldface type.

The present report describes the characterization of four putative peptide permeases in *S. agalactiae*. Defined mutants in the putative peptide uptake systems were constructed and used to define the role of these permeases in the uptake of di- and oligopeptides and in the growth of *S. agalactiae* in various complex media. The transcriptional organization of the peptide permease genes was analyzed in *S. agalactiae*, and additionally, the expression of these genes was determined during growth in various media. Finally, we addressed the importance of the peptide permeases in the binding of *S. agalactiae* to host proteins, in the attachment to eukaryotic cells, and in the expression of a virulence gene in *S. agalactiae*.

MATERIALS AND METHODS

Bacterial strains, eukaryotic cells, and growth conditions. The *S. agalactiae* strain O90R (ATCC 12386) is a capsule mutant of the serotype Ia clinical isolate O90. Strain O90R was used for the construction of insertion and deletion mutants in genes encoding putative peptide transporters in *S. agalactiae*. *S. agalactiae* strains belonging to the serotypes Ia, Ib, II, III, IV, and V, respectively, are clinical isolates that were described previously (53). *S. agalactiae* was generally grown in Todd-Hewitt broth supplemented with 1% yeast extract (THY). Recombinant *S. agalactiae* strains were selected in the presence of erythromycin (5 µg/ml). Growth experiments with *S. agalactiae* were performed in THY, in fetal calf serum (FCS), and in amniotic fluid. Amniotic fluid was obtained by amniocentesis from a patient suffering from polyhydramnios, and was kindly provided by the children's hospital in Ulm, Germany. Growth of *S. agalactiae* in THY or FCS was monitored by determining the optical density of the culture at 600 nm (OD₆₀₀). Due to the turbid nature of amniotic fluid, growth of *S. agalactiae* in this medium was determined by measuring the viable cell counts of the culture. In addition to growth in complex media, *S. agalactiae* was also grown in chemically defined medium (CDM) (65). Depleted CDM (dCDM) was prepared without the essential amino acid isoleucine but containing isoleucine-containing di-, tri-,

and oligopeptides at a final concentration of 0.1 mg ml⁻¹. The isoleucine-carrying peptides were purchased from Bachem (Bubendorf, Switzerland). The amino acid requirements of different *S. agalactiae* strains were tested with dCDM lacking, in each case, 1 of the 20 proteinogenic amino acids. Amino acids in the growth medium were detected as their *o*-phthalaldehyde derivatives via high-pressure liquid chromatography as described elsewhere (68).

E. coli DH5α was grown in Luria-Bertani medium, and pG⁺host6- or pTCV-lac-carrying clones were selected with ampicillin (100 µg/ml) or kanamycin (50 µg/ml).

The cell line A549 (ATCC CCL-185) was obtained from the American Type Culture Collection. A549 is a human lung carcinoma cell line which has many characteristics of type I alveolar pneumocytes. A549 cells were propagated in RPMI tissue culture medium (Gibco BRL) supplemented with 10% FCS. Tissue cultures were incubated in a humid atmosphere at 37°C with 5% CO₂.

Construction of insertion and deletion mutants in *S. agalactiae*. Primers used for the construction of insertion or deletion mutants of *S. agalactiae* O90R are listed in Table 1. Insertional inactivation of genes in *S. agalactiae* O90R was performed as described previously (48). Briefly, an internal fragment of the target gene was amplified by PCR and cloned into the thermosensitive plasmid pG⁺host6. The resulting plasmid was transformed into *S. agalactiae* according to the method described by Ricci et al. (49), and transformants were selected on erythromycin agar at 30°C. Cells carrying the plasmid within the chromosome were selected at 37°C under erythromycin pressure as described previously (31). The locations of the plasmid insertions were confirmed by Southern blot hybridization. The disruption of the *oppA2* gene in *S. agalactiae* was confirmed by *Cl*I digestion of genomic DNA of the parental strains and of putative *oppA2* integration mutants. Insertional inactivation of the *gbs1577* gene was tested with *Hpa*I-digested chromosomal DNA of the parental strains and of putative *gbs1577* integration mutants. Digoxigenin-labeled probes of the genes *oppA2* and *gbs1577* were obtained with the primers 5'-GGATTGGAATGGTTCAATGG and 5'-AGAGTCCGAATCAGCTGTG and with the oligonucleotides 5'-TTGAAGA GTCTAAAGGTGG and 5'-ACTTATTCCTGCGAACTC, respectively. Integration mutants were constructed in the *S. agalactiae* O90R wild type and in *S. agalactiae* mutants that already carried deletions in the genes *oppA1*, *oppB*, *dppB*, and/or *dpsA*. The genes *dppA*, *dppB*, *oppA1*, *oppB*, and/or *dpsA* were deleted

TABLE 2. Primers used for RT-PCR and for quantitative real-time PCR to analyze transcriptional organization and expression of genes *dppA-E*, *oppA1-F*, and *dpsA* in *S. agalactiae*

Transcriptional region or gene tested	Gene(s) hybridized	First primer (5'→3')	Second primer (5'→3')
<i>dpp</i> region	<i>gbs0183-dppA</i>	ATGACTATGGACACCTTG	GCAGGATTAGGTCTAGGG
	<i>dppA-dppC</i>	CCTTGAGCACCTTTCCCAAC	TAGCGGAGCAAGCTAAAGC
	<i>dppB-dppE</i>	CTCGACTTGGGTAATAAG	GTGATACACCGCTACTTC
	<i>dppD-gbs0189</i>	GGTATCATTCTGTGTCAGAGGC	AAATCTCATGCGTGTGGCAC
<i>opp</i> region	<i>gbs0143-oppA1</i>	CAGTATAGCGCCTTTTTTC	GCAACTGAAGCAAGTGTAAAG
	<i>oppA1-oppB</i>	ATCATACGTGATACCTGGCTG	ACTCTACTTCCGTAGTGG
	<i>oppB-oppC</i>	ACGTTGACGTTTCACTGCC	GCTATCTTGATAACAGACG
	<i>oppC-oppF</i>	GATGGACTTCTATGTCTCG	ACGTTGACGTTCACTCTCC
	<i>oppF-rDNA</i>	AGCTATTGAGCAAGACGGAC	TCAGACTTCCGTCCATTCG
	<i>dpsA</i> region	<i>gbs1512-dpsA</i>	TGGACATTAGCAACTACG
	<i>dpsA-dpsA</i>	GGTATGCGTGCTATTTTGC	CTGATAATCCAACCTGGTGAG
	<i>dpsA-gbs1514</i>	CTCACCAGTTGGATTATCAG	CATTGGTACACAGATGG
<i>dppA</i> gene	<i>dppA</i>	TAGCGGAGCAGCTAAAGC	GCAGATTCATCCCAAGTC
<i>dppB</i> gene	<i>dppB</i>	GATCGAATTGTAAGGTGG	AGAAGTAGCGGTGTATCAG
<i>dppE</i> gene	<i>dppE</i>	CTCCTAGATCTGGTGAAG	CCATTGAAAGATACGATACG
<i>oppA1</i> gene	<i>oppA1</i>	TATGTTGCCCCAGGTTATG	GTTATAAGCGCCTTGCTC
<i>oppB</i> gene	<i>oppB</i>	CGCTAGGGATTCTATGC	CGTCTGTTATCAAGATAGC
<i>oppF</i> gene	<i>oppF</i>	CGTGATATCGTAGCAGAAGG	ACGTTGACGTTCACTCTCC
<i>dpsA</i> gene	<i>dpsA</i>	GGTATGCGTGCTATTTTGC	CCAATAGCAGCAAGTGAG

from *S. agalactiae* according to a procedure described by Schubert et al. (53). In brief, two DNA fragments flanking the target gene were amplified by PCR with the primers listed in Table 1. Due to 21 bases of cDNA in the two primer sets used for the amplification, the resulting PCR products possessed a stretch of 21 identical base pairs. This sequence identity was subsequently used to combine the two PCR products in a crossover PCR. The resultant PCR product consisted of a single DNA fragment that carried the flanking regions of the target gene. The crossover PCR product was cloned into the plasmid pG⁺host6, and the construct was used to transform *S. agalactiae* with subsequent erythromycin selection at 30°C. Cells in which the plasmid had integrated into the chromosome were selected at 37°C under erythromycin pressure. Six such integrant strains were serially passaged for 6 days in liquid medium at 30°C without erythromycin selection to facilitate the excision of the pG⁺host6 construct, leaving the desired target gene deletion in the chromosome. Dilutions of the serially passaged cultures were plated onto agar, and single colonies were tested for erythromycin sensitivity to identify pG⁺host6 excisants. Successful deletion of the genes *oppA1*, *oppB*, *dppA*, and/or *dppB* was confirmed by Southern blotting with *EcoRI*-digested chromosomal DNA of the *S. agalactiae* parental strains and of the putative deletion mutants. A digoxigenin-labeled probe for the detection of deletions in *oppA1* and/or *oppB* was obtained by PCR with the primers 5'-GTA TCTTGATAACAGACG and 5'-GAATACCTACGATGATGCGC. Deletions in the genes *dppA* and/or *dppB* were detected with a digoxigenin-labeled probe that had been obtained by PCR with the primers 5'-GTGATACACCGCTA CTTC and 5'-CCTTGAGCACCTTTCCCAAC. The successful deletion of the *dpsA* gene was confirmed by Southern blot analysis with *XbaI*-digested chromosomal DNA from *S. agalactiae* and a digoxigenin-labeled probe obtained by PCR with the primers 5'-AACTTTTGTACTGCGTGAC and 5'-TAGGTGCTTTGA CCGAGATG. Deletion mutants that had been confirmed by Southern blot analysis were subsequently used for the construction of double and triple mutants in genes encoding other putative peptide transporters.

