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Three Oligopeptide-binding Proteins Are Involved in the Oligopeptide Transport of *Streptococcus thermophilus**

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The functions necessary for bacterial growth strongly depend on the features of the bacteria and the components of the growth media. Our objective was to identify the functions essential to the optimum growth of *Streptococcus thermophilus* in milk. Using random insertional mutagenesis on a *S. thermophilus* strain chosen for its ability to grow rapidly in milk, we obtained several mutants incapable of rapid growth in milk. We isolated and characterized one of these mutants in which an *amiA1* gene encoding an oligopeptide-binding protein (OBP) was interrupted. This gene was a part of an operon containing all the components of an ATP binding cassette transporter. Three highly homologous *amiA* genes encoding OBPs work with the same components of the ATP transport system. Their simultaneous inactivation led to a drastic diminution in the growth rate in milk and the absence of growth in chemically defined medium containing peptides as the nitrogen source. We constructed single and multiple negative mutants for *AmiA*s and cell wall proteinase (PrtS), the only proteinase capable of hydrolyzing casein oligopeptides outside the cell. Growth experiments in chemically defined medium containing peptides indicated that *AmiA1*, *AmiA2*, and *AmiA3* exhibited overlapping substrate specificities, and that the whole system allows the transport of peptides containing from 3 to 23 residues.

Oligopeptide transport systems are key channels between the environment and the inner part of micro-organisms, which have been described in numerous Gram-negative and Gram-positive bacteria. They generally internalize peptides with an ATP-driving force and belong to the ABC transporter family (1). They are composed of oligopeptide-binding proteins (OBP),¹ which are periplasmic in Gram-negative bacteria and membrane-associated in Gram-positive bacteria; transmembrane proteins that form a channel for the passage of oligopeptides and inner membrane-associated ATPases, which provide the energy for transport.

The oligopeptide transport system of Gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) transports peptides up to hexapeptides. This size limit seems to be imposed by the outer membrane pores rather than by the trans-

porter itself (2). In Gram-positive bacteria, the size of the peptides transported is more variable. Peptides from 2 to 7 residues and of 6–7 residues are transported by the oligopeptide transport system of *Streptococcus pneumoniae* (3) and *Streptococcus gordonii* (4), respectively. In *Lactococcus lactis* and *Listeria monocytogenes*, the oligopeptide transport system is capable of internalizing peptides composed of 4–18 amino acids (5) and of 5–8 amino acids (6). Thirty years ago, Desmazeaud and Hermier (7) demonstrated that peptides having a mass included between 1000 and 2500 and containing lysine or arginine have a stimulatory effect on the growth of *S. thermophilus*, whereas larger peptides (masses of 5000) inhibit it. Mixtures of amino acids contained in stimulatory peptides have the same stimulant effect on growth, which demonstrates that stimulatory peptides act as amino acid source. The requirements of *S. thermophilus* for peptides with specific length were explained by the likely presence of a peptide transport system different from that described in *E. coli*.

The most obvious role of these systems is to supply bacteria with essential amino acids in a low energy fashion. In the case of lactic acid bacteria, which are auxotrophic for several amino acids (8–10) and which are used to growing in milk, a medium containing a low level of free amino acids (11), the oligopeptide transport system is fundamental to optimal growth. In addition to two di-tripeptide transport systems in *L. lactis* (a proton motive force-driven di-tripeptide carrier (DtpT) (12–14), and an ATP-driven di-tripeptide transporter (DtpP) (15, 16), an oligopeptide transport system (Opp) (13, 17), which internalizes oligopeptides released from caseins by the action of cell wall proteinase, allows *L. lactis* to grow in milk.

In the present work, we have identified and characterized the oligopeptide transport system of *S. thermophilus*. We demonstrate that it works with three functional oligopeptide-binding proteins, that it is capable of transporting entities as large as 23 amino acid peptides, and that it plays a major role in nutrition.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Culture Conditions—The strains used in this work were described in Table I. All *E. coli* strains were grown on Luria-Bertani medium (18) at 37 °C with shaking and in the presence of erythromycin (Em, 150 µg/ml) when required. Three media were used for *S. thermophilus* cultures. The first medium was reconstituted, low heat skim milk (10% w/v) (Nilac, Nederlands Instituut von Zuivelonderzoek, Ede, The Netherlands), autoclaved at 110 °C for 12 min, buffered with 0.75 mM sodium glycerophosphate and, in some cases, containing bactotryptone at 3 g/liter (pancreatic digest of casein; Difco Laboratories, Detroit, MI). Bacterial growth was monitored by measuring optical density (OD) at 480 nm after clarification of the milk by a 10-fold dilution in 2 g/L EDTA pH 12 (19). Two other media were used for general cultures and growth rate experiments. The first was M17Lac medium (20), in which bacterial growth was monitored by measuring the OD at 600 nm. The second was a chemically defined medium (CDM) containing nucleotides, vitamins, salts, potassium phosphate buffer

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¹ The abbreviations used are: OBP, oligopeptide-binding protein; Em, erythromycin; OD, optical density; CDM, chemically defined medium; ORF, open reading frame; IS, insertion sequence.

