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Identification of a Differentially Expressed Oligopeptide Binding Protein (OppA2) in *Streptococcus uberis* by Representational Difference Analysis of cDNA

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***Streptococcus uberis* is an increasingly significant cause of intramammary infection in the dairy cow, presently responsible for approximately 33% of all cases of bovine mastitis in the United Kingdom. Following experimentally induced infection of the lactating mammary gland, *S. uberis* is found predominantly in the luminal areas of secretory alveoli and ductular tissue, indicating that much of the bacterial growth occurs in residual and newly synthesized milk. With the objective of identifying potential virulence determinants in a clinical isolate of *S. uberis*, we have used representational difference analysis of cDNA to identify genes that show modified expression in milk. We have identified a number of differentially expressed genes that may contribute to the overall pathogenicity of the organism. Of these, a transcript encoding a putative oligopeptide binding protein (OppA) was further characterized. We have found that *S. uberis* possesses two *oppA*-like open reading frames, *oppA1* and *oppA2*, which are up-regulated to different degrees following growth in milk. Mutants lacking either *oppA1* or *oppA2* are viable and have an increased resistance to the toxic peptide derivative aminopterin; however, only mutants lacking *oppA1* display a lower rate of growth in milk. In addition, expression of the *oppA* genes appears to be coordinated by different mechanisms. We conclude that the *oppA* genes encode oligopeptide binding proteins, possibly displaying different specificities, required for the efficient growth of *S. uberis* in milk.**

The prevalence of bovine mastitis caused by bacteria that show a contagious route of transmission has declined in the United Kingdom over the past 25 years as a result of the implementation of a five-point control plan aimed at reducing exposure, duration, and transmission of intramammary infections. However, these improvements in dairy husbandry have had little impact on the incidence of mastitis due to bacteria that infect the bovine udder from an environmental reservoir, such as *Streptococcus uberis* and *Escherichia coli* (12). As a consequence, mastitis remains the most common and costliest disease of dairy cattle (22). It is estimated that the average cost of a case of mastitis in the United Kingdom is presently £180 and is equivalent nationally to an annual loss of around £170 million (http://www.fawc.org.uk/dairycow/dcowrtoc.htm).

Following experimentally induced infection of the lactating mammary gland, *S. uberis* is found predominantly in the luminal areas of secretory alveoli and ductular tissue, indicating that much of the bacterial growth occurs in residual and newly synthesized milk (48). This is likely to provide a medium that is deficient in free- and peptide-associated amino acids and as such represents a nutritionally challenging environment for the infecting bacteria (4). To colonize the udder, survive, and grow under such conditions requires that *S. uberis* is able to obtain nutrients, initially from the milk itself, and following activation of the immune system, from damaged host tissues.

In order to facilitate their survival and growth within the host, successful pathogens have evolved a variety of specific gene products, as well as mechanisms to regulate expression of these factors in response to particular environmental stimuli. The response to these cues is the coordinately regulated expression of multiple virulence-associated genes that facilitate the organisms' survival in an environment that can be nutritionally challenging and immunologically hostile (30, 31). As a consequence, identifying such differentially expressed genes has been a major focus of scientific research and a number of specialized techniques have been developed to this purpose. Recently several PCR-based screening methods have been described that avoid the need for large amounts of starting material, a factor that has often hampered more traditional subtractive hybridization techniques (40).

In this study representational difference analysis (RDA) of cDNA (cDNA RDA) has been used (16) to identify genes in a clinical isolate of *S. uberis* whose expression is increased in response to growth in milk, with the objective of identifying putative virulence-associated genes. cDNA RDA has an advantage over similar PCR-coupled approaches in that a subtractive hybridization step allows the removal of sequences common to both populations, thereby greatly simplifying the interpretation of results and identification of the differentially expressed genes. In addition, the exponential degree of enrichment achieved by the use of PCR in cDNA RDA enables the detection of rare transcripts. We have identified a number of differentially expressed genes that may contribute to the overall pathogenicity of the organism. Of these, a transcript encoding a putative membrane-bound protein with homology to oligopeptide binding proteins (OppA-like) was isolated.

The Opp proteins are part of the family 5 extracellular solute binding proteins, high-affinity transport systems involved in the active transport of solutes across the cytoplasmic membrane

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(for review see reference 45). The components of these oligopeptide binding systems include a high-affinity periplasmic or extracellular membrane-bound solute-binding protein (OppA), two integral membrane proteins (OppB and -C), and two membrane-associated ATP-binding proteins (OppD and -F) localized to the cytoplasmic side of the membrane. In the gram-positive streptococci, the solute-binding proteins are bound to the extracellular surface side of the cytoplasmic membrane via an N-terminal lipid anchor. Family 5 presently contains proteins, including periplasmic oligopeptide binding proteins of gram-negative bacteria (OppA) and the homologous lipoproteins in gram-positive bacteria (OppA, AmiA, or AppA), periplasmic dipeptide binding proteins of *E. coli* and *Bacillus subtilis* (DppA and DppE, respectively 45). The substrate specificity of OppA of *Salmonella enterica* serovar Typhimurium has been extensively characterized and is capable of binding peptides of 2 to 5 amino acids in length regardless of sequence (46). In contrast to this, OppA of *Lactococcus lactis* is capable of binding peptides of up to 35 amino acids in length (10), displaying a preference for hydrophobic residues and not transporting either di- or tripeptides (19). Recent evidence from studies with *L. lactis* suggests that the ability to transport certain peptides is not solely dependent on OppA and that the OppBCDF complex plays a role in conferring some substrate specificity (8).

The transport of dipeptides in the group A streptococci (GAS) is performed by the well-defined Dpp system, and its expression appears to be under the control of the Mga virulence regulator (37). Emerging genome sequence data (http://www.sanger.ac.uk/Projects/S_uberis/) suggest that the Dpp system may be absent in *S. uberis*.

The most obvious role for Opp is in acquisition of essential amino acids (20, 23). *S. uberis* is auxotrophic for several amino acids (21), and accordingly during growth in milk, which is deficient in free or peptide-associated amino acids (4), import of oligopeptides probably constitutes the primary source of nitrogen for the cell. However, recent evidence proposes an assortment of alternative or other functions for Opp, including the induction of competence in *Streptococcus pneumoniae* (1, 33) sporulation in *Bacillus* (14, 27, 44), and adherence to host proteins and epithelia (9, 11). A possible role in the initiation of sensory transduction pathways, detecting and transmitting signals to cytoplasmic constituents, which then signal a change in state by alteration of specific gene expression, has also been suggested (1, 14, 27, 44). Processes such as these can be used to indicate to the pathogen that it has entered a host and should modify its gene expression accordingly.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains were routinely grown in Todd-Hewitt (TH) broth (Difco Laboratories) at 37°C or on TH agar plates supplemented with 5% sheep blood (TCS Biosciences).

For cDNA RDA analysis, strain 0140J in TH broth was grown overnight, washed in HEPES-buffered saline, and used to inoculate either fresh TH broth (Difco), tryptic soy broth (Difco) containing 46 g of lactose per liter, or sterile skim milk. Cultures were grown to mid-exponential phase as determined by our previous growth rate analyses.