RNA preparation, RT-PCR, and LightCycler real-time PCR. *S. agalactiae* O90R was grown to mid-log phase in 50 ml of THY, CDM, dCDM plus dipeptide (Ile-Val), or dCDM plus oligopeptide (Val-Tyr-Ile-His-Pro-Phe). Total RNA was prepared from the bacterial culture with the RNeasy midi extraction kit (Qiagen) and treated for 1 h with 150 U of RNase-free DNase (Promega). RNA samples were checked for DNA contamination by PCR without prior reverse transcription (RT). As no amplicons were obtained, DNA contamination during RNA preparation could be excluded. The transcriptional organization of the genes *dppA-E*, *oppA1-F*, and *dpsA* in *S. agalactiae* was elucidated by RT-PCR analysis with the primers listed in Table 2 by using 1-μg RNA samples prepared from THY-grown culture.

Quantitative real-time PCR experiments were performed after RT of RNA with random hexanucleotides and the RevertAid first-strand cDNA synthesis kit (MBI Fermentas) according to the instructions of the manufacturer. Expression analysis of the genes *dppA*, *dppB*, *dppE*, *oppA1*, *oppB*, *oppF*, and *dpsA*, respectively, was performed with the primers listed in Table 2 in a LightCycler as

described previously (21). The quantity of cDNA for the investigated genes was normalized to the quantity of *gyrA* cDNA in each sample. Each experiment was performed at least four times with two independent RNA preparations.

Construction of the *fbxA-lacZ* transcriptional fusion plasmid pT_{fbxA}. Plasmid pTCV-*lac* (46) was used for expression analysis of the *fbxA* gene in *S. agalactiae*. A DNA fragment of 747 bp containing the *fbxA* promoter (unpublished data) was amplified by PCR from the genome of *S. agalactiae* with the primers 5'-CCGG AATTCAACTATTGCTCCCTCTGC and 5'-GCCGGATCCTTTCCTGATTTCAAGTTC. The *EcoRI* and *BamHI* sites used for cloning are underlined. After digestion of the PCR product and of plasmid pTCV-*lac* with *EcoRI* and *BamHI*, the *fbxA* promoter region was ligated into pTCV-*lac*, resulting in plasmid pT_{fbxA}. The plasmids pTCV-*lac* and pT_{fbxA} were subsequently transformed into *S. agalactiae* by electroporation (49), and recombinant clones were obtained by erythromycin selection.

Binding of fluorescein isothiocyanate-labeled group B streptococci to immobilized human proteins. Terasaki microtiter plates were coated with the human proteins fibrinogen, fibronectin, and haptoglobin, respectively, and the binding of fluorescein isothiocyanate-labeled *S. agalactiae* to the immobilized fibrinogen was measured as described previously (21). Results were measured in triplicate, and each assay was repeated at least four times.

Adherence and internalization assays. Adherence of *S. agalactiae* to A549 epithelial cells and internalization into this cell line were assayed as described previously (21). Briefly, A549 cells were transferred to 24-well tissue culture plates at approximately 4×10^5 cells per well and cultivated overnight in RPMI tissue culture medium supplemented with 10% FCS. After replacement of the medium with 1 ml of fresh medium, the cells were infected with *S. agalactiae* at a multiplicity of infection of 10:1 and incubated at 37°C for 2 h. The infected cells were subsequently washed three times with phosphate-buffered saline. The number of cell-adherent bacteria was determined by lysis of the eukaryotic cells with distilled water and subsequent determination of CFU by plating appropriate dilutions of the lysates on THY agar. Intracellular bacteria were determined after a further incubation of the infected cells for 2 h with RPMI medium containing penicillin G (10 U) and streptomycin (0.01 mg) to kill extracellular bacteria. After three washes with phosphate-buffered saline, the epithelial cells were lysed with distilled water and the amount of intracellular bacteria was quantitated by plating serial dilutions of the lysate onto THY agar plates. All samples were tested in triplicate, and experiments were repeated at least three times.

Determination of β-galactosidase activity. Recombinant *S. agalactiae* strains harboring the plasmid pTCV-*lac* or pT_{fbxA} were grown aerobically in THY broth at 37°C with shaking (100 rpm). At different time points, 1-ml samples were withdrawn to determine the OD₆₀₀ and β-galactosidase activity of the culture. Prior to measuring β-galactosidase activity (36), cells were permeabilized with a 0.5% toluene–4.5% ethanol mixture as described previously (46). Enzymatic activity is expressed in Miller units, which were calculated as follows: $(10^3 \times$

$OD_{420}/(\text{time of the reaction [in minutes]} \times OD_{600} \times \text{dilution of the cells in the assay})$.

Nucleotide sequence accession number. The genomic DNA sequence of *S. agalactiae* NEM316 can be accessed in the EMBL database under accession number AL732656. In the SwissProt TREMBL database, the polypeptides described in this report possess the following accession numbers: DppA, Q8E7H0; DppB, Q8E7G9; DppC, Q8E7G8; DppD, Q8E7G7; DppE, Q8G7G6; OppA1, Q8E7L0; OppA2, Q8E5L7; OppB, Q8E7K9; OppC, Q8E7K8; OppD, Q8E7K7; OppF, Q8E7K6; DpsA, Q8E489.

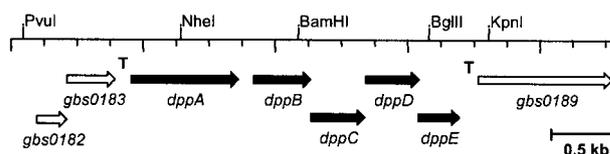
RESULTS

The genome of *S. agalactiae* possesses several putative peptide permease genes. The annotated genome sequence of the *S. agalactiae* strains NEM316 (<http://genolist.pasteur.fr/SagaList/index.html>) and 2603 V/R (<http://www.pnas.org/cgi/data/182380799/DC1/1>) indicated the presence of three ABC transporters and one proton-driven symporter that are possibly involved in the import of peptides into the cell. In the genome of *S. agalactiae* NEM316, the putative ABC peptide transporters are encoded by the genes *gbs0144* to *gbs0148*, *gbs0184* to *gbs0188*, and *gbs1573* to *gbs1577*. According to the functional analysis of these genes (see below), the *S. agalactiae* genes *gbs0144* to *gbs0148* were termed *oppA1-F* and the genes *gbs0184* to *gbs0188* were named *dppA-E* (Fig. 1). As the *S. agalactiae* genome carries two *oppA* homologs (63), one carried by *gbs0144* and the other carried by *gbs0966*, the two genes were termed *oppA1* and *oppA2*, respectively. The putative function of each of the gene products of the ABC transporters was inferred based on homology to functionally characterized ABC transporters (24). Thus, the proteins DppA, OppA1, OppA2, and Gbs1577 represent putative substrate-binding proteins, the polypeptides DppB,C, OppB,C, and Gbs1575,1576 represent hydrophobic integral membrane proteins, and DppD,E, OppD,F, and Gbs1573,1574 represent putative ATPases that couple ATP hydrolysis to substrate transport across the membrane.

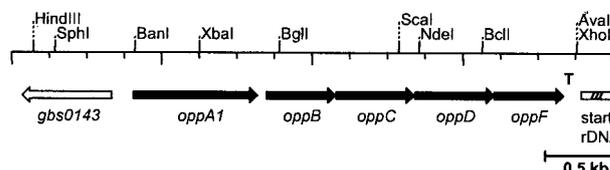
The genome of *S. agalactiae* also contains a gene, *gbs1513*, whose derived polypeptide sequence reveals 49% identity to the di- and tripeptide permease DtpT from *L. lactis* (23). Functional analysis of *gbs1513* in *S. agalactiae* indicated that it encodes a dipeptide symporter (see below), and therefore, *gbs1513* was termed *dpsA*.