TABLE I
Strains and plasmids

Strains	
<i>E. coli</i>	
TIL206: TG1 supE <i>hsdΔ5 thiΔ (lac-proAB)F[traD 36- proAB⁺ lacI^qlacZΔM15] + repA</i>	Provided by P. Renault
TIL401: TIL206 containing pG ⁺ h9::ISS1	Provided by D. Obis
TIL287: TIL206 containing pG ⁺ h9	Provided by M. Nardi
<i>S. thermophilus</i>	
St18: wild type strain, PrtS ⁺ , plasmid-free	Provided by Rhodia-Food
St18 <i>amiA1</i> ⁻ : St18 strain <i>amiA1Δ</i> 861 bp <i>AvaII-AvaII</i>	This study
St18 <i>amiA2</i> ⁻ : St18 strain <i>amiA2Δ</i> 93 bp <i>PshAI-BstSNI</i>	This study
St18 <i>amiA3</i> ⁻ : St18 strain <i>amiA3Δ</i> 313 bp <i>MslI-StuI</i>	This study
St18 <i>prtS</i> ⁻ : St18 strain <i>prtSΔ</i> 2054 bp <i>HpaI-NruI</i>	This study
St18 <i>amiA1, prtS</i> ⁻ : St18 strain <i>amiA1Δ(AvaII-AvaII); prtSΔ(HpaI-NruI)</i>	This study
St18 <i>amiA2, prtS</i> ⁻ : St18 strain <i>amiA2Δ(PshAI-BstSNI); prtSΔ(HpaI-NruI)</i>	This study
St18 <i>amiA3, prtS</i> ⁻ : St18 strain <i>amiA3Δ(MslI-StuI); prtSΔ(HpaI-NruI)</i>	This study
St18 <i>amiA1, A2</i> ⁻ : St18 strain <i>amiA1Δ(AvaII-AvaII); amiA2Δ(PshAI-BstSNI)</i>	This study
St18 <i>amiA1, A2, A3</i> ⁻ : St18 strain <i>amiA1Δ(AvaII-AvaII); amiA2Δ(PshAI-BstSNI); amiA3ΔMslI-StuI</i>	This study
St18 <i>amiA1, A3, prtS</i> ⁻ : St18 strain <i>amiA1Δ(AvaII-AvaII) amiA3Δ (MslI-StuI); prtSΔ(HpaI-NruI)</i>	This study
IS mutant: St18 insertional mutant containing pG ⁺ h9::ISS1 in <i>amiA1</i> gene	This study
Plasmids	
pGEMt	Promega
TopoXL	Invitrogen
pG ⁺ h9	Ref. 27
pG ⁺ h9ISS1	Ref. 23

(0.05 mol·liter⁻¹, pH 6.7) and 0.5% lactose (w/v) as described by Letort and Juillard (21), and sterilized by filtration. The nitrogen source of the CDM was provided by amino acids, a mix of amino acids associated with a single peptide (Sigma) or α ₂-casein trypsin hydrolysate, sterilized by filtration. Peptides MK, MH, EA, ED, EPET, PQFY, DYM, DYMG, YGGFM, RPKPQQFFGLM, MKRPPGFSPFR, ACTH-(1–17) fragment (SYSMEMFRWKGKPVGKKR), ACTH-(1–24) fragment (SYSMEMFRWKGKPVGKKRRPVKQYYP), and oxidized B chain of insulin (FVNQHLCGSHLVEALYLVCGERGFFVTPKA) were used at rate of 100 μ mol·liter⁻¹ in the culture medium. Growth rate experiments were then performed at 37 °C using a Microbiology Reader Bioscreen C (Lab-systems, Helsinki, Finland) in 100-well, sterile, covered microplates. Each well contained 200 μ l of the culture medium. Overnight M17Lac cultures of *S. thermophilus* were washed twice and resuspended in a volume of sterile potassium phosphate buffer (0.05 mol·liter⁻¹, pH 6.7) equal to the culture volume. 4 μ l of the suspension were used to inoculate each well. The optical density was measured at 600 nm every 20 min, after gentle shaking. The apparent growth rate (μ_{max}) was defined as the maximum slope of a semi-logarithmic representation of growth curves, assessed by OD measurements.

DNA Manipulations and Sequencing—Plasmid DNA manipulations and transformations of *E. coli* were performed as described previously (18). RNA was prepared as previously described from *S. thermophilus* grown in M17Lac (18). The total DNA of integrants obtained by insertional mutagenesis (see below) were digested by *EcoRI* or *HindIII*, and then ligated. TIL206 electrocompetent cells were transformed with ligation products, and Em^R colonies were screened by PCR after 24-h incubation at 37 °C.