Milk samples. Sterile skim milk was kindly provided by T. Field (Institute for Animal Health [IAH], Compton, United Kingdom). Samples were collected aseptically from the mammary glands of uninfected cows of the herd at IAH, skimmed, and stored at −20°C for up to 3 months.

RNA isolation. Total RNA was isolated by a procedure described by (28) with some modification. The protocol used was as follows: cells were harvested at 10^4 CFU/ml by centrifugation at 8,000 × *g* for 5 min at 4°C. The cell pellet was resuspended in 200 μl of RNase-free water (Ambion) and was added to an RNase-free 2-ml centrifuge tube containing approximately 0.4 g of zirconia or silica beads (Biospec Products Inc.), 500 μl of acid phenol, pH 4.3 (Sigma), 100 μl of chloroform, and 500 μl of detergent solution (9.6% Decon 90 [Decon Labs Ltd.]–120 mM sodium acetate, pH 4.0). The tube was then processed immediately in a Fastprep machine (Q-biogene) three times at 20 s at full speed and cooled on ice for 10 min. The sample was centrifuged for 10 min at 16,000 × *g* in an Eppendorf 1545R at 4°C, and the aqueous phase was taken to an fresh Eppendorf tube containing 500 μl of 100% ethanol. The samples were precipitated at −70°C for 1 h, and the RNA was harvested by centrifugation at 16,000 × *g* for 30 min in an Eppendorf 1545R at 4°C. The RNA pellet was washed in 70% ethanol, dried, and resuspended in 30 μl of RNase-free water (Ambion) containing 1 U of prime RNase inhibitor (Eppendorf)/μl. Quantification and crude quality assessment were done by visual examination on a 1% nondenaturing agarose gel in Tris-borate-EDTA buffer stained with ethidium bromide. Aliquots of total RNA were stored at −80°C until use.

Removal of DNA from total RNA preparations. DNA was removed from RNA samples by using the DNA-free kit (Ambion) according to the manufacturer's instructions.

cDNA synthesis. cDNA synthesis was performed by random priming with hexamers, on total RNA by using the Timesaver cDNA synthesis kit as described by the manufacturer (Amersham Pharmacia).

Isolation of *S. uberis* rRNA genes and generation of rRNA representations. Synthetic oligonucleotide primers (pn16Sup/pn16Sdn and pn23Sup/pn23Sdn) to published streptococcal rRNA sequences were used to amplify the 16S and 23S rRNA genes from 0140J chromosomal DNA (15). The rRNA genes were restricted by using *Dpn*II and R-Bgl-12 and -24 adapters ligated as described elsewhere. Twenty cycles of amplification were used to generate the rRNA representations.

cDNA RDA. Generation of representations. RDA was performed (47). Double-stranded cDNA (1 to 2 μg) was digested with *Dpn*II, phenol extracted, ethanol precipitated by using 15 μg of glycogen as carrier (Ambion), and resuspended in 20 μl of Tris-EDTA. To this were added 24 μl of H_2O, 6 μl of ligase buffer (as supplied with T4 DNA ligase), 4 μl of 0.25 mM R-Bgl-12, and 4 μl of 0.5 mM R-Bgl-24. The oligonucleotides were annealed to each other in a PCR machine by heating to 50°C for 5 min and cooling to 10°C at 1°C/min. Two microliters of T4 DNA ligase was then added and incubated at 14°C for 18 h. Ligations were diluted by the addition of 120 μl of Tris-EDTA, and multiple PCRs were set up to generate the representations. Each 200-μl reaction contained 3 μl of the diluted ligation, 139 μl of H_2O, 40 μl of 5× PCR buffer (335 mM Tris-HCl, pH 8.9, 20 mM $MgCl_2$, 80 mM $[NH_4]_2SO_4$, and 166 μg of bovine serum albumin/ml), 16 μl of 4 mM deoxynucleoside triphosphate (dNTP) mix (Amersham Pharmacia), and 1 μl of 0.5 mM R-Bgl-24 adaptor. The R-Bgl-12 adaptor was melted away (5 min, 72°C) and the 3′ ends were filled by the addition of 5 U of AmpliTaq (Applied Biosystems) (5 min, 72°C). Reaction mixtures were then amplified by using 17 cycles of amplification (1 min, 95°C; and 3 min, 72°C); the products were combined, phenol extracted, and resuspended in a final Tris-EDTA concentration of 0.5 mg/ml. The R adaptors were removed from the driver and tester populations by digestion with *Dpn*II, followed by phenol extraction and purification through a spin column such as MicroSpin S-300 HR columns according to the manufacturer's instructions and ethanol precipitation. Driver populations were taken directly for hybridization. To the tester population, J-Bgl-12 and -24 adaptors were then ligated in the manner described above and the ligation was diluted to a final concentration of 10 ng/μl with Tris-EDTA.

Subtractive hybridization and amplification. Five micrograms (10 μl) of driver was combined with 5 μg (10 μl) of the rRNA-derived representation and 0.1 μg (10 μl) of J-Bgl ligated tester in a 0.5-ml microcentrifuge tube, and 70 μl of distilled water (ddH_2O) was added to generate a driver-to-tester ratio of 100:1 (50:50:1). The reaction mixtures were phenol extracted and ethanol precipitated by using 1 μl of glycogen carrier (Ambion). The pellet was air dried and was thoroughly resuspended in 4 μl of 3× EE buffer (30 mM *N*-[2-hydroxyethyl]piperazine-*N*′-3-propanesulfonic acid, pH 8.0; and 3 mM EDTA, pH 8.0). The solution was overlaid with a few drops of mineral oil (Sigma) and was heated in a PCR machine to 98°C for 5 min and was then incubated at 67°C for 24 h to allow complete annealing. The hybridization mix was then diluted to 200 μl, and two PCRs were set up comprising 122 μl of ddH_2O, 40 μl of 5× PCR buffer (335 mM Tris-HCl, pH 8.9, 20 mM $MgCl_2$, 80 mM $[NH_4]_2SO_4$, and 166 μg of bovine serum albumin/ml), 16 μl of 4 mM dNTP mix (Amersham Pharmacia), and 20 μl of diluted hybridization mix. The J-Bgl-12-mer was melted away (72°C, 5 min), and 5 U of AmpliTaq (Perkin-Elmer) was added to fill in the 3′ DNA ends (72°C,