Amino acid auxotrophies of *S. agalactiae* isolates. Studying peptide uptake in *S. agalactiae* requires knowledge regarding amino acid auxotrophies of the strains in use. In a 1943 report, *S. agalactiae* strains were shown to be auxotrophic for the amino acids valine, leucine, isoleucine, phenylalanine, glutamic acid, arginine, lysine, histidine, and tryptophan (38). We tested 12 different *S. agalactiae* strains, belonging to six different serotypes, for growth in CDM lacking, in each case, one of the 20 proteinogenic amino acids. All of the *S. agalactiae* isolates investigated, including strain O90R, were auxotrophic for the amino acids valine, leucine, isoleucine, phenylalanine, tyrosine, arginine, lysine, histidine, and tryptophan. The tested *S. agalactiae* isolates therefore differ from the previously described strains (38) in that they require tyrosine but not glutamic acid for growth. Analysis of metabolic pathways that can be predicted from the genome sequence (<http://www.genome.ad.jp/kegg/metabolism.html>) of the *S. agalactiae* strains NEM316 and 2603 R/V confirmed for these strains the amino auxotro-

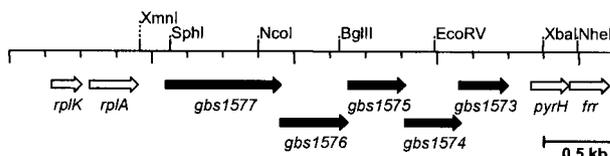
dppA-E region



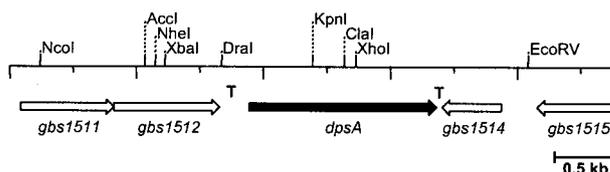
oppA1-F region



gbs1573-1577 region



dpsA region



oppA2 region

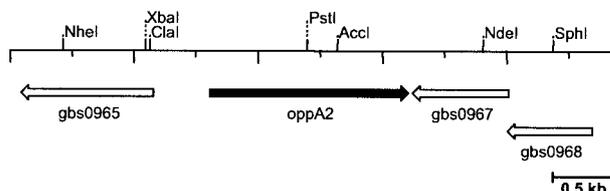


FIG. 1. Restriction maps of genomic regions in *S. agalactiae* encoding putative peptide permeases. According to the functional analysis of these regions, the genes *dppA* to *dppE* were shown to encode a dipeptide permease, the genes *oppA1* to *oppF* code for di- and oligopeptide permeases, the *dpsA* gene encodes a dipeptide symporter, and the *oppA2* gene represents a homolog of the *oppA1* gene. The gene designations of the other open reading frames were adapted from the genome sequence of *S. agalactiae* NEM316. The arrow labeled "start rDNA" indicates the start of a ribosomal DNA operon in *S. agalactiae*. T indicates transcriptional terminators, as identified by RT-PCR analysis.

phies identified by the growth experiments. Peptide uptake was further investigated in *S. agalactiae* strain O90R by making use of its isoleucine auxotrophy.

Identification of the oligopeptide uptake system in *S. agalactiae*. Oligopeptide import in *S. agalactiae* was studied by deleting the genes *oppA1* and *oppB* and by insertionally inactivating the *oppA2* gene in the genome of *S. agalactiae* O90R, resulting in the mutant strains $\Delta oppA1$ $\Delta oppB$ and *oppA2*-int, respectively. Furthermore, a double mutant of the genes *oppA1*

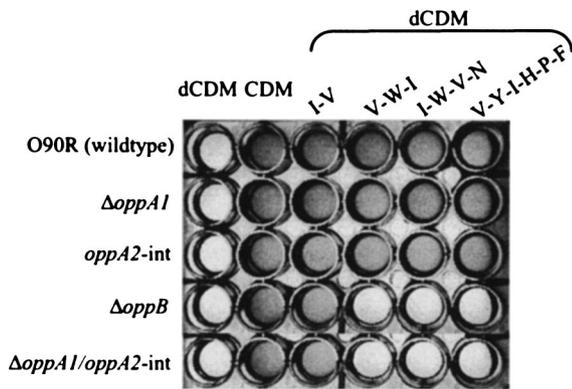


FIG. 2. Growth of the *S. agalactiae* wild-type strain O90R and of different oligopeptide permease mutants in CDM or dCDM lacking the amino acid isoleucine. dCDM was supplemented with different isoleucine-containing peptides whose sequences are depicted in single letter code. Growth of the different strains was visually inspected.

and *oppA2* was constructed and named $\Delta oppA1 oppA2$ -int. In THY complex medium, *S. agalactiae* O90R and its isogenic mutants revealed identical growth rates and final ODs (data not shown), suggesting that the genes *oppA1*, *oppA2*, and *oppB* are dispensable for growth in this medium. The role of these genes for the uptake of peptides was studied by growing strain O90R and its isogenic mutants in various formulations of CDM. All strains grew in complete CDM (Fig. 2) and, as determined by monitoring the OD of the cultures during growth, showed approximately identical growth rates and final ODs (data not shown). When CDM was depleted of the essential amino acid isoleucine (dCDM), there was no apparent growth of the strains (Fig. 2), confirming the isoleucine auxotrophy of *S. agalactiae* O90R. Addition of the isoleucine-containing dipeptide Ile-Val to dCDM restored growth of all the strains tested (Fig. 2), suggesting that the isoleucine auxotrophy can be supplemented by an isoleucine-containing dipeptide. This finding also indicates that the genes *oppA1*, *oppA2*, and *oppB* are not essential for the uptake of dipeptides. Also

demonstrated in Fig. 2, *S. agalactiae* O90R revealed growth in dCDM with isoleucine-containing tri-, tetra-, and hexapeptides. However, no growth of the mutant $\Delta oppB$ was observed in these media (Fig. 2 and Table 3), indicating an essential function of *oppB* for the uptake of oligopeptides by *S. agalactiae*. Interestingly, the mutant strain $\Delta oppA1$ and *oppA2*-int exhibited growth in dCDM with different tri-, tetra-, and hexapeptides while the double mutant $\Delta oppA1 oppA2$ -int did not grow on isoleucine-containing oligopeptides (Fig. 2). These data suggest that the substrate-binding proteins OppA1 and OppA2 are both involved in oligopeptide uptake and that they can substitute for each other functionally.

Identification of dipeptide import systems in *S. agalactiae*. Dipeptide uptake was studied by deleting the genes *dppA*, *dppB*, and *dpsA* and insertionally disrupting the gene *gbs1577* in the chromosome of *S. agalactiae* O90R, resulting in the mutant strains $\Delta dppA$, $\Delta dppB$, $\Delta dpsA$, and *gbs1577*-int. In THY complex medium, the mutants revealed no difference in growth rate and final OD compared to *S. agalactiae* O90R (data not shown). All of the mutants, however, exhibited growth in dCDM that contained isoleucine-carrying dipeptides (Table 3), indicating that the import of dipeptides in *S. agalactiae* is mediated by different uptake systems with overlapping substrate specificities. This hypothesis was tested by constructing *S. agalactiae* double and triple mutants with different combinations of defects in the genes *dppB*, *oppB*, *gbs1577*, and *dpsA*. All double mutants revealed growth in dCDM with isoleucine-containing dipeptides (Table 3), suggesting that more than two uptake systems are involved in the import of dipeptides in *S. agalactiae*. Finally, a triple mutant with deletions in the genes *dppB*, *oppB*, and *dpsA* did not grow in dCDM with the isoleucine-carrying dipeptides Ile-Val, Ile-Glu, and Ile-Pro, respectively. This finding shows that dipeptide uptake is mediated by the oligopeptide permease OppA1-F, the dipeptide permease DppA-E, and the dipeptide symporter DpsA. Additionally, it shows that any of these permeases is sufficient for growth of *S. agalactiae* with dipeptides as an amino acid source. Our data also indicate that the putative ABC transporter genes *gbs1573* to *gbs1577* are not re-

TABLE 3. Growth characteristics of different *S. agalactiae* strains in complete CDM or dCDM^a lacking the amino acid isoleucine

Strain (genotype)	Growth on ^b :									
	CDM	dCDM	dCDM supplemented with:						Ile-Trp-Val-Asn	Val-Tyr-Ile-His-Pro-Phe
			Ile-Val	Ile-Glu	Ile-Pro	Ile-Phe	Val-Trp-Ile			
O90R (wild type)	+	-	+	+	+	+	+	+	+	
$\Delta dppA$	+	-	+	+	+	+	+	+	+	
$\Delta dppB$	+	-	+	+	+	+	+	+	+	
$\Delta oppB$	+	-	+	+	+	+	-	-	-	
<i>gbs1577</i> -int	+	-	+	ND ^b	ND	+	+	+	+	
$\Delta dpsA$	+	-	+	+	+	+	+	+	+	
$\Delta dppB gbs1577$ -int	+	-	+	ND	ND	+	+	+	+	
$\Delta oppB gbs1577$ -int	+	-	+	ND	ND	+	-	-	-	
$\Delta dppB \Delta dpsA$	+	-	+	+	+	+	+	+	+	
$\Delta dppB \Delta oppB$	+	-	+	+	+	+	-	-	-	
$\Delta oppB \Delta dpsA$	+	-	+	+	+	+	-	-	-	
$\Delta dppB \Delta oppB gbs1577$ -int	+	-	+	ND	ND	+	+	+	+	
$\Delta dppB \Delta oppB \Delta dpsA$	+	-	-	-	-	+	-	-	-	

^a dCDM was supplemented with the indicated peptides, and growth was visually inspected.

^b ND, not determined.