PCR amplifications were performed with the Gene Amp PCR system 2400 (PerkinElmer Life Sciences Inc.) using *Taq* polymerase (Appligene Oncor, Illkirch, France) and oligonucleotides from pG⁺h9::ISS1 sequences (5'-ACTACTGACAGCTTCCAAGGA-3' and 5'-ATAGTTCATTGATATATCCTC-3' for *EcoRI* digestion and 5'-GTAAACGACGGCCAGTG-3' and 5'-TATCTACTGAGATTAAGGTCT-3' for *HindIII* digestion). The Dye Terminator kit and a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) were used for DNA sequencing; each strand was sequenced twice on independent PCR products. DNA sequences were analyzed with Genetics Computer Group (GCG) sequence analysis software from the University of Wisconsin (22) and Mail Fasta (National Center for Biotechnology Information). Internal *amiA2* and *amiA3* fragments were amplified using degenerated oligonucleotides (5'-TTGTWTACWTCWGAWGGHGAAGA-3'; 5'-ACTATCWRTYAACCAWGCTTG-3') corresponding to conserved sequences of streptococcal oligopeptide-binding proteins (Refs. 3 and 4; this work). Annealing was performed at 54 °C. Additional reverse PCRs were performed to amplify fragments flanking the known parts of *amiA1/amiA2/amiA3* genes. The total DNA of the St18 strain was completely digested by *HindIII*, *EcoRI*, *TaqI*, *HaeII*, *HhaI*, *PstI*, or *NsiI*, ligated in a dilute form (1 μ g/ml), and amplified by PCR.

Southern and Northern hybridizations were performed using a Positive nylon membrane for transfer (Appligene Oncor, Illkirch, France) according to the instructions in the ECL detection system (Amersham

Biosciences, Inc., Buckinghamshire, United Kingdom).

***S. thermophilus* Insertional Mutagenesis, Construction of Oligopeptide-binding Protein, and Protease Mutants**—Insertional mutagenesis with pG⁺h9::ISS1 in *S. thermophilus* St18 had previously been adapted from the method described by Maguin *et al.* (23, 24). Integrants affected for their growth in milk were selected on Fast Strain Differencing Agar medium (25).

The genes encoding oligopeptide-binding proteins *amiA1*, *amiA2*, *amiA3*, and the gene encoding cell wall proteinase *prtS* (26) were inactivated in the St18 strain using the pG⁺h9 gene replacement system. The mutants obtained are listed in Table I. Deletions were made in the middle of target genes amplified from the DNA St18 strain, as follows. PCR fragments of *amiA* genes were cloned into the pGEMt easy vector (Promega) according to the manufacturer's instructions. *amiA2* deletion was obtained by double digestions with *PshAI* and *BstSNI* followed by a ligation step. The partially deleted *amiA2* gene fragment was then cloned into pG⁺h9. Fragments of *amiA1* and *amiA3* genes were cloned in pG⁺h9, digested by *AvaII* for *amiA1* and *Bsp120I* and *BstXI* for *amiA3*, and ligated to obtain *amiA1* and *amiA3* deletions. For the *prtS* mutant, a PCR fragment gene was first cloned into the TopoXL vector (Invitrogen), according to the manufacturer's instructions. The *prtS* gene fragment was cloned in pG⁺h9, and a deletion was obtained by *HpaI* and *NruI* digestion followed by ligation. The procedures for *S. thermophilus* electroporation, pG⁺h9 integration, and excision were similar to those used for insertional mutagenesis, as described previously (24, 27).

Experimental Growth in the Presence of a Toxic Peptide—The ability of a toxic peptide analog (aminopterin) to inhibit bacterial growth on M17Lac plates was quantified by determining the extent of the inhibitory zone surrounding a filter paper disc saturated with 30 μ g of aminopterin (Sigma).

Mass Spectrometry Analysis—Cells were grown in CDM with α ₂-casein trypsin hydrolysate as the nitrogen source containing more than 30 different peptides (28). Cells were cultured for 13 h and then centrifuged (5 min, 5000 \times g). Culture supernatants were concentrated and desalted with ZipTip (Millipore). Mass spectra were recorded in the positive-ion reflectron mode on a Voyager DE-STR mass spectrometer (Perspective Biosystems, Framingham, MA). All experiments were performed using a 20-kV acceleration voltage, a 337-nm laser, and 100-ns delayed extraction. The matrix solution was prepared freshly by dissolving 10 mg of α -cyano-4-hydroxycinnamic acid (Sigma) in 70/30 acetonitrile/trifluoroacetic acid, 0.3%. 0.5 μ l of the sample was mixed with 0.5 μ l of the matrix solution, spotted on a stainless steel sample plate, and air-dried.