5 min). One microliter of 0.5 mM J-Bgl-24 adaptor was added and amplified by 11 cycles of 95°C, 1 min; and 70°C, 3 min. The two reactions were combined, phenol extracted, ethanol precipitated by using 1 μl of carrier tRNA (Sigma), and resuspended in 20 μl of Tris-EDTA. Unhybridized single-stranded templates were removed by digestion with mung bean nuclease (20 μl of hybridized DNA, 4 μl of mung bean nuclease buffer, and 2 μl of mung bean nuclease) (New England Biolabs) and 14 μl of ddH_2O at 30°C for 30 min. The digest was terminated by the addition of 160 μl of 50 mM Tris-HCl, pH 8.9, and by heating to 98°C for 5 min and was chilled on ice. For final amplification a PCR comprising 20 μl of the mung bean nuclease-treated DNA, 122 μl of ddH_2O, 40 μl of 5× PCR buffer (335 mM Tris-HCl, pH 8.9, 20 mM $MgCl_2$, 80 mM $[NH_4]_2SO_4$, and 166 μg of bovine serum albumin per ml), 16 μl of 4 mM dNTP mix (Amersham Pharmacia), and 1 μl of 0.5 mM J-Bgl-24 adaptor was set up. Reaction mixtures were heated to 95°C for 1 min; 5 U of AmpliTaq was added and amplified for 18 cycles of 95°C, 1 min; and 70°C, 3 min. DNA concentration was estimated by agarose gel electrophoresis on a 1.5% gel. The DNA was phenol extracted, ethanol precipitated, and resuspended at 0.5 μg/μl. This is the first difference product (DP1). J-Bgl adaptors on DP1 were changed to N-Bgl-12 and -24 adaptors, and subtractive hybridization was repeated by using a driver/tester ration of 400:1 to generate DP2. The N-Bgl adaptors on DP2 were replaced with J-Bgl adaptors, and a third round of subtractive hybridization was performed by using a driver/tester ratio of 8,000:1 to generate DP3. Driver/tester ratios were as follows: DP1, 100:1 (50:50:1); DP2, 400:1; and DP3, 8,000:1.

Cloning and sequencing of difference products. Final difference products (DP3) were resolved by electrophoresis with high-resolution agarose (agarose 1000; Invitrogen), excised, and purified by using a Qiaquick spin kit (Qiagen). Products were cloned into the pCR4-TOPO vector (Invitrogen) according to the manufacturer's instructions and were sequenced with oligonucleotides M13Uni (−21) and M13 Rev (−29) by MWG-Biotech. Sequences were compared to the GenBank database by using the BLAST program (3).

Genomic DNA isolation from *S. uberis*. Cells were harvested from a 3-ml culture, washed in 0.5 ml of 10 mM Tris and 5 mM EDTA (pH 7.8), resuspended in 375 μl of the same buffer containing 30 U of mutanolysin/ml and 10 mg of lysozyme /ml, and incubated at 37°C for 30 min. Cells were lysed by addition of 20 μl of sodium dodecyl sulfate (20% [wt/vol] in 50 mM Tris–20 mM EDTA, pH 7.8) containing 3 μl of proteinase K (20 mg/ml) and by incubation at 37°C for 1 h. Two hundred microliters of saturated NaCl (approximately 6.0 M) was added to precipitate protein and cell wall material and was centrifuged (12,500 × *g*) for 10 min to obtain a firm pellet. Four hundred fifty microliters of the supernatant was taken to a fresh tube, phenol extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and centrifuged (12,500 × *g*, 10 min). The aqueous phase was taken to a fresh tube; 2 volumes of cold ethanol were added and incubated at 4°C for 2 h. Genomic DNA was harvested by centrifugation (12,000 × *g*, 5 min), washed with 70% ethanol, air dried, and resuspended in 100 μl of Tris-EDTA buffer and stored at −20°C until use.

Southern blotting. DNA was resolved by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Hybond; Amersham) as described by (41). DNA was cross-linked to the membrane by UV in a Stratalinker (Stratagene). DNA probes were produced by excision of the DNA fragment from plasmid pDub3 and purification by the Qiaquick kit (Qiagen). Labeling and detection were performed by using the Gene Images-CPD star labeling and detection kit (Amersham Pharmacia) according to the manufacturer's instructions.

Quantification of specific transcripts with Stratagene MX4000 Q-RT-PCR. Quantitative reverse transcriptase PCR (Q-RT-PCR) was performed in an MX4000 Multiplex Quantitative PCR system (Stratagene) by using Stratagene Brilliant Q-RT-PCR reagent kits and SYBR green I (Molecular Probes). Master mixes were prepared by following the manufacturer's instructions, using 500 ng of total RNA and primers directed to *oppA2* (oppA2QF/oppA2QR), *oppA1* (oppA1-QF/oppA1-QR), *oppF* (oppFQF/oppFQR), *glnA* (glnAQF/glnAQR), and *23S* (23SQF/23SQR) (Table 1). Primers and hybridization probes were designed by using Primer3 software (39). The samples were amplified under the following profile: reverse transcription at 45°C for 40 min, denaturation at 95°C for 10 min, and 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s with fluorescence acquisition at 55°C. Sequence-specific standard curves were generated by using serial dilutions of *S. uberis* genomic DNA. The specificity of the reaction was checked postamplification by melt curve analysis on the MX4000 and with agarose gel electrophoresis and ethidium bromide staining.

Cloning N and C termini of OppA2. To clone the C-terminal regions of the *oppA2* ORF, primers oppA through -F and a random 21-mer were used in a PCR with ExTaq DNA polymerase (TaKaRa) and were cycled for 28 cycles of 94°C, 1 min; 50°C, 1 min; and 72°C, 3 min. To isolate the N terminus, primers oppA4 and oppA5′3 were used in a PCR with AmpliTaq DNA polymerase (ABI) and were cycled for 25 cycles of 94°C, 30 s; 48 to 56°C, 30 s; and 72°C, 2 min. All products were resolved on a 0.8% agarose gel, and Southern blotting was performed with pDub3 as a probe as described above. Strongly hybridizing bands were excised from a second agarose gel and were cloned into pCR4-TOPO following the manufacturer's instructions (Invitrogen).

TABLE 1. Primers used in this study

Primer	Sequence
oppAF	ACGCCTATCTTACAACAGATGC
oppA4	CTTTGTAAGGTTTAGCAGACC
oppA6	ACCTTATCCAGAGGGTTT
oppA5′3	TGKTAYGARGARGGWTATATG
oppA2QF	TCGAGCTGACTACCTTGTTT
oppA2QR	ACTAAAGCTTCCCGATCAGT
glnAQF	TTTGATTTAGCACCAACAGAT
glnAQR	TACTTCATGGTGACTTGCTTC
R-Bgl-12	GATCTGCGGTGA
R-Bgl-24	AGCACTCTCCAGCCTCTCACCGCA
J-Bgl-12	GATCTGTTGATG
J-Bgl-24	ACCGACGTCGACTATCCATGAACA
N-Bgl-12	GATCTTCCCTCG
N-Bgl-24	AGGCAACTGTGCTATCCGAGGGAA
pn16Sup	CTTGTTACGACTTCACCCCA
pn16Sdn	TGGCTCAGGACGAACGCT
pn23Sup	CCTGATCATCTCTCAGGGCT
pn23Sdn	CCTTGGCACTAGAAGCCGA
P247	GCTCTTCGGATTTTCGGTATC
P250	CATTTTCCACGACGAATAGAAGGACTGTC
ISS1F	TGTGATTATTGTCGCTGTTGG
ISS1R	GATACCGAAAATCCGAAGAGC
oppA1-3	TTGTTGCTTCTAATACGGGA
oppB1	TAGACGTACTCGTGGATCC
oppA1QF	GACGTTGTTCCCGTATTAGA
oppA1QR	ACGAATCTTAAGGTTGCTCA
oppFQF	TCAAGCAAGTTTGAATGGTC
oppFQR	AACCCTATCTTCACGCTCTT
RP1	ATGCACGTCTCCGAATTACA
RP2	CCGCGCATCAGTTCAAAGAAATC

PCR screening of *S. uberis* 0140J pGh9:IS*S1* mutant bank. Screening was performed (49). Overnight cultures from individual 96-well plates were pooled, and genomic DNA was prepared for use as template in a PCR containing a gene-specific primer, oppA6 (oppA2), oppA1-3 or oppB1, and an IS*S1*-specific primer, P247 or P250. Thirty-five cycles of 95°C for 20 s, 54°C for 1 min, and 72°C for 3 min were performed with AmpliTaq Gold master mix (ABI), and the products were visualized by gel electrophoresis. Following plate identification, a well location was identified by column and row PCR screening of individual plates with the same primer combination. Vector excision was promoted by growth at the permissive temperature (28°C) without antibiotic selection. Loss of the vector and retention of IS*S1* were confirmed by Southern blotting with a probe directed against IS*S1* generated with primers IS*S1*-F and IS*S1*-R.