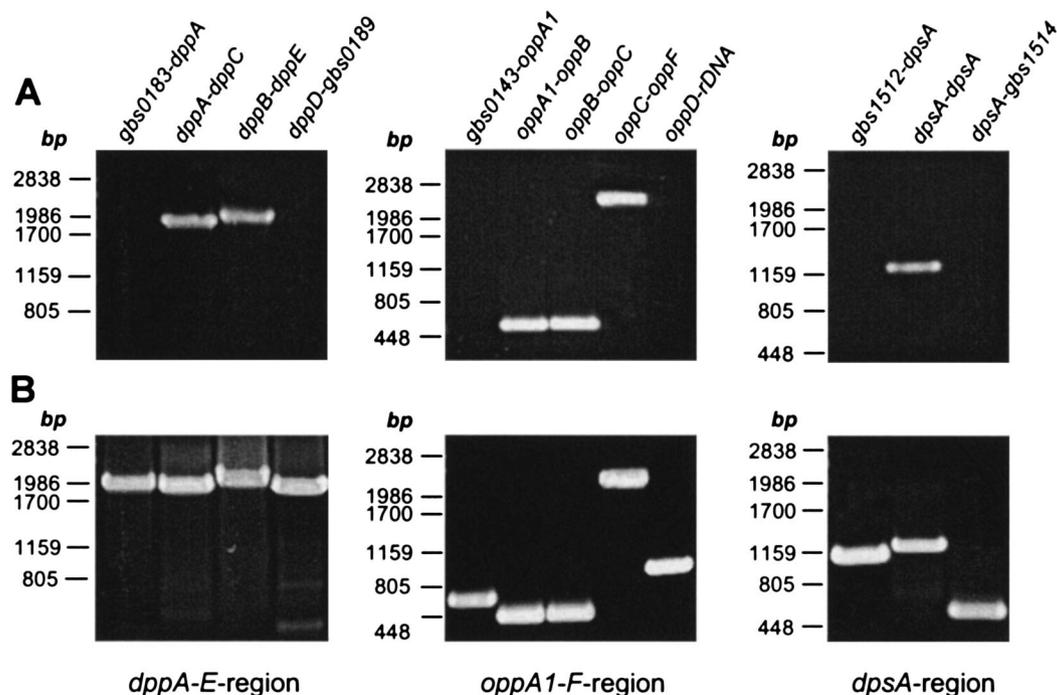


FIG. 3. Transcriptional organization of the genes *dppA-E*, *oppA1-F*, and *dpsA* in *S. agalactiae*. The gene names on top indicate the genes to which the primer pairs annealed during RT-PCR with total RNA (A) or during PCR with chromosomal DNA (B) from *S. agalactiae* O90R.

quired for dipeptide import. However, the triple mutant $\Delta dppB \Delta oppB \Delta dpsA$ was still capable of growth on the dipeptide Ile-Phe, suggesting the presence of at least one more dipeptide uptake system in *S. agalactiae*. High-pressure liquid chromatography analysis did not detect free isoleucine during growth of *S. agalactiae* in Ile-Phe-containing dCDM (data not shown), indicating that this dipeptide is not externally hydrolyzed by *S. agalactiae*.

Transcriptional organization and expression control of *dppA-E*, *oppA1-F*, and *dpsA*. The transcriptional organization of the genes *dppA-E*, *oppA1-F*, and *dpsA* was investigated by RT-PCR with total RNA from *S. agalactiae* O90R. As shown in Fig. 3A, RT-PCR amplified the regions *dppA* to *dppC* and *dppB* to *dppE* from *S. agalactiae* RNA, suggesting that the genes *dppA-E* comprise an operon. No amplification products were obtained with primer pairs specific to the genes *gbs0183* to *dppA* and *dppD* to *gbs0189*, indicating transcriptional initiation upstream of *dppA* and transcriptional termination downstream of *dppE*, respectively. Similarly, RT-PCR amplified the regions *oppA1* to *oppB*, *oppB* to *oppC*, and *oppC* to *oppF* from O90R RNA (Fig. 3A), suggesting transcriptional coupling of the genes *oppA1-F*. Here again, no amplification products were obtained with primer pairs specific to the genes *gbs0143* to *oppA1* and *oppD* to rDNA, indicating a promoter in front of *oppA1* and a transcriptional terminator downstream of *oppF*. Analysis of the transcriptional organization of *dpsA* by RT-PCR revealed a *dpsA*-specific amplicon with *dpsA*-specific primers (Fig. 3A). No amplification products were obtained by RT-PCR with primers specific to the regions *gbs1512* to *dpsA* and *dpsA* to *gbs1514*, indicating a monocistronic organization of the *dpsA* gene in *S. agalactiae*. All of the primer pairs used for RT-PCR analysis amplified products of the expected size from chromosomal *S. agalactiae* DNA (Fig. 3B). As no PCR

product was amplified from total *S. agalactiae* RNA (data not shown), DNA contaminations in the RNA preparation could be excluded.

In several organisms, transcriptional attenuation behind *dppA* or *oppA* results in an increased amount of *dppA*- or *oppA*-specific mRNA compared to the polycistronic mRNA (2, 10, 44). Therefore, quantitative real-time PCR was performed in a LightCycler to determine the ratio of expression between *dppA* and the downstream genes, *dppB* and *dppE*, and between *oppA1* and the downstream genes, *oppB* and *oppF*. The impact of medium components on the expression of *dppA*, *dppB*, *dppE*, *oppA1*, *oppB*, *oppF*, and *dpsA* was also studied by quantitative real-time PCR. Expression analysis was performed with total RNA from *S. agalactiae* O90R after growth in THY, CDM, or dCDM supplemented with the dipeptide Ile-Val or the oligopeptide Val-Tyr-Ile-His-Pro-Phe. Under different growth conditions, the expression of the *dppA* gene was similar to that of *dppE* and even weaker than that of *dppB* (Fig. 4A). Similarly, the amount of *oppA1* mRNA was, under different growth conditions, nearly identical to the amount of transcript from the genes *oppB* and *oppF* (Fig. 4B). These findings suggest that in the operons *dppA-E* and *oppA1-F* in *S. agalactiae*, transcriptional attenuation does not take place behind the genes *dppA* and *oppA1*.

As depicted in Fig. 4, the expression of the *oppA1-F* operon significantly exceeded the expression of the *dppA-E* operon and that of the *dpsA* gene (note the different scale of the transcript copy number between the graphs), indicating that the oligopeptide permease might be required in higher amounts by the cell. Growth of *S. agalactiae* under different culture conditions revealed a moderately increased transcription of the genes *dppA*, *dppB*, and *dppE* in THY complex medium. In contrast, expression of the *dpsA* gene remained

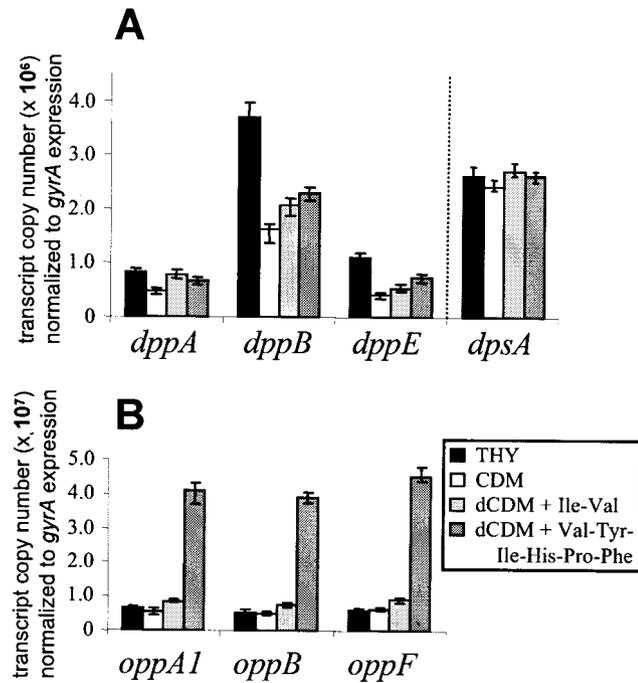


FIG. 4. Expression analysis of the genes *dppA*, *dppB*, *dppE*, and *dpsA* (A) and of *oppA1*, *oppB*, and *oppF* (B) during growth of *S. agalactiae* O90R in THY complex medium, CDM, or dCDM containing the dipeptide Ile-Val or the hexapeptide Val-Tyr-Ile-His-Pro-Phe. The amount of mRNA of the different genes was measured by quantitative real-time PCR in a LightCycler and normalized to the expression of the *gyrA* gene. The expression of the different genes is presented as the transcript copy number per 1.7×10^6 copies of *gyrA* transcript. The dotted line indicates distinct transcription of the genes *dppA*, *dppB*, *dppE*, and *dpsA*. Note the different scales for the transcript copy number in panels A and B.

constant under the growth conditions tested (Fig. 4A). Interestingly, the transcription of the genes *oppA1*, *oppB*, and *oppE* was about fivefold induced in dCDM containing the hexapeptide Val-Tyr-Ile-His-Pro-Phe (Fig. 4B). However, growth of *S. agalactiae* in dipeptide-containing dCDM had no effect on the expression of either of the peptide permease-encoding genes. These findings indicate complex regulatory mechanisms that control the expression of peptide permease-encoding genes in *S. agalactiae*.