RESULTS

Characterization of One Mutant with Slower Growth in Milk—The insertional mutagenesis in *S. thermophilus* St18 produced 1.183×10^4 Em^R integrants. Based on their phenotype on Fast Strain Differencing Agar, we selected 75 of them. After Southern analysis of the digested chromosomal DNAs of

integrants growing slowly in milk, we selected 14 clones in which pG⁺h9::ISS1 was integrated at only one locus, distinct in each one of them. In 12 clones, pG⁺h9::ISS1 was tandemly integrated, exhibiting two hybridization bands using pG⁺h9 as a probe, whereas the 2 remaining clones contained only one copy of pG⁺h9::ISS1. The growth rate of one of the mutants, called the insertion sequence (IS) mutant throughout this paper, was significantly lower in milk (0.19 h⁻¹) than that of the wild type strain (0.79 h⁻¹). Rapid growth was restored by the addition of bactotryptone (growth rate of 0.75 h⁻¹ for the mutant and of 0.85 h⁻¹ for the wild type strain), suggesting that the affected function was related to nitrogen nutrition.

The sequence of the interrupted gene of the IS mutant was determined using oligonucleotides from pG⁺h9::ISS1. We obtained a 392-bp sequence for the IS mutant, which formed part of an ORF exhibiting homologies with fragments of genes encoding oligopeptide-binding proteins (OBPs) from *Streptococci*, *Bacilli*, and *L. monocytogenes* (Refs. 3, 4, 29, and 30; accession no. AF305387). By applying additional reverse PCRs, we obtained a single 7032-bp DNA fragment containing the entire ORF corresponding to the interrupted gene of the IS mutant, together with four additional ORFs displaying a high level of homology with *amiC*, *amiD*, *amiE*, and *amiF* from different streptococci. These five ORFs, called *amiA*, *amiC*, *amiD*, *amiE*, and *amiF*, constituted the five proteins of ATP-binding cassette transporters (31); by homology, we named the *S. thermophilus* genes *amiA1*, *amiC*, *amiD*, *amiE*, and *amiF*. Protein sequences deduced from the entire DNA sequence exhibited the greatest homology with similar proteins from *S. pneumoniae* (ranging from 62% identity for AmiA1 to 86% identity for AmiE), *S. gordonii* (56% identity for AmiA1), and *S. pyogenes* (48% identity for AmiA1). Analysis of the sequence revealed the presence of a putative -10 extended promoter sequence situated 35 bp upstream of the ATG start codon of *amiA1*, and of a putative terminator situated 8 bp downstream of the stop codon of *amiF*.

Presence of Three Homologous Oligopeptide-binding Proteins in *S. thermophilus*—The Southern hybridization under non-stringent conditions of *Hind*III- and *Eco*RI-digested St18 strain DNA, using a 1400-bp fragment of *amiA1* as a probe, revealed two and three bands, respectively, suggesting the presence of at least two homologous genes (Fig. 1). Using PCR with degenerated oligonucleotides deduced from conserved regions of OBPs from streptococci and DNA from the IS mutant to avoid the amplification of an *amiA1* gene fragment, we obtained two 1400-bp PCR products corresponding to two fragments of genes, named *amiA2* and *amiA3*, homologous to each other and to *amiA1*. With additional PCRs, we obtained 2938- and 3089-bp sequences containing entire *amiA2* and *amiA3* genes, respectively. Comparisons of AmiA1, AmiA2, and AmiA3 protein-deduced sequences revealed a very strong identity between the three proteins (97.6% identity between AmiA1 and AmiA2, 87.1% identity between AmiA1 and AmiA3). *amiA1*, *amiA2*, and *amiA3* encode proteins with 655, 655, and 657 residues, respectively. Their primary sequences contain a putative membrane lipoprotein lipid attachment site (VLAACS) (32), an extracellular peptide and nickel-binding protein family signature sequence (A₇D₂TYIIRKGIKW) (1). These features indicate the probable covalent attachment of AmiA proteins to the bacterial membrane.

Analysis of the DNA sequences revealed the presence of putative -10 extended promoter sequences upstream of the *amiA2* and *amiA3* start codons, and of putative terminators downstream of the stop codons of the same genes. No open reading frames homologous to other genes encoding oligopeptide transport components were located either 590 and 1130 bp upstream or 500 and 400 bp downstream of the *amiA2* and