Measurement of growth sensitivity to aminopterin. Microtiter plate wells containing 90 μl of TH broth and twofold dilutions of 100 μM aminopterin were inoculated with bacterial cells and incubated at 37°C overnight, and culture densities were measured at an optical density of 620 nm by using an iEMS microtiter plate reader (Thermo LifeSciences).

Analysis of peptide utilization by *opp* mutants. Chemically defined media (CDM) minus glutamic acid and/or valine was prepared according to (25). Peptides (Sigma and in-house synthesis) were supplemented to 300 mg/liter and inoculated, and growth was measured at an optical density of 620 nm by using an iEMS microtiter plate reader (Thermo LifeSciences).

Primers used during this study. Primers used are shown in Table 1.

RESULTS

RDA of cDNA. To amplify genes differentially expressed during growth in milk, RNA samples were isolated from mid-exponential-phase cultures of *S. uberis* 0140J (Fig. 1a) grown in either rich media TH broth (driver component) or in sterile skim milk (tester component). Following RNA isolation (Fig. 1b), cDNA synthesis, and restriction with *Dpn*II, three rounds

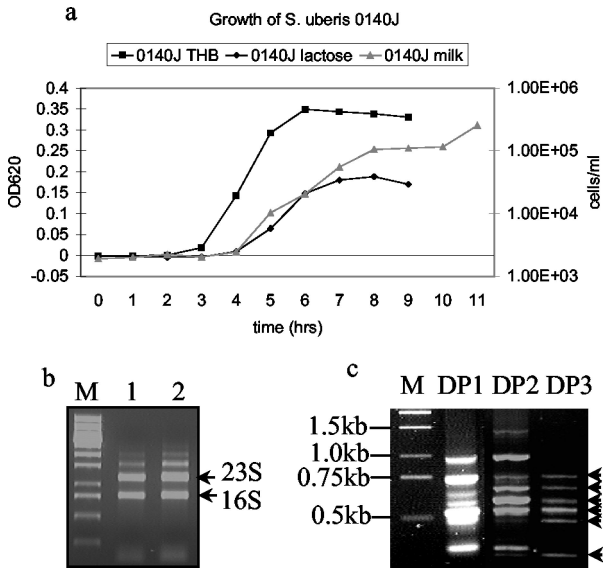

FIG. 1. (a) Growth of 0140J in TH broth, milk, and tryptic soy broth lactose. Growth in milk plotted on left *y* axis. (b) RNA preparation from 0140J. Lanes: M, GeneRuler 1-kb ladder (MBI), 1; 0140J grown in TH broth, and 2; 0140J grown in milk. (c) Analysis of difference products produced by cDNA RDA. Arrows indicate bands from DP3 isolated and cloned.

of cDNA RDA were performed (47). Additional representations, derived from *Dpn*II-restricted *S. uberis* 16S and 23S rRNA genes, were used to supplement the normal total RNA-derived driver component in a final ratio of 1:1:2. This measure effectively increases the selection against amplification of the high-abundance rRNA-derived sequences likely to be present in the tester representations (5).

The amplicons resulting from three rounds of RDA (DP3) were finely resolved by electrophoresis and six well-defined bands of approximate sizes: 350, 470, 490, 520, 550, and 600 bp were generated (Fig. 1c). These were gel purified and were cloned into the cloning vector pCR4-TOPO (Invitrogen).

To confirm that these products were the result of genuine differences in gene expression, Southern blot analysis was performed against the original driver- and tester-derived representations, using the cloned DP3 amplicons as probes. A number of independent clones were taken from each ligation, as it has been reported previously that each band may contain heterogeneous amplicons of similar size (5). Of 26 Southern blots performed, 11 revealed that the amplicon hybridized exclusively in the tester representation (data not shown), suggesting that this amplicon was derived from a transcript expressed or up-regulated during growth in milk but not when grown in rich media. These 11 amplicons (designated pDub*x*, for "differences in uberis") were taken for sequence analysis and yielded six discrete sequences. These sequences were used in tBlastX searches of the GenBank database (3) and revealed homologies with other streptococcal sequences.

Further analysis of differentially expressed determinants. Of the sequences isolated during this analysis, five showed significant homology to genes encoding known streptococcal metabolic proteins, some of which are involved in lactose metabolism (Table 2). Amplicons pDub7, -9, and -19 contained sequences that encode peptides with homology to the 6-phospho-β-galactosidase (*lacG*) of *S. pneumoniae* and tagatose 6-phosphate kinase (*lacC*) of *Streptococcus pyogenes*. These are glucose-repressible members of the *lacABCDFEGX* operon that regulates lactose metabolism and are likeliest to be differentially expressed in milk in response to utilization of lactose as the major carbon and energy source. pDub15 contained an orthologue with homology to the *S. pyogenes* putative formylglycinamidine ribonucleotide (FGAM) synthetase II gene (*purL*). *purL* catalyzes the conversion of formylglycinamide ribonucleotide, glutamine, and MgATP to FGAM, glutamate, ADP, and phosphate during the biosynthesis of purine molecules. pDub22 encoded an orthologue of the putative mannose-specific phosphotransferase system component IIC (*manM*) of *Streptococcus salivarius.* The orthologue is also glucose repressible, being involved in the transport of other sugars across the cell membrane during conditions of glucose limitation. The final metabolic gene isolated, pDub23, showed significant homology to the argininosuccinate lyase gene (*argH*) of *S. pneumoniae*, which is involved in the biosynthesis of the amino acid arginine.