The *dpsA* gene plays an important role during growth in amniotic fluid. *S. agalactiae* is known to infect the placenta and grow in amniotic fluid to high cell densities (15, 50, 66). The importance of peptide uptake for growth of *S. agalactiae* in amniotic fluid was studied with the strain O90R and the triple mutant $\Delta dppB \Delta oppB \Delta dpsA$. As shown in Fig. 5, the mutant $\Delta dppB \Delta oppB \Delta dpsA$ grew to significantly lower cell numbers in amniotic fluid than *S. agalactiae* O90R, suggesting an important role of dipeptide and/or oligopeptide uptake during growth of *S. agalactiae* in amniotic fluid. To investigate this effect in more detail, growth in amniotic fluid was monitored for the *S. agalactiae* single mutants $\Delta dppB$, $\Delta oppB$, and $\Delta dpsA$ (Fig. 5). Interestingly, the mutant $\Delta dpsA$ revealed the same growth defect in amniotic fluid as observed for the triple mutant $\Delta dppB \Delta oppB \Delta dpsA$, suggesting that the *dpsA* deficiency caused the growth impairment in amniotic fluid. The different

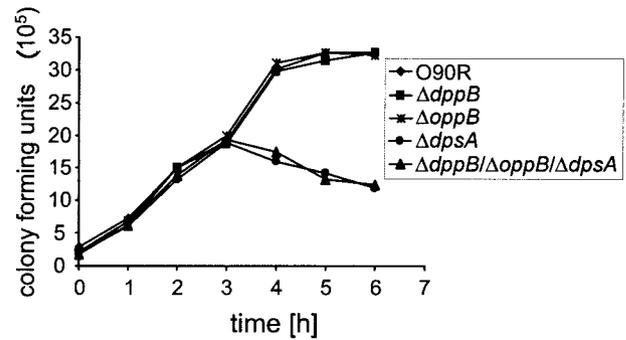


FIG. 5. Growth of *S. agalactiae* O90R and isogenic peptide permease mutants in human amniotic fluid. The growth of the strains was monitored by plating serial dilutions onto THY agar plates and counting the CFU.

peptide permease mutants and strain O90R were also tested for growth in various complex media. However, the tested strains did not reveal differences in growth rates and final ODs in either THY or FCS (data not shown). These findings suggest that *S. agalactiae* specifically requires the dipeptide permease DpsA to successfully proliferate in human amniotic fluid.

The oligopeptide permease is important for the binding of *S. agalactiae* to host proteins. In *Streptococcus gordonii* and *Streptococcus pneumoniae*, the respective oligopeptide permeases control the interaction of the bacteria with host proteins (11, 27, 34). Therefore, the importance of the three peptide uptake systems for the binding of *S. agalactiae* to different host proteins was investigated. *S. agalactiae* strain O90R revealed significant binding to immobilized fibrinogen, fibronectin, and haptoglobin (Table 4). However, the fibrinogen binding of the *S. agalactiae* mutants $\Delta oppB$ and $\Delta dppB \Delta oppB \Delta dpsA$ was reduced by 42%. Similarly, both mutants revealed a 33% reduction in binding to human fibronectin. None of the tested mutants was impaired in its interaction with human haptoglobin. These findings indicate that the oligopeptide permease in *S. agalactiae* plays a role in the binding of the bacteria to human fibrinogen and fibronectin.

The influence of peptide permeases on the bacterial adherence to and invasion of eukaryotic cells. In *S. pneumoniae*, the oligopeptide permease was shown to be required for efficient adherence of the bacteria to human cells (11). The *S. agalactiae* wild-type strain O90R and the peptide permease mutants $\Delta dppB$, $\Delta oppB$, $\Delta dppB \Delta oppB \Delta dpsA$, and $\Delta dppB \Delta oppB \Delta dpsA$ were

TABLE 4. Binding of *S. agalactiae* O90R and isogenic peptide permease mutants to immobilized fibrinogen, fibronectin, and haptoglobin^a

Strain	% Bacteria bound to immobilized protein		
	Fibrinogen	Fibronectin	Haptoglobin
O90R (wild type)	52 ± 6	27 ± 7	24 ± 5
$\Delta dppB$	44 ± 5	28 ± 6	25 ± 4
$\Delta dpsA$	43 ± 7	25 ± 5	22 ± 5
$\Delta oppA1$	49 ± 7	26 ± 7	26 ± 5
$\Delta oppB$	30 ± 5	18 ± 4	23 ± 4
$\Delta dppB \Delta dpsA$	42 ± 7	28 ± 5	27 ± 5
$\Delta dppB \Delta oppB \Delta dpsA$	29 ± 4	19 ± 4	22 ± 5

^a Each assay was performed at least four times in triplicate.

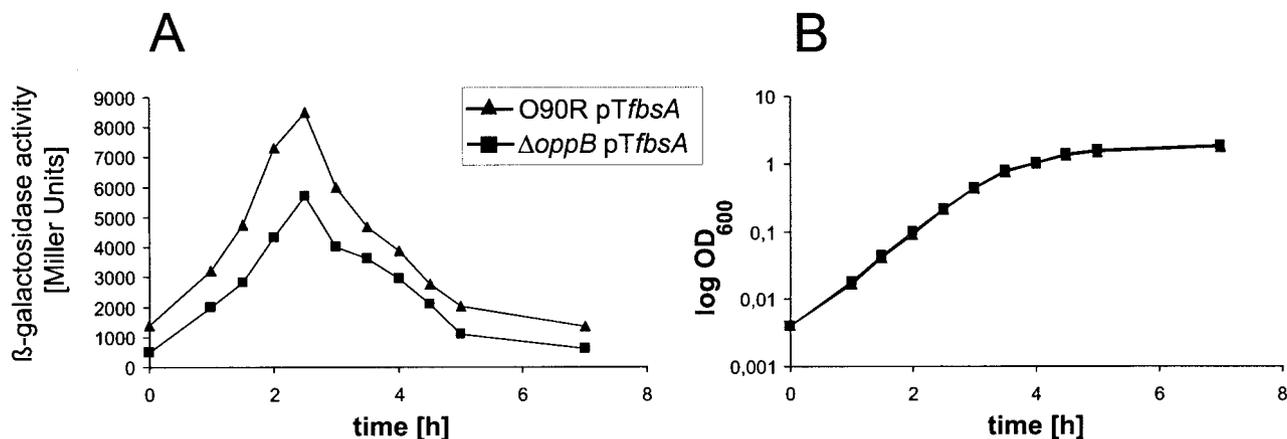


FIG. 6. Growth and expression profiles of an *fbsA-lacZ* transcriptional fusion in the *S. agalactiae* strains O90R and $\Delta oppB$. The bacteria were grown aerobically in THY liquid medium, and at various time points, samples were withdrawn for the determination of β -galactosidase activity (A) and OD₆₀₀ (B) of the culture. β -Galactosidase activity was expressed in Miller units.

therefore tested for their ability to adhere to and invade cells of the human lung epithelial cell line A549. Among the tested strains, the mutants $\Delta oppB$ and $\Delta dppB \Delta oppB \Delta dpsA$ revealed a 26% reduced adherence to host cells. However, none of the mutants exhibited altered invasion into host cells (data not shown), indicating that a deficiency in the oligopeptide permease only moderately reduces adherence of *S. agalactiae* to human cells.

The oligopeptide permease controls expression of the *fbsA* gene. In *S. agalactiae*, the FbsA protein represents the major fibrinogen-binding protein (53), which also plays an important role in the adherence of the bacteria to host cells (unpublished data). As the previous result demonstrated an influence of the oligopeptide permease on fibrinogen binding and adherence of *S. agalactiae* to human cells, the expression of the *fbsA* gene was analyzed in *S. agalactiae* O90R and its isogenic mutant $\Delta oppB$. Both strains were transformed with the vector pTCV-*lac* and with the plasmid pTfbsA, which carries the *fbsA* promoter in front of the promoterless *lacZ* gene. In pTfbsA-carrying strains, *fbsA* expression can be monitored by measuring the β -galactosidase activity. Strains that harbored the vector pTCV-*lac* did not possess β -galactosidase activity during growth (data not shown). However, both pTfbsA-carrying strains revealed a growth-phase-dependent expression of *fbsA*, peaking in the middle of the exponential growth phase with a moderate decline during late logarithmic and stationary growth (Fig. 6). The obtained results are identical to the expression analysis of *fbsA* in the *S. agalactiae* strain 6313-*fbsA-luc* (21), which carries in its chromosome a promoterless luciferase gene fused to the *fbsA* gene. This result indicates that plasmid-mediated expression of *fbsA* does not influence its growth-phase-dependent regulation. Growth experiments revealed identical growth of the *S. agalactiae* strains O90R(pTfbsA) and $\Delta oppB$ (pTfbsA) (Fig. 6). However, in strain $\Delta oppB$ (pTfbsA), the *fbsA* expression was approximately 35% reduced compared to the wild-type strain O90R(pTfbsA), indicating that the oligopeptide permease stimulates the expression of the *fbsA* gene in *S. agalactiae*.