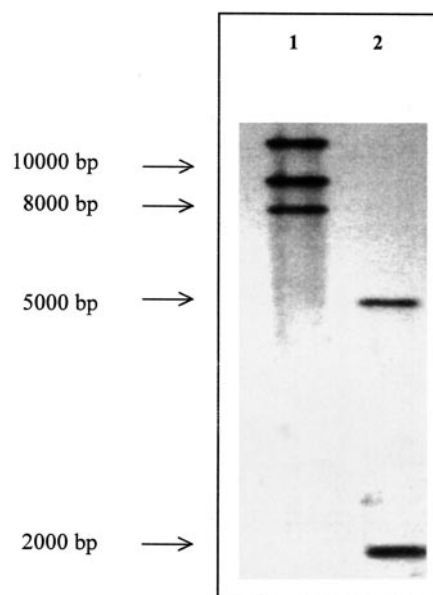


FIG. 1. Demonstration of the presence of additional oligopeptide-binding proteins by Southern hybridization of St18 strain DNA with an *amiA1* probe. Chromosomal DNA of St18 strain was digested by *Eco*RI or *Hind*III (lanes 1 and 2), with the *amiA1* gene being used as a probe. The SMART ladder marker (Eurogentec) was used as a size reference.

amiA3 genes, respectively. The *amiA3* promoter region differed from that of the two other *amiA* genes because of the presence of four potential -10 extended promoter sequences, including two inverted repeat sequences (Fig. 2).

Upstream of the three *amiAs*, we found part of insertion sequences or transposable elements (Fig. 3). A shuffled IS1193 (GenBank[®] accession no. STIS1193) was found upstream of the *amiA1* and the *amiA2* sequences. The environment upstream of *amiA3* differed from that of the other two *amiA* genes because of the presence of a *L. lactis* IS904 (33). Downstream of the *amiA2* and *amiA3* genes, we sequenced a part of *S. thermophilus* IS1193.

PCR screening of 21 industrial and three CNRZ collection *S. thermophilus* strains, using the same degenerated oligonucleotides as those used to search for *amiA2* and *amiA3*, demonstrated the presence of at least one copy of an *amiA* gene in all strains. Southern analysis of 12 *S. thermophilus* strains, using *Eco*RI-digested DNA and *amiA2* as a probe, highlighted the presence of several large hybridization bands (some larger than 6000 bp; data not shown). These results suggested that the presence of several *amiA* in *S. thermophilus* is a general characteristic of this species.

The Three Oligopeptide-binding Proteins Are Functional—The first prerequisite for oligopeptide-binding protein to be functional is expression of the corresponding genes. Northern blot analysis revealed the presence of a 7000-bp transcript hybridizing with a 1860-bp *amiA2* fragment (Fig. 4). This demonstrated that the potential promoter and terminator sequences identified upstream of *amiA1* and downstream of *amiF*, respectively, were functional, and that the *amiA1*, -C, -D, -E, and -F genes were organized into an operon. Northern blot analysis revealed another 2000-bp transcript hybridizing with the 1860-bp *amiA2* fragment, indicating that the potential promoter and terminator sequences identified upstream and downstream of *amiA2* and/or *amiA3* genes are functional. This result was confirmed by Northern blot analysis after RNA preparation of the IS mutant, which revealed the same 2000-bp transcript hybridizing with the same 1860-bp *amiA2* probe. As expected in this case, no 7000-bp transcript corresponding to an

FIG. 2. Sequence analysis of the promoter regions of the *amiA* genes. A, sequence comparison of the promoter regions of *amiA* genes. *pn*, potential -10 extended promoter sequences (in boxes); inverted repeats are indicated by arrows. Bold type indicates consensus sequences of -10 extended promoters, RBS sequences, and start codons. B, inverted repeat sequences identified upstream of the *amiA3* gene.

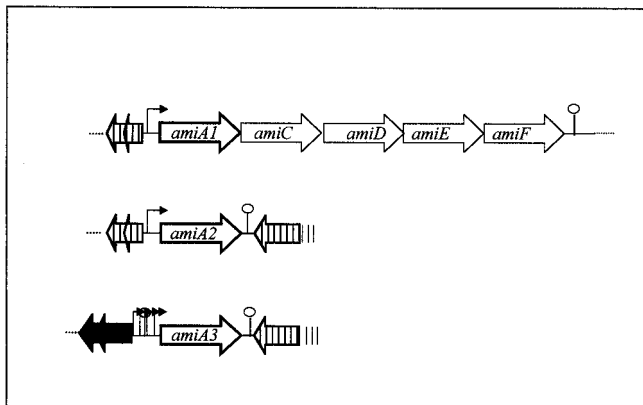
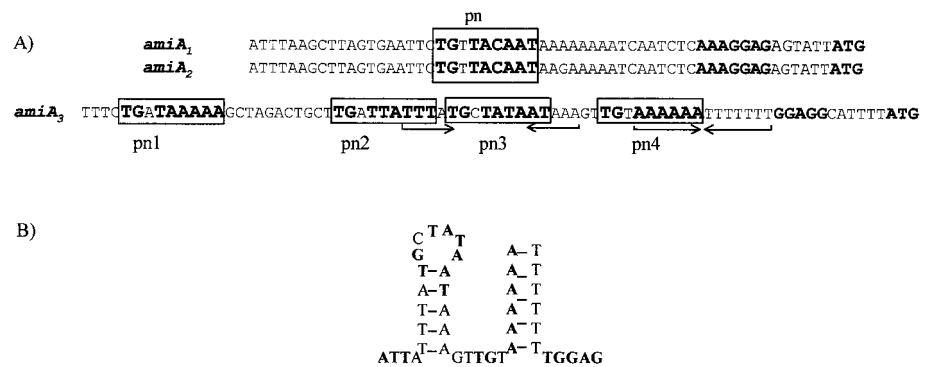