Identification of a putative membrane-associated protein, OppA2. The two remaining sequences were found to be nonoverlapping, although each encoded 600- and 490-bp fragments of the same open reading frame. tBlastX searches against the GenBank database (3) revealed that the peptide sequence encoded by the 600-bp fragment clones (pDub1, -2, and -3) and the 490-bp fragment clones (pDub15 and -14) showed 62% identity (71% similarity) to a *Streptococcus equisimilis* hyaluronate-associated protein (HAP) (7, 24). This protein in turn had homology with family 5 bacterial extracellular oligopeptide binding proteins (45). Together, the 600- and 490-bp fragments isolated during cDNA RDA represented approximately two-thirds of the predicted open reading frame

TABLE 2. Differentially expressed determinants isolated during cDNA RDA

Clone	Orthologous protein	Gene	Putative function	Closest homologue
pDub1, -2, and -3	HAP?	*oppA2*	Capsule synthesis/release?	HAP *S. equi* AF100456
pDub7 and -9	6-Phospho-β-galactosidase	*lacG*	Lactose metabolism	*lacG S. pneumoniae* NC_003098
pDub13 and -14	HAP	*oppA2*	Capsule synthesis/release?	HAP *S. equi* AF100456
pDub15	Putative phosphoribosylformyl-glycinamidine synthase II	*purL*	De novo purine biosynthesis	*purL S. pyogenes* NC_004070
pDub19	Tagatose 6-phosphate kinase	*lacC*	Lactose metabolism	*lacC S. pyogenes* NC_004070
pDub22	Putative mannose-specific phosphotransferase system component IIC	*manM*	Phosphoenolpyruvate:mannose phosphotransferase system	*manM S. salivarius* AF130465
pDub23	Argininosuccinate lyase	*argH*	Arginine biosynthesis	*argH S. pneumoniae* NC_003098

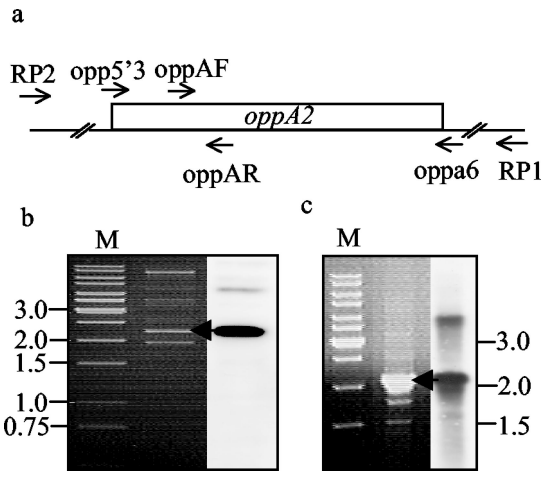

FIG. 2. Cloning the full-length *oppA2* gene. (a) Schematic of how 5′ and 3′ regions of the *oppA2* gene were cloned. Gene-specific primers SP1 and SP2 (HAP F and HAP R) were used in concert with primers directed against pneumococcal sequences RP1 or RP2 and were used in a PCR at low annealing temperature to encourage mispriming of the pneumococcal primers. (b) Cloning the 3′ *oppA2* region. Primer RP2 misprimed close to the *oppA2* gene and generated a product that was detected by Southern blotting by using pDub3 insert as a probe (lane M, GeneRuler 1-kb ladder; center lane, ethidium bromide-stained agarose gel of PCR products; and rightmost lane, Southern blot of PCR products). (c) Cloning the 5′ sequence of *oppA2* (lane M, GeneRuler 1-kb ladder; center lane, ethidium bromide-stained agarose gel of PCR products; and rightmost lane, Southern blot of PCR products probed with pDub3). Arrowheads indicate DNA bands taken for cloning. Molecular mass of GeneRuler 1-kb ladder (MBI) shown in kilobases.

based on comparisons with orthologous and emerging genome sequence data.

Cloning the full-length transcript of *oppA2*. Using a specific primer, oppAF, and a selection of nonspecific 21-bp primers directed to pneumococcal sequences, we amplified the 3′ region encoding the C terminus by low-stringency PCR (Fig. 2a) at an annealing temperature of 45°C. Southern blot hybridization of these products identified a 2-kb DNA fragment that hybridized to the pDub3 probe (Fig. 2b). Cloning and sequencing of this fragment provided an additional 1.1 kb of sequence, including the termination codon. To amplify the missing 5′, N-terminal encoding sequence, a degenerate primer, oppA5′3, was designed based on the *S. equisimilis* HAP gene, immediately upstream of and encompassing the initiation codon. This was used in a low-stringency PCR with a specific primer, oppA4, followed by Southern blot hybridization and cloning and sequencing of positive fragments (Fig. 2c). This yielded a clone containing the missing N-terminal sequence, including the initiation codon. An additional primer, oppA6, was then designed upstream of the ATG and was used in a PCR with primer oppA4 and a proofreading DNA polymerase, *Pfu* Turbo (Stratagene), to amplify the full-length gene product, *oppA2*.

The 1,650-nucleotide open reading frame (accession no. AY256913) encodes a 549-amino-acid protein with a predicted mass of 60.4 kDa. A signal peptide was identified in the N terminus in addition to a bacterial lipoprotein motif (LXXC) and an extracellular peptide and nickel binding protein family signature sequence($\underline{A}X_7\underline{D}X_4TX_3\underline{R}X_3\underline{K}$) (45) (Fig. 3). These features indicate the probable covalent attachment of this protein to the external side of the bacterial membrane, and its similarity to other family 5 proteins (OppA/AmiA like proteins) suggests that it may be an oligopeptide binding protein (OppA), most likely involved in transport of oligopeptides across the bacterial membrane.

The expression of *oppA2* is up-regulated during growth in milk. To directly analyze the induction of the putative *oppA* transcript during growth in milk, a Q-RT-PCR (real-time) assay was designed by using SYBR green detection methodology and was analyzed on an MX4000 Q-PCR system (Stratagene). Q-RT-PCR allows analysis of accumulating PCR products during the logarithmic phase of the amplification. The point at which a fluorescent signal was detected, or cycle threshold (Ct), allows the calculation of the quantity of template DNA in each sample.

Having established that the expression of *glnA* (glutamine synthase) is invariant during growth in milk (Fig. 4a and b), we were able to use this gene as a reference gene to determine the relative amounts of the putative *oppA2* transcripts present in milk-grown cultures compared to the amounts present in TH broth-grown cultures. Quantitation of gene amplification from three separate populations of milk-grown cultures was made following Q-RT-PCR by determining the threshold cycle (Ct) number for FAM fluorescence within the semilog plot generated during PCR. The relative quantity of *oppA2* gene expression was calculated by using the comparative Ct method. The ΔCt value was determined by subtracting the *glnA* Ct value for each sample from the *oppA2* Ct value of that sample. Changes (*n*-fold) in the relative gene expression of *oppA2* were then determined by evaluating the expression ΔΔCt (35). Analysis showed that the expression levels of *oppA2* in mid-log-phase cultures grown in sterile milk were significantly increased compared to those grown in rich media (Fig. 4c). This induction was not observed in cultures grown in tryptic soy broth with lactose as a sole carbon source (46 g/liter, equivalent to that of milk), suggesting that the differential regulation of the putative *oppA2* transcript occurs not in response to carbon source or osmolality but to an alternative factor in milk.

In addition to this sequence, a second *oppA*-like open reading frame identified by similarity searches in *S. uberis* is part of an *oppABCDF*-like operon. It has been found in some streptococcal species that multiple *oppA* genes are present in the genome as separate sequences, not coupled to a transporter operon (1, 13). As this second sequence is part of a clear operon, we have termed it *oppA1* and the sequence isolated by cDNA RDA *oppA2*. The mature proteins display 70% identity at the amino acid level.