DISCUSSION

S. agalactiae is a fastidious organism that requires 9 of the 20 proteinogenic amino acids for growth (reference 38 and the present report). To meet its amino acid requirements, *S. agalactiae* takes up amino acids in their free or peptide-bound form. Peptides are imported by the cell with the aid of specific peptide permeases and are subsequently cleaved by intracellular peptidases to their single amino acids (41). Four putative peptide permeases with homology to known peptide transport systems have been noted in the genomes of the two sequenced *S. agalactiae* strains (18, 63). However, none of these putative peptide permeases had been tested for functionality. In the present report, defined mutants in the four putative peptide permease systems were constructed and used to define the roles of these uptake systems in the overall process of peptide utilization and the expression of virulence traits in *S. agalactiae*.

Feeding experiments with defined *S. agalactiae* mutants demonstrated that peptide uptake in these bacteria is mediated by two ABC transporters and one putative proton-driven permease, as is also the case for *L. lactis* and *E. coli* (23, 41, 52, 60, 64). The ABC transporter Gbs1573-1577, however, appears not to be required for the uptake of peptides in *S. agalactiae*. As Gbs1573-1577 reveals similarity to peptide transporters but also to Ni²⁺ permeases (18), it is possibly involved in the uptake of Ni²⁺. Like other microorganisms (41), *S. agalactiae* requires an ABC transporter system (OppA1-F) for the import of oligopeptides of two to six residues. In the genome of *S. agalactiae*, two *oppA* homologs, termed by us *oppA1* and *oppA2*, have been noted. The presence of two *oppA* genes readily explains the apparent dispensability of either for the transport of oligopeptides by the oligopeptide permease system. Indeed, the two proteins appear to have overlapping specificities for oligopeptides, as only the simultaneous inactivation of *oppA1* and *oppA2* abolished transport of oligopeptides. The presence of several oligopeptide binding proteins that serve the same membrane complex is rather common in microorganisms. *S. pneumoniae*, *S. gordonii*, and *E. coli* each possess three genes that encode oligopeptide binding proteins

(3, 26, 40). In *Borrelia burgdorferi*, five *oppA* homologs have been identified (9). Finally, *Enterococcus faecalis* produces two peptide binding proteins with different affinities for the peptide sex pheromone cCf10, which is required for conjugative transfer of certain plasmids (29). It has been suggested that, as OppA1 and OppA2 from *S. agalactiae* are closely related to each other on the amino acid level, they presumably evolved from a common ancestor following gene duplication (63).

Dipeptide import by *S. agalactiae* appears to be a complex process, involving the two ABC transporters DppA-E and OppA1-F and the putative proton-driven symporter DpsA. DpsA and DppA-E homologs of several microorganisms have been shown to transport both dipeptides and tripeptides (6, 22, 37, 41, 67). The tripeptide Val-Trp-Ile was not transported in oligopeptide permease mutants of *S. agalactiae*, demonstrating that the dipeptide permeases DppA-E and DpsA are not involved in the transport of this tripeptide. However, this finding does not rule out other tripeptides as substrates for either of the two dipeptide permeases.

Unexpectedly, feeding experiments with *S. agalactiae* peptide permease mutants demonstrated the uptake of the dipeptide Ile-Phe even in the absence of functional DppA-E, OppA1-F, and DpsA permeases. Cleavage of the dipeptide Ile-Phe by extracellular peptidases is unlikely, as isoleucine was not detectable in Ile-Phe-containing dCDM and other isoleucine-containing dipeptides also were not cleaved by *S. agalactiae*. Our findings therefore indicate the presence of a further dipeptide permease that mediates the uptake of the dipeptide Ile-Phe in *S. agalactiae*. Interestingly, *L. lactis* triple mutants in the two ABC peptide transporters and the proton-driven peptide symporter also still grow on different dipeptides (52). The presence of four different dipeptide permeases is therefore discussed for *L. lactis* as well.

The presence of different dipeptide uptake systems in *S. agalactiae* indicates their involvement in the import of different types of dipeptides. However, the *S. agalactiae* mutants $\Delta dppB$, $\Delta oppB$, and $\Delta dpsA$ exhibited unaltered growth in dCDM with different isoleucine-containing dipeptides, suggesting overlapping substrate specificities of the three dipeptide uptake systems. In contrast, mutant $\Delta dpsA$ was significantly impaired for growth in human amniotic fluid, whereas the mutants $\Delta dppB$ and $\Delta oppB$ exhibited identical growth compared to the parental strain O90R. Amniotic fluid has a low concentration of free amino acids (55) but contains significant amounts of proteins and peptides (47, 56). It is therefore tempting to speculate that during growth of *S. agalactiae* in human amniotic fluid, the permease DpsA plays an essential role in the import of specific dipeptides to satisfy the bacterial amino acid requirements. Similarly, *L. lactis* also depends on the DpsA ortholog DtpT for growth in a mixture of caseins (59). A frequent cause of *S. agalactiae* infection is aspiration of infected amniotic fluid by the fetus. Amniotic fluid only has low titers of antibody against *S. agalactiae* (20) and supports growth of *S. agalactiae* to high cell densities (14, 66). As the present study demonstrates an important role of DpsA for growth of *S. agalactiae* in amniotic fluid, DpsA represents an interesting target for the prevention or treatment of in utero *S. agalactiae* infections.

In several organisms, the expression of genes encoding peptide permeases is induced by the presence of peptides (7, 25, 44) or repressed in the absence of substrate (39, 57). As shown

by quantitative real-time PCR, the transcription of the *S. agalactiae* genes *dppA* to *dppE* was not affected in di- or hexapeptide-containing dCDM. However, the expression of the *dppA-E* operon increased about twofold during growth of the bacteria in THY medium, which is a complex mixture of free amino acids, dipeptides, and oligopeptides. This indicates that components in THY medium, putatively peptides, cause an induction of *dppA-E* gene expression in *S. agalactiae*. In contrast, the expression of the *dppA* gene in *E. coli* is repressed during growth of the bacteria in a peptide-rich complex medium (39). In *S. pyogenes* as well, the transcription of the *dppA-E* operon is down-regulated during growth of the bacteria in THY medium (44). This suggests different regulatory mechanisms in expression control of the *dppA-E* operon among these microorganisms. As demonstrated by our studies, the expression of the *oppA1-F* operon was induced about eightfold in dCDM containing the hexapeptide Val-Tyr-Ile-His-Pro-Phe. However, no induction of *oppA1-F* expression was observed during growth of *S. agalactiae* in THY complex medium, which also contains dipeptides and free amino acids in addition to oligopeptides. It can therefore be speculated that in *S. agalactiae* the transcription of the *oppA1-F* operon is repressed by the presence of free amino acids and/or dipeptides in the medium. In *L. lactis*, the expression of the *oppA* gene is also significantly repressed during growth of the bacteria in a complex medium that is rich in free amino acids and peptides (13), indicating a similar transcription control of the *oppA-F* operons in these bacteria. In our studies, the expression of the *dpsA* gene was not altered during growth of the bacteria in different media, whereas in *L. lactis*, transcription of the *dpsA* ortholog *dtpT* is regulated by the peptide content of the medium (22). Taken together, our findings suggest that the transcriptional regulation of genes encoding peptide permeases in *S. agalactiae* is complex and partially distinct from the regulatory mechanisms of peptide permease-encoding genes in other microorganisms.

In several gram-positive bacteria, peptide uptake systems are not only essential for nutrient accumulation but they are also involved in determining other cellular functions, including virulence mechanisms. In pneumococci and *S. pyogenes*, the oligopeptide permease modulates the adherence of the bacteria to human host cells (11, 12). As revealed by our studies, the oligopeptide permease from *S. agalactiae* also stimulates the adherence of the bacteria to human epithelial cells and modulates their binding to fibrinogen and fibronectin. In addition, the oligopeptide permease was shown to control the expression of the *fbxA* gene, which encodes a fibrinogen-binding adhesin. Similarly, the oligopeptide permease from *S. gordonii* modulates the expression of the *csxA* gene, encoding a 259-kDa protein that mediates the binding of the bacteria to immobilized fibronectin and to other microorganisms (33, 34). In *S. pyogenes*, the oligopeptide and the dipeptide permease both control the transcription of the *speB* gene, encoding a cysteine protease which is important for the virulence of the bacteria (30, 44, 45). Furthermore, in *Bacillus cereus* and *Bacillus thuringiensis*, the oligopeptide permease controls the expression of the *plcR* regulon, which is essential for the virulence of these bacteria (19, 58). As demonstrated by these examples, oligopeptide permeases are involved in the expression control of virulence genes in various organisms. At present, the molecu-

lar basis for the oligopeptide permease-dependent regulation of *fbxA* expression in *S. agalactiae* remains unknown. However, a putative quorum-sensing system was recently identified in *S. agalactiae* O90R (61). Interestingly, the inactivation of this quorum-sensing system also changed the fibrinogen-binding properties of the resultant mutant. As quorum sensing in gram-positive bacteria is mediated by small peptides, it is tempting to speculate that in *S. agalactiae* the oligopeptide permease is involved in the quorum-sensing-dependent regulation of *fbxA* expression. In fact, in several gram-positive bacteria, oligopeptide permeases are involved in quorum-sensing-dependent gene regulation and control cellular properties like sporulation in *Bacillus subtilis* (43, 51), development of competence in *B. subtilis* and *S. pneumoniae* (5, 42), and conjugation in *E. faecalis* (29). All these studies point to an involvement of peptide permeases in the transport of signaling molecules that in turn modulate gene expression by interacting with transcriptional regulators. The precise mechanism of oligopeptide permease-dependent gene regulation in *S. agalactiae* and the full complement of molecules that participate in this signaling process remain to be identified. Studies are therefore under way to unravel peptides with signaling function in *S. agalactiae* and to identify further genes revealing an oligopeptide permease-dependent expression.