FIG. 3. Schematic representation of *amiA* genes and respective environment. Positions of potential promoters (\rightarrow) and terminators (\circ). \odot , shuffled IS1193; \square , IS1193; \blacktriangle , *L. lactis* IS904.

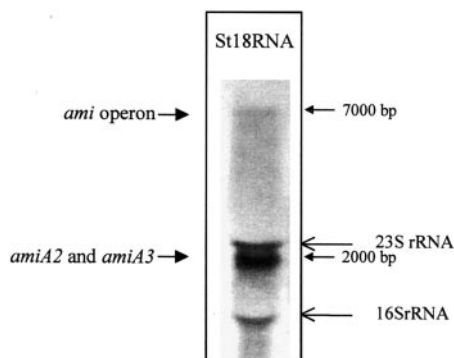


FIG. 4. Northern analysis of the *S. thermophilus* wild type strain. RNA was prepared from bacteria grown in M17Lac medium, and *amiA2* probe was used. RNA marker (0.2–10 kb; Sigma) was used as size reference.

ami operon was visible for the RNA preparation of the IS mutant (data not shown).

As a second stage, we constructed stable negative mutants for oligopeptide-binding proteins by gene replacement (23) and measured their growth rate in milk. Mutations in the three *AmiA*-encoding genes were achieved to obtain an *AmiA* triple negative mutant.

Growth of the *AmiA1*⁻ and *AmiA2*⁻ mutants was comparable with that of the wild type strain in milk, whereas that of the *AmiA3*⁻ mutant was significantly lower (Fig. 5). The *AmiA1/A2/A3*⁻ triple mutant exhibited very slow, limited growth in milk, similar to that seen for the IS mutant. This observation confirmed that there are probably no other oligopeptide-binding proteins working with the same system. In addition, we concluded that the mutation in the IS mutant affected the whole operon of oligopeptide transport. This result explained

why the *AmiA1* mutant and IS mutant exhibited different phenotypes. This was confirmed by the absence of an *ami* operon transcript on the Northern blot performed with the RNA of the IS mutant. The significant difference in the growth rates of the *AmiA3*⁻ and *AmiA1/A2/A3*⁻ mutants indicated that, in addition to *AmiA3*, at least one other *AmiA* was functional. At this stage in our work, we concluded that at least two of the three *AmiA* were functional and functioned with the same permease.

The St18 strain is endowed with a cell wall proteinase, PrtS, that degrades proteins and peptides in smaller peptides (26). We constructed each *AmiA*/PrtS double mutant as well as the *AmiA1/A3*/PrtS⁻ triple mutant to study the functionality of *AmiA* proteins independently of extracellular peptide degradation by the cell wall proteinase, PrtS. For technical reasons, we were unable to obtain the *AmiA1/A2/A3*/PrtS⁻ quadruple mutant. We used CDM containing a tryptic hydrolysate of α ₂-casein as the nitrogen source to compare the effects of *AmiA* mutations on *AmiA*/PrtS⁻ mutants. The growth of all *AmiA*/PrtS⁻ mutants was slower than that of the single mutant, PrtS⁻. More specifically, growth of the *AmiA1*/PrtS⁻ and *AmiA2*/PrtS⁻ mutants was half and one third less rapid, respectively, than that of the PrtS⁻ mutant, indicating that *AmiA1* and *AmiA2* are functional (data not shown). Based on growth experiments in milk and CDM, we concluded that the three *AmiA* oligopeptide-binding proteins were functional.

The Three Oligopeptide-binding Proteins Have Overlapping Substrate Specificities—The simplest way to measure peptide uptake is based on the ability of an auxotrophic strain to utilize peptides as an amino acid source when all the peptidases have an intracellular location, as is the case for *S. thermophilus* (34). Internalized peptides are then rapidly hydrolyzed by a battery of highly active intracellular peptidases. The rate-limiting step to peptide utilization in *Ami* mutants is their transport into the cytoplasm because the St18 strain has the same pool of peptidases as *AmiA* mutants of the ST18 strain.