Analysis of *opp* and *dpp* operon transcripts in GAS has shown that the binding protein transcript (*oppA*/*dppA*) is expressed at a higher level than the downstream transcripts of the operon (37, 38), as would be expected for the rate-limiting step of a transport process. This may be controlled by a putative Rho-independent transcriptional terminator located between OppA and OppB (38). To determine if this also occurs with the *S. uberis oppABCDF* operon, we designed a SYBR green Q-RT-PCR assay to analyze the induction of the *oppA1* and *oppF* transcripts during growth in milk. This analysis shows that *oppA1* is expressed at an approximately threefold-higher rate than is *oppF* during growth in milk (Fig. 4c), indicating

```
OppA2           -----MKNLKKYSSLGLLVISATTLVACGTKSASGNSESSDVINWYTPTEIITLDVSKNT 55
OppA1           MTVAQKSTFKRFGLGAVTLASAALLMACGNKTAAKN-DNKNEINWYTPTELITLDISKNT 59
                      ..:*::.  .: : **: *:***.*:*: * :..: ********:****:****

OppA2           DRYSAMAIGNSGSNLLRVGKNGKLQPDLATKVDISKDGKTYTATLRDGLKWSDGSDLTAD 115
OppA1           DTYSGLAIGNSESNLLRVDGKGKLQPDLAKKVEVSEDGLTYTATLRDGLKWSDGSELTAE 119
                * **.:***** ******. :******** .**::*:** ****************:***:

OppA2           DFVYTWQRMVDPKTASEYAYLTTDAHLLNADKIISGQETDLSKLGVKAEGNKVIFTLETP 175
OppA1           DFVYTWKRIVDPATASEYAYLATESHLLNAEDINTGKNTDLNSLGVKAEGNKVIFTLSAP 179
                ******:*:*** ********:*::*****:.* :*::***..**************.:*

OppA2           APQFESLLSFSNFVPQKKSFVEKEGKNYGTNSEKLLYSGPYKVSNWNGTSGSFKLVKNKY 235
OppA1           APQFKSLLSFANFMPQKEEFVTKTGKDYGTSSEKQIYSGPYIVKDWNGTSGSFKLVKNDK 239
                ****:*****:**:***:.** * **:***.*** :***** *.:**************.

OppA2           YWDAKNVKTKTVKVQAVKKPDTAVQMYKQGQLDFANISATSAIYNANKNNKDNVKIAEAR 295
OppA1           YWDAKNVKMKTINVQTVKKPDTAVQMYKQGKLDYANISGTSAIYNANKNNKDVVPVLEAT 299
                ******** **::**:***************:**:****.************* * : **

OppA2           ADYLVYNETGNVKALSNLKIRQALNLATDREALVKAAVDTGSKAAKTLVPYGLSTLSDGT 355
OppA1           TAYMVYNQTGSVPALSNLKIRQALNLATDRKGIVSAAVDTGSKPATAIAPTGLAKLKDGS 359
                : *:***:**.* *****************:.:*.********.*.:::.* **:.*.**:

OppA2           DLAEHTAQPYTYDKEKAAKLFKEGLAELGTDKLTLTLLADSDNPAKKAAVDYLKEAWEKN 415
OppA1           DLTKFVAPGYKYDEKEAGKLFKEGLAELGKDSLKITITADADAPAAKSSVDYIKETWEKA 419
                **:::.*  *.**:::*.***********.*.*.:*: **:* ** *::***:**:***

OppA2           LPGFTLEEKFVTFKQRLKDTSEQNFDIAFVSWGGDYPEGSTFYGLFTSTSDYNYGKINSP 475
OppA1           LPGLTVEEKFVPFKQRLEDTKNQNFEVAVALWGGDYPEGSTFYGLFKSGSAYNYGKFTNA 479
                ***:*:*****.*****:**.:***::*.. ***************.* * *****:...

OppA2           EYDAAYQKALTTDALDKDAAADDYKDAEKALYEGAHYSPLYNLSNEGLQNPKIKGLVRNS 535
OppA1           KFDAAYEKALTTDALDTDAAANDYKEAEKALYDEANYNPLYFRSGEGLQNPSIKGLIRNS 539
                ::****:*********.****:***:******: *:*.***  *.******.****:***

OppA2           TGLDVDFTHAYKTK 549
OppA1           TGLNVDFTYAYKK- 552
                ***:****:***.
```

FIG. 3. ClustalW alignment of the *S. uberis* OppA2 and OppA1 proteins. Putative lipoprotein motif (LXXC) and extracellular peptide and nickel binding protein family signal sequence have been highlighted.

that a similar transcriptional termination process could be functioning in *S. uberis*. The *opp* operon has a 74-bp noncoding region between *oppA1* and *oppB* that could also function as a stem-loop transcriptional terminator.

In comparison to *oppA2*, the expression of *oppA1* is not significantly increased during growth in milk, suggesting that the products of these transcripts may have different substrates or have different regulation and therefore different roles. This would also account for our failure to detect *oppA1* during our initial cDNA RDA screen. *dpp* genes have also failed to be identified in *S. uberis* by Southern blot analysis with a GAS *dpp* probe (A. Smith, unpublished data) and by emerging genome analysis, suggesting that *S. uberis* does not possess the genes for these proteins.

OppA1 and OppA2 are components of an oligopeptide transport mechanism. *S. uberis* is a genetically intractable organism, and routine molecular techniques such as directed gene deletions are unavailable at present for use in this microbe. To isolate mutants in these genes, a bank of approximately 8,800 random insertion mutants (49) was screened by PCR. Mutants in *oppA2* (DLT003), *oppA1* (DLT004), and *oppB* (DLT005) (to prevent *oppBCDF* translation) were isolated, confirmed as single insertions by Southern blotting (data not shown), and taken for characterization.

An increased resistance to the toxic peptide derivative aminopterin has previously been used to confirm a role in peptide transport of OppA proteins from other species (17, 38). Any increase in resistance correlates with a reduction in peptide import. All three *S. uberis opp* mutants also displayed an increased resistance to aminopterin (Fig. 5a) compared to the parental strain, confirming their role in peptide transport in this organism. The *oppA1* and *oppB* mutants appeared to have a slightly higher resistance to aminopterin than did the *oppA2* mutant, suggesting that *oppA2* is less important for peptide transport, despite its expression in milk being more significantly up-regulated.