REFERENCES

- Abouhamad, W. N., M. Manson, M. M. Gibson, and C. F. Higgins. 1991. Peptide transport and chemotaxis in *Escherichia coli* and *Salmonella typhimurium*: characterization of the dipeptide permease (Dpp) and the dipeptide-binding protein. *Mol. Microbiol.* **5**:1035–1047.
- Abouhamad, W. N., and M. D. Manson. 1994. The dipeptide permease of *Escherichia coli* closely resembles other bacterial transport systems and shows growth-phase-dependent expression. *Mol. Microbiol.* **14**:1077–1092.
- Alloing, G., P. de Philip, and J. P. Claverys. 1994. Three highly homologous membrane-bound lipoproteins participate in oligopeptide transport by the Ami system of the gram-positive *Streptococcus pneumoniae*. *J. Mol. Biol.* **241**:44–58.
- Alloing, G., C. Granadel, D. A. Morrison, and J. P. Claverys. 1996. Competence pheromone, oligopeptide permease, and induction of competence in *Streptococcus pneumoniae*. *Mol. Microbiol.* **21**:471–478.
- Alloing, G., B. Martin, C. Granadel, and J. P. Claverys. 1998. Development of competence in *Streptococcus pneumoniae*: pheromone autoinduction and control of quorum sensing by the oligopeptide permease. *Mol. Microbiol.* **29**:75–83.
- Alves, R. A., and J. W. Payne. 1980. The number and nature of the peptide-transport systems of *Escherichia coli*: characterization of specific transport mutants. *Biochem. Soc. Trans.* **8**:704–705.
- Andrews, J. C., T. C. Blevins, and S. A. Short. 1986. Regulation of peptide transport in *Escherichia coli*: induction of the *trp*-linked operon encoding the oligopeptide permease. *J. Bacteriol.* **165**:428–433.
- Baker, C. J., and M. S. Edwards. 1995. Group B streptococcal infections, p. 980–1054. In J. S. Remington and J. O. Klein (ed.), *Infectious disease of the fetus and newborn infant*. W. B. Saunders Company, Philadelphia, Pa.
- Bono, J. L., K. Tilly, B. Stevenson, D. Hogan, and P. Rosa. 1998. Oligopeptide permease in *Borrelia burgdorferi*: putative peptide-binding components encoded by both chromosomal and plasmid loci. *Microbiology* **144**:1033–1044.
- Borezee, E., E. Pellegrini, and P. Berche. 2000. OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. *Infect. Immun.* **68**:7069–7077.
- Cundell, D. R., B. J. Pearce, J. Sandros, A. M. Naughton, and H. R. Masure. 1995. Peptide permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. *Infect. Immun.* **63**:2493–2498.
- Darmstadt, G. L., L. Mentele, A. Podbielski, and C. E. Rubens. 2000. Role of group A streptococcal virulence factors in adherence to keratinocytes. *Infect. Immun.* **68**:1215–1221.
- Detmers, F. J., E. R. Kunji, F. C. Lanfermeijer, B. Poolman, and W. N. Konings. 1998. Kinetics and specificity of peptide uptake by the oligopeptide transport system of *Lactococcus lactis*. *Biochemistry* **37**:16671–16679.
- Eickhoff, T. C., J. O. Klein, K. L. Daly, D. I. Ingall, and M. Finland. 1964. Neonatal sepsis and other infections due to group B beta-hemolytic streptococci. *N. Engl. J. Med.* **271**:1221–1228.
- Eidelman, A. I., A. Nevet, B. Rudensky, R. Rabinowitz, C. Hammerman, D. Raveh, and M. S. Schimmel. 2002. The effect of meconium staining of amniotic fluid on the growth of *Escherichia coli* and group B streptococcus. *J. Perinatol.* **22**:467–471.
- Fang, G., W. N. Konings, and B. Poolman. 2000. Kinetics and substrate specificity of membrane-reconstituted peptide transporter DtpT of *Lactococcus lactis*. *J. Bacteriol.* **182**:2530–2535.
- Gibson, M. M., M. Price, and C. F. Higgins. 1984. Genetic characterization and molecular cloning of the tripeptide permease (*tpg*) genes of *Salmonella typhimurium*. *J. Bacteriol.* **160**:122–130.
- Glaser, P., C. Rusniok, C. Buchrieser, F. Chevalier, L. Frangeul, T. Msadek, M. Zouine, E. Couve, L. Lalioui, C. Poyart, P. Trieu-Cuot, and F. Kunst. 2002. Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol. Microbiol.* **45**:1499–1513.
- Gominet, M., L. Slamti, N. Gilois, M. Rose, and D. Lereclus. 2001. Oligopeptide permease is required for expression of the *Bacillus thuringiensis plcR* regulon and for virulence. *Mol. Microbiol.* **40**:963–975.
- Gray, B. M., J. D. Springfield, and H. C. Dillon, Jr. 1987. Type-specific streptococcal antibodies in amniotic fluid. *Am. J. Obstet. Gynecol.* **156**:666–669.
- Gutekunst, H., B. E. Eikmanns, and D. J. Reinscheid. 2003. Analysis of RogB-controlled virulence mechanisms and gene expression in *Streptococcus agalactiae*. *Infect. Immun.* **71**:5056–5064.
- Hagting, A., J. Knol, B. Hasemeier, M. R. Streutker, G. Fang, B. Poolman, and W. N. Konings. 1997. Amplified expression, purification and functional reconstitution of the dipeptide and tripeptide transport protein of *Lactococcus lactis*. *Eur. J. Biochem.* **247**:581–587.
- Hagting, A., E. R. Kunji, K. J. Leenhouts, B. Poolman, and W. N. Konings. 1994. The di- and tripeptide transport protein of *Lactococcus lactis*. A new type of bacterial peptide transporter. *J. Biol. Chem.* **269**:11391–11399.
- Higgins, C. F. 1992. ABC transporters: from microorganism to man. *Annu. Rev. Cell Biol.* **8**:67–113.
- Jamieson, D. J., and C. F. Higgins. 1984. Anaerobic and leucine-dependent expression of a peptide transport gene in *Salmonella typhimurium*. *J. Bacteriol.* **160**:131–136.
- Jenkinson, H. F., R. A. Baker, and G. W. Tannock. 1996. A binding-lipoprotein-dependent oligopeptide transport system in *Streptococcus gordonii* essential for uptake of hexa- and heptapeptides. *J. Bacteriol.* **178**:68–77.
- Jenkinson, H. F., and R. A. Easingwood. 1990. Insertional inactivation of the gene encoding a 76-kilodalton cell surface polypeptide in *Streptococcus gordonii* Challis has a pleiotropic effect on cell surface composition and properties. *Infect. Immun.* **58**:3689–3697.
- Jones, A. L., K. M. Knoll, and C. E. Rubens. 2000. Identification of *Streptococcus agalactiae* virulence genes in the neonatal rat sepsis model using signature-tagged mutagenesis. *Mol. Microbiol.* **37**:1444–1455.
- Leonard, B. A., A. Podbielski, P. J. Hedberg, and G. M. Dunny. 1996. *Enterococcus faecalis* pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. *Proc. Natl. Acad. Sci. USA* **93**:260–264.
- Lukomski, S., C. A. Montgomery, J. Rurangirwa, R. S. Geske, J. P. Barrish, G. J. Adams, and J. M. Musser. 1999. Extracellular cysteine protease produced by *Streptococcus pyogenes* participates in the pathogenesis of invasive skin infection and dissemination in mice. *Infect. Immun.* **67**:1779–1788.
- Maguin, E., H. Prevost, S. Ehrlich, and A. Gruss. 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J. Bacteriol.* **178**:931–935.
- Manson, M. D., V. Blank, G. Brade, and C. F. Higgins. 1986. Peptide chemotaxis in *E. coli* involves the Tap signal transducer and the dipeptide permease. *Nature* **321**:253–256.
- McNab, R., H. Forbes, P. S. Handley, D. M. Loach, G. W. Tannock, and H. F. Jenkinson. 1999. Cell wall-anchored CshA polypeptide (259 kilodaltons) in *Streptococcus gordonii* forms surface fibrils that confer hydrophobic and adhesive properties. *J. Bacteriol.* **181**:3087–3095.
- McNab, R., and H. F. Jenkinson. 1998. Altered adherence properties of a *Streptococcus gordonii hppA* (oligopeptide permease) mutant result from transcriptional effects on *cshA* adhesin gene expression. *Microbiology* **144**:127–136.
- Mesavage, W. C., S. F. Suchy, D. L. Weiner, C. S. Nance, D. B. Flannery, and B. Wolf. 1985. Amino acids in amniotic fluid in the second trimester of gestation. *Pediatr. Res.* **19**:1021–1024.
- Miller, J. H. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Nakajima, H., A. Hagting, E. R. Kunji, B. Poolman, and W. N. Konings. 1997. Cloning and functional expression in *Escherichia coli* of the gene encoding the di- and tripeptide transport protein of *Lactobacillus helveticus*. *Appl. Environ. Microbiol.* **63**:2213–2217.
- Niven, C. F. 1943. The nutrition of group B streptococcus. *J. Bacteriol.* **46**:573–574.
- Olson, E. R., D. S. Dunyak, L. M. Jurss, and R. A. Poorman. 1991. Identification and characterization of *dppA*, an *Escherichia coli* gene encoding a periplasmic dipeptide transport protein. *J. Bacteriol.* **173**:234–244.
- Park, J. T., D. Raychaudhuri, H. Li, S. Normark, and D. Mengin-Lecreulx.