We studied the specificities of *AmiA* proteins in two stages. First, they were compared by analyzing the external medium of each *AmiA*/PrtS mutant in CDM, in which nitrogen was supplied by a mixture of peptides. We grew PrtS and *AmiA* mutants in CDM with a tryptic hydrolysate of α ₂-casein as the nitrogen source. After growth, the culture supernatants were analyzed by mass spectrometry. The presence or absence of a peptide in the supernatant indicated complete or incomplete utilization of a peptide by a mutant. Analysis of the culture supernatants revealed differences in peptide composition. Several peptides were totally consumed by the PrtS⁻ mutant but not by *AmiA*/PrtS⁻ mutants. Their identification provided an indication of the specificities of OBPs. Most differences were found with *AmiA3*⁻ mutants where some peptides were still present in the medium after growth, although they had completely disappeared from the culture medium of other *AmiA* and PrtS mutants. Among the most demonstrative examples,

FIG. 5. Growth rate (μ_{\max}) of *S. thermophilus* wild type strain and *amiA* derivative mutants in milk.

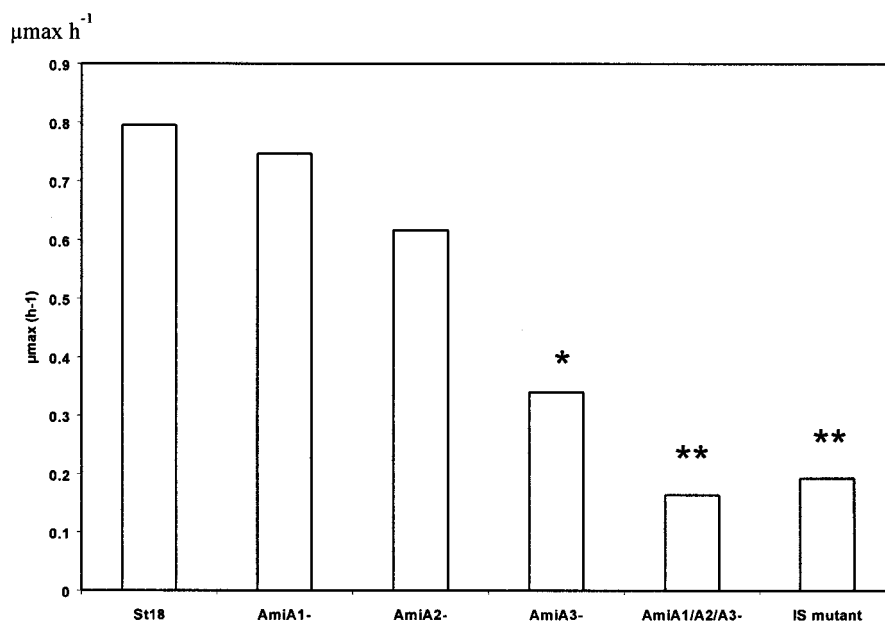


TABLE II

Presence or absence of peptides after the growth of *AmiA* and *PrtS* negative mutants in CDM with peptides from α_s -casein hydrolysate as the sole amino acid source

Peptide hydrophobicity values were calculated according to Kyte and Doolittle (45).

Peptide mass	Sequence and average hydrophobicity (in parentheses) of peptides	Presence in culture supernatant of				
		<i>PrtS</i> ⁻	<i>AmiA1/PrtS</i> ⁻	<i>AmiA2/PrtS</i> ⁻	<i>AmiA3/PrtS</i> ⁻	<i>AmiA1/A3/PrtS</i> ⁻
1098	AMPKPWIQPK (-1.15)	-	-	-	-	-
746	VIPYVR (0.14)	-	+	-	+	+
979	FALPQYLK (-0.22)	-	-	-	+	+
2709	FPQYLQYLYQGPIVLNPWDQVK (-0.51)	-	-	-	+	+
2865	FPQYLQYLYQGPIVLNPWDQVKR (-0.7)	-	-	-	+	+
1367	ALNEINQFYQK (-1.14)	+	+	+	+	+
1633	LTEEEKNRNFLK (-1.84)	+	+	+	+	+