The individual *opp* genes were not required for growth of *S. uberis* in milk; however, interruption of either the *oppA1* or *oppB* gene resulted in a elongated lag phase before the culture entered the exponential phase of growth, suggesting that OppA1 may be the more significant peptide transporter, as interruption of *oppA2* had no discernible difference in growth

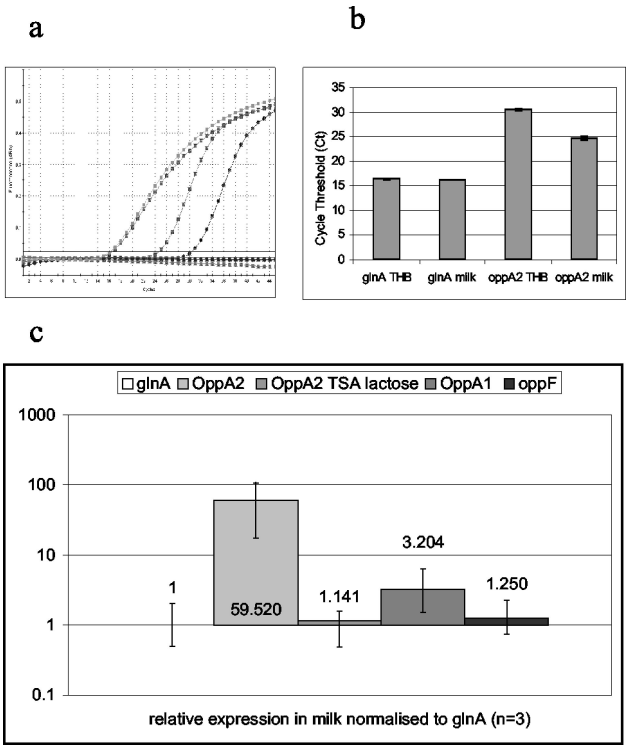

FIG. 4. Expression of *oppA2* is up-regulated in milk. (a) Example of raw Q-RT-PCR data showing amplification curves of *glnA* and *oppA2* in TH broth and milk from 500 ng of total RNA. The *glnA* curves in TH broth and milk have the same Ct, demonstrating its invariant expression under these conditions. (b) Bar chart of Ct data from panel a. (c) Relative induction (*n*-fold) of *oppA2*, *oppA1*, and *oppF* transcripts in milk or tryptic soy broth lactose ($n = 3$) by Q-RT-PCR. All expression is normalized to the invariant reference gene *glnA*.

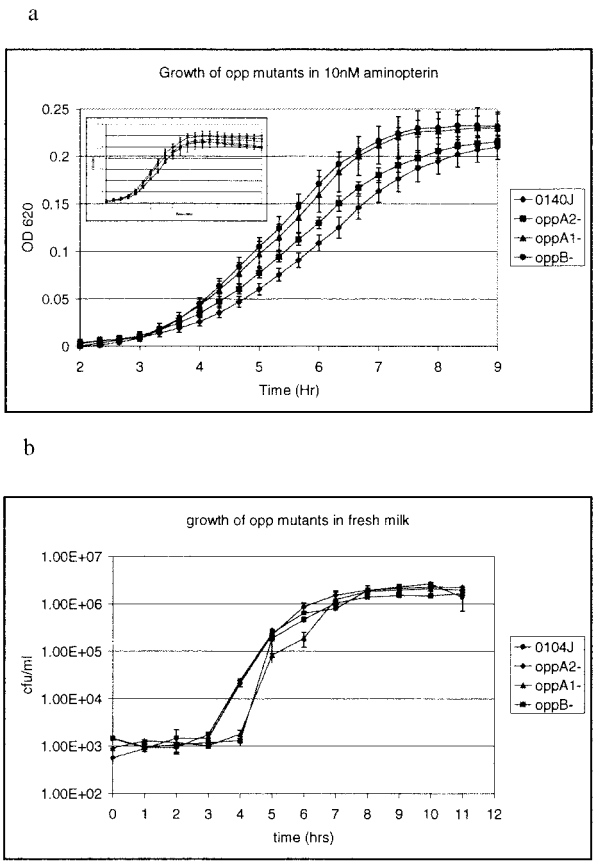

FIG. 5. (a) Growth of *opp* mutants in the presence of 10 nM aminopterin. Inset figure shows growth of mutants in TH broth. (b) Growth of *opp* mutants in fresh raw milk.

kinetics from those of the wild type, presumably being able to import peptides by using the OppABCDF unit (Fig. 5b).

OppA1 is the primary oligopeptide transporter in *S. uberis*. To determine if OppA1 and OppA2 bind peptides of different lengths, we analyzed the ability of the mutant strains to utilize the essential amino acids glutamic acid and valine from small peptides. The *opp* mutants and the parental strain 0140J were grown in CDM (25) deprived of valine and/or glutamic acid in the free form and these amino acids supplemented in small peptide form (Table 3). In all cases the loss of *oppA2* did not alter the phenotype, indicative that OppA1 can utilize peptides of 3 to 8 amino acids in length, whereas the disruption of *oppA1* or *oppB* resulted in a complete lack of growth in CDM-Glu-Val supplemented with the small peptides WE+EW+EV, EVF+VYV, EHIWLMVR, VKEAMAPK, LMHVRIEWC, and LMHVRIEWCY. However, it was able to utilize glutamic acid from the hexapeptide EAMAPK, suggesting that OppA2 can transport hexapeptides perhaps by using an alternative transport machinery. The parental strain failed to utilize Glu or Val from di-, nona-, and decapeptides, suggesting that perhaps *S. uberis* Opp proteins cannot utilize peptides of this length. However, previous evidence suggests that the length composition and arrangement of amino acids in a peptide could be important factors for the acquisition of essential amino acids from peptides by *S. uberis* (43a) and that this may need to be investigated in more detail.

***oppA1* and *oppA2* are regulated by different mechanisms.** Previous evidence has suggested that peptide binding proteins may be directly or indirectly regulated by the positive gene regulator *mga* (multigene activator in GAS). Previously known as *mry* or *virR* (6, 29), *mga* functions as a DNA binding protein that interacts with promoter regions and has been shown to

TABLE 3. Peptide utilization of *opp* mutants

CDM supplement	Utilization by:			
	0140J	OppA2$^-$	OppA1$^-$	OppB$^-$
All amino acids	+	+	+	+
Minus E	−	−	−	−
Minus EV	−	−	−	−
WE, **E**W, **EV**[a]	−	−	−	−
EVF, **VY**V	+[c]	+[c]	−	−
EAMAPK	+[b]	+[b]	+[b]	+[b]
EHIWLMVR	+[b]	+[c]	−	−
VKEAMAPK				
LMH**V**RIE**W**C	−	−	−	−
LMH**V**RIE**W**CY	−	−	−	−

[a] Boldface indicates amino acids being tested for utilization from the given peptide or mix of peptides.
[b] All strains efficiently utilized glutamic acid from EAMAPK.
[c] Wild-type 0140J and the *oppA2* mutant efficiently utilized glutamic acid and valine from the peptide mixture. OppA1 and OppB mutants were unable to grow under these conditions.