1998. MppA, a periplasmic binding protein essential for import of the bacterial cell wall peptide L-alanyl- γ -D-glutamyl-meso-diaminopimelate. *J. Bacteriol.* **180**:1215–1223.
41. Payne, J. W., and M. W. Smith. 1994. Peptide transport by micro-organisms. *Adv. Microb. Physiol.* **36**:1–80.
 42. Pearce, B. J., A. M. Naughton, and H. R. Masure. 1994. Peptide permeases modulate transformation in *Streptococcus pneumoniae*. *Mol. Microbiol.* **12**: 881–892.
 43. Perego, M., C. F. Higgins, S. R. Pearce, M. P. Gallagher, and J. A. Hoch. 1991. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. *Mol. Microbiol.* **5**:173–185.
 44. Podbielski, A., and B. A. Leonard. 1998. The group A streptococcal dipeptide permease (Dpp) is involved in the uptake of essential amino acids and affects the expression of cysteine protease. *Mol. Microbiol.* **28**:1323–1334.
 45. Podbielski, A., B. Pohl, M. Woischnik, C. Korner, K. H. Schmidt, E. Rozdzinski, and B. A. Leonard. 1996. Molecular characterization of group A streptococcal (GAS) oligopeptide permease (*opp*) and its effect on cysteine protease production. *Mol. Microbiol.* **21**:1087–1099.
 46. Poyart, C., and P. Trieu-Cuot. 1997. A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to beta-galactosidase in gram-positive bacteria. *FEMS Microbiol. Lett.* **156**:193–198.
 47. Queenan, J. T. 1971. Amniotic fluid analysis. *Clin. Obstet. Gynecol.* **14**:505–536.
 48. Reinscheid, D. J., B. Gottschalk, A. Schubert, B. J. Eikmanns, and G. S. Chhatwal. 2001. Identification and molecular analysis of PcsB, a protein required for cell wall separation of group B streptococcus. *J. Bacteriol.* **183**:1175–1183.
 49. Ricci, M. L., R. Manganelli, C. Berneri, G. Orefici, and G. Pozzi. 1994. Electrotransformation of *Streptococcus agalactiae* with plasmid DNA. *FEMS Microbiol. Lett.* **119**:47–52.
 50. Rubens, C. E., H. V. Raff, J. C. Jackson, E. Y. Chi, J. T. Bielitzki, and S. L. Hillier. 1991. Pathophysiology and histopathology of group B streptococcal sepsis in *Macaca nemestrina* primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. *J. Infect. Dis.* **164**:320–330.
 51. Rudner, D. Z., J. R. LeDeaux, K. Ireton, and A. D. Grossman. 1991. The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. *J. Bacteriol.* **173**:1388–1398.
 52. Sanz, Y., F. C. Lanfermeijer, P. Renault, A. Bolotin, W. N. Konings, and B. Poolman. 2001. Genetic and functional characterization of dpp genes encoding a dipeptide transport system in *Lactococcus lactis*. *Arch. Microbiol.* **175**:334–343.
 53. Schubert, A., K. Zakikhany, M. Schreiner, R. Frank, B. Spellerberg, B. E. Eikmanns, and D. J. Reinscheid. 2002. A fibrinogen receptor from group B streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. *Mol. Microbiol.* **46**:557–569.
 54. Schuchat, A. 1998. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. *Clin. Microbiol. Rev.* **11**:497–513.
 55. Schulman, J. D., J. T. Queenan, and L. Doores. 1972. Gas chromatographic analysis of concentrations of amino acids in amniotic fluid from early, middle and late periods of human gestation. *Am. J. Obstet. Gynecol.* **114**:243–249.
 56. Schulman, J. D., J. T. Queenan, L. Doores, and B. Holland. 1973. Molecular weight distribution of amniotic fluid proteins during normal gestation. *Obstet. Gynecol.* **42**:344–348.
 57. Slack, F. J., J. P. Mueller, M. A. Strauch, C. Mathiopoulos, and A. L. Sonenshein. 1991. Transcriptional regulation of a *Bacillus subtilis* dipeptide transport operon. *Mol. Microbiol.* **5**:1915–1925.
 58. Slamti, L., and D. Lereclus. 2002. A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. *EMBO J.* **21**:4550–4559.
 59. Smid, E. J., R. Plapp, and W. N. Konings. 1989. Peptide uptake is essential for growth of *Lactococcus lactis* on the milk protein casein. *J. Bacteriol.* **171**:6135–6140.
 60. Smith, M. W., D. R. Tyreman, G. M. Payne, N. J. Marshall, and J. W. Payne. 1999. Substrate specificity of the periplasmic dipeptide-binding protein from *Escherichia coli*: experimental basis for the design of peptide prodrugs. *Microbiology* **145**:2891–2901.
 61. Spellerberg, B., E. Rozdzinski, S. Martin, J. Weber-Heynemann, and R. Luticken. 2002. *rgf* encodes a novel two-component signal transduction system of *Streptococcus agalactiae*. *Infect. Immun.* **70**:2434–2440.
 62. Steiner, H. Y., F. Naider, and J. M. Becker. 1995. The PTR family: a new group of peptide transporters. *Mol. Microbiol.* **16**:825–834.
 63. Tettelin, H., V. Masignani, M. J. Cieslewicz, J. A. Eisen, S. Peterson, M. R. Wessels, I. T. Paulsen, K. E. Nelson, I. Margarit, T. D. Read, L. C. Madoff, A. M. Wolf, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, J. F. Kolonay, R. Madupu, M. R. Lewis, D. Radune, N. B. Fedorova, D. Scanlan, H. Khouri, S. Mulligan, H. A. Carty, R. T. Cline, S. E. Van Aken, J. Gill, M. Scarselli, M. Mora, E. T. Iacobini, C. Brettoni, G. Galli, M. Mariani, F. Vegni, D. Maione, D. Rinaldo, R. Rappuoli, J. L. Telford, D. L. Kasper, G. Grandi, and C. M. Fraser. 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. *Proc. Natl. Acad. Sci. USA* **99**:12391–12396.
 64. Tynkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A. Haandrikman. 1993. Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. *J. Bacteriol.* **175**:7523–7532.
 65. van de Rijn, I., and R. E. Kessler. 1980. Growth characteristics of group A streptococci in a new chemically defined medium. *Infect. Immun.* **27**:444–448.
 66. Varner, M. W., J. W. Turner, C. R. Petzold, and R. P. Galask. 1985. Ultrastructural alterations of term human amniotic epithelium following incubation with group B beta-hemolytic streptococci. *Am. J. Reprod. Immunol. Microbiol.* **8**:27–32.
 67. Verheul, A., A. Hagting, M. R. Amezaga, I. R. Booth, F. M. Rombouts, and T. Abee. 1995. A di- and tripeptide transport system can supply *Listeria monocytogenes* Scott A with amino acids essential for growth. *Appl. Environ. Microbiol.* **61**:226–233.
 68. Vrljic, M., J. Garg, A. Bellmann, S. Wachi, R. Freudl, M. J. Malecki, H. Sahm, V. J. Kozina, L. Eggeling, M. H. Saier, Jr., L. Eggeling, and M. H. Saier, Jr. 1999. The LysE superfamily: topology of the lysine exporter LysE of *Corynebacterium glutamicum*, a paradigm for a novel superfamily of transmembrane solute translocators. *J. Mol. Microbiol. Biotechnol.* **1**:327–336.
 69. Willett, N. P., and G. E. Morse. 1966. Long-chain fatty acid inhibition of growth of *Streptococcus agalactiae* in a chemically defined medium. *J. Bacteriol.* **91**:2245–2250.
 70. Willett, N. P., G. E. Morse, and S. A. Carlisle. 1967. Requirements for growth of *Streptococcus agalactiae* in a chemically defined medium. *J. Bacteriol.* **94**:1247–1248.