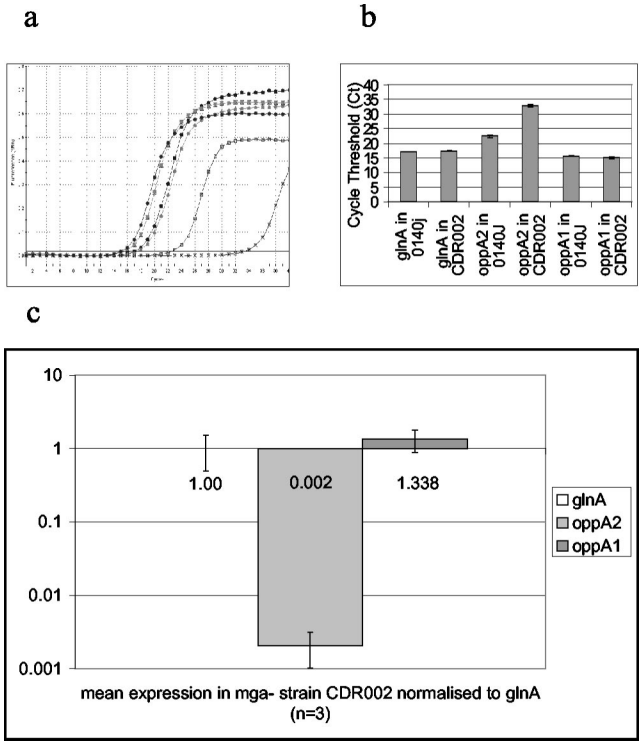

FIG. 6. Expression of *oppA2* is regulated by an *mga*-like protein. (a) Example of raw Q-RT-PCR data showing amplification curves of *glnA, oppA2*, and *oppA1* in 0140J and the *mga* mutant CDR002 from 500 ng of total RNA. The *glnA* curves in each strain have the same Ct, demonstrating its invariant expression under these conditions. (b) Bar chart of Ct data from panel a. (c) Expression of *oppA1* and *oppA2* in TH broth in CDR002 normalized to the invariant reference gene *glnA*.

directly regulate a number of known virulence determinants in streptococci, including M protein, C5a peptidase, and serum opacity factor (6, 36, 42). By using an *S. uberis* mutant in which an *mga*-like gene has been inactivated by insertion of pGhost9::IS*S1* at the start codon (C. D. Rapier, unpublished data), we analyzed the expression levels of *oppA1* and *oppA2* in TH broth compared to that of the wild-type parental strain 0140J by Q-RT-PCR. Again using *glnA* as an invariant reference gene (Fig. 6a and b), we found a significant decrease (approximately 500-fold) in the levels of *oppA2* in the absence of *mga*; however, in marked contrast to this the levels of *oppA1* were unchanged (Fig. 6c). This suggests that the two OppA proteins have alternative mechanisms of regulation, one being clearly controlled by a regulator of known virulence determinants in other streptococci. Given the different regulatory mechanisms and the questionable significance of Opp2 to peptide transport, it appears that the functional role of OppA2 remains to be fully elucidated. *oppA1* appears not to be regulated to any great degree under the conditions used here, and it is possible that it is constitutively expressed or regulated by an alternative mechanism.

DISCUSSION

The active transport of solutes across membranes is performed by transporters driven by ATP hydrolysis. Termed ATP-binding cassette-type (ABC-type) transporters, traffic ATPases, or permeases, these multicomponent systems share a common structural and genetic organization and a common mechanism of action. These systems can be involved in the uptake of a wide range of substrates, including sugars, peptides, metallic cations, and vitamins (45). In this study we have used RDA of cDNA to identify a number of genes, some glucose repressible, that are up-regulated during growth in milk. The basis for observing possible differential expression of these transcripts during such growth is that the precursors required for the biosynthesis of these moieties are likely to be limited in this medium. Although these transcripts are not classical virulence determinants, they almost certainly contribute to the overall pathogenicity of *S. uberis* during infection of the bovine udder, as de novo synthesis of many biological precursors must be initiated for survival in limiting nutrient conditions. Of principal interest was a transcript that encodes a putative oligopeptide binding protein that is greatly up-regulated during growth in milk, although this was not found to be part of a peptide transport operon.

The role of Opp proteins in nitrogen acquisition in bacteria has been considerably characterized, and they are essential for peptide acquisition for many of these organisms. *S. uberis* is auxotrophic for 8 to 10 amino acids, and during growth in milk the primary source of these essential nutrients is likely to be from hydrolyzed casein peptides present in the medium (21).

Other streptococci are known to possess additional *oppA*-like genes that are found at discrete loci under the control of their own promoters (2, 13). *Lactobacillus delbrueckii* subsp. *bulgaricus* has a second *oppA2* gene located downstream of the *oppABCDF* operon and is expressed as a separate transcript under the control of its own promoter (34). Unlike the *L. delbrueckii* subsp. *bulgaricus oppA1*, its *oppA2* is unable to complement an *L. lactis oppA* mutant, suggesting it does not have a role in peptide transport despite its similarity to *oppA1* (34). During preparation of this report, advances in the assembly of the *S. uberis* genome (http://ww.sanger.ac.uk/Projects/S_uberis/blast_server.shtml) have allowed us to confirm that *oppA2* is also located as a lone gene in the genome, although its proximity to the *oppABCDF* operon remains to be determined.

The marked variation in expression level of the two *S. uberis oppA* genes during growth in milk suggests that they have different functions or substrate specificities, although the absence of the OppA2 protein did not have any observable effect on growth in milk or on valine and glutamic acid acquisition from small peptides. In contrast to this, the disruption of *oppA* resulted in an initial delay in growth in milk and a failure to acquire these amino acids from most peptides, suggesting that it has a primary role in nutrient acquisition for *S. uberis*. In the absence of OppA2, the OppABCDF unit appears competent to transport all the amino acids required for growth. In the absence of OppABCDF, it is possible that OppA2 can still function to bind hexapeptides and uses the membrane and ATPase machinery from an alternative transporter to carry them across the membrane. This scenario could account for the delay in the growth of the *oppA* and *oppB* mutants in milk. tBlastX screens of the *S. uberis* genome database (3) using the GAS *dpp* genes initially indicate the absence of dipeptide and tripeptide transporter genes, although the presence of *abp*, a putative polar amino acid and opine binding ABC transport system, may be sufficient for importing single amino acids (18).

Analysis of aminopterin resistance in the *opp* mutants revealed a possible partial phenotype for the *oppA2* mutant, as there was an increase in resistance to the toxic peptide derivative in this background, suggesting some involvement of *oppA2* in peptide uptake. However, the degree of resistance conferred was less than that for either the *oppA1* or *oppB* mutant. This probably reflects the OppABCDF transporter continuing to actively transport peptides, although to a lower level than in the presence of both proteins. Overall, the data presented here suggest that OppA2 plays a relatively minor role in amino acid acquisition in *S. uberis*.

So what exactly is the function of OppA2 and why is its expression increased to such a high level in milk if not required for peptide uptake? The differential regulation by an Mga-like protein suggests that OppA2 may play a role in virulence in some manner. It is possible that OppA2 binds small peptides involved in quorum sensing or some other cell-cell signaling mechanism. It is conceivable that OppA2 is part of an environmental sensing mechanism that determines entry into the host by binding host-specific factors. Recent evidence has shown that in *Bacillus* Opp is involved in the uptake of a small cell-cell signaling peptide that activates transcription of a virulence regulon (43). In addition, OppA2 has 29 and 30% identity with the *Enterococcus faecalis* pheromone binding proteins PrgZ and TraC, respectively. PrgZ and TraC bind small signaling peptides with high specificity and then possibly recruit the OppBCDF components of the *opp* operon to transport the molecules into the cell (26, 32). Given the homology between these proteins, it is also feasible that Opp-like proteins may function in similar environmental sensing pathways in *S. uberis*. Further characterization on the peptide binding preferences of purified OppA1 and OppA2 proteins is needed to determine if *S. uberis* OppA2 does indeed possess a similar role.

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