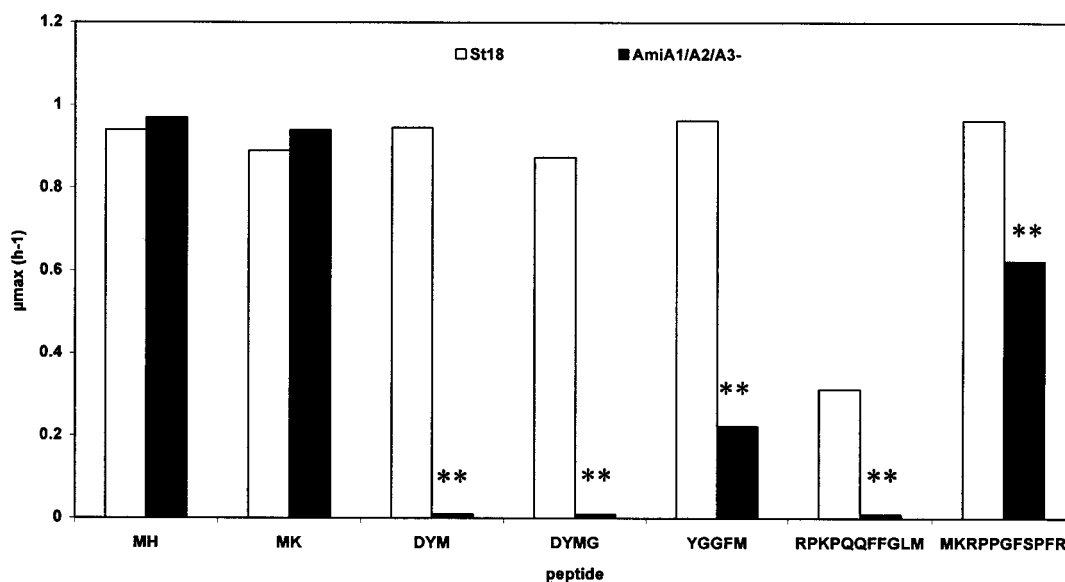


FIG. 6. Growth rate (μ_{\max}) of *S. thermophilus* wild type strain and *amiA* triple negative mutant in CDM containing a peptide as the methionine source.

presented in Table II, the 92–114 α_s -casein fragment (FPQYLQYLYQGPIVLNPWDQVKR) was still present in the culture supernatants of *AmiA3/PrtS*⁻ and *AmiA1/A3/PrtS*⁻ mutants, but not in that of the *PrtS*⁻ strain or *AmiA1/PrtS*⁻ and *AmiA2/PrtS*⁻ mutants. From these MS analyses, we therefore concluded that large peptides were used by the St18 strain

and that the *AmiA3* protein was capable of binding the peptides of at least 23 residues.

During a second stage, St18 wild type strain, *PrtS*, *AmiA1/PrtS*, and *AmiA1/AmiA2/AmiA3* negative mutants were cultured in CDM containing a single peptide as a source of methionine or glutamate (the St18 strain is auxotrophic for

TABLE III
Inhibition of *S. thermophilus* and *AmiA* negative mutants by aminopterin

The radius of *S. thermophilus* growth-inhibitory zone surrounding a filter paper disc saturated with 30 µg of aminopterin was measured. Two independent experiments were performed and gave identical radius values, which are reported in the table.

Mutant	Radius
	mm
St18	2
PrtS ⁻	2
AmiA1 ⁻	3
AmiA2 ⁻	4
AmiA3 ⁻	2.5
AmiA1/A2 ⁻	4
AmiA1/A2/A3 ⁻	0
AmiA1/A3/PrtS ⁻	1.75

methionine and glutamate). None of the strains was able to grow with EA and ED as the source of glutamate or with ACTH-(1–17) or -(1–24) fragments, the oxidized B chain of insulin as the sole source of methionine. The wild type, PrtS⁻, and AmiA/PrtS⁻ strains were capable of growing, without significant differences in their growth rates, with the other peptides tested. The AmiA1/A2/A3⁻ negative mutant was the only one whose growth was really affected. It grew on CDM containing MH, MK, YGGFM, or MKRPPGFSPFR but not in CDM containing DYM, DYMG, or RPKPQQFFGLM (Fig. 6). With YGGFM and MKRPPGFSPFR, the growth rate achieved was less than 60% of that seen in the St18 wild type strain. The residual growth observed with this peptide and the triple mutant was probably caused by the action of the proteinase and subsequent transport of the peptide degradation products. The comparable growth rates in different media for all Ami/PrtS⁻ mutants (data not shown) indicated that the three Ami proteins have overlapping specificities. The growth rates obtained with MH or MK dipeptides were the same as for the wild type strain and AmiA1/A2/A3⁻, indicating the probable existence of a dipeptide transport system in the St18 strain and the absence of exclusive transport of these dipeptides by the Ami system of the St18 strain.

Ability of *AmiA* Mutants to Grow on Aminopterin—We tested the different Ami mutants for their ability to grow in the presence of a toxic peptide analog, aminopterin (Table III). Aminopterin was transported by the Ami system because the AmiA1/A2/A3⁻ mutant was not sensitive to aminopterin, whereas St18 was sensitive. At least AmiA2 and AmiA3 are involved in the transport of aminopterin because the growth of both the AmiA1/A2⁻ and AmiA1/A3/PrtS⁻ mutants was inhibited by aminopterin. AmiA2⁻ and AmiA1/A2⁻ mutants were more sensitive to aminopterin than the St18 wild type strain, suggesting an increase in the transport of aminopterin by AmiA3 protein.

DISCUSSION

Oligopeptide Uptake Is Essential to the Growth of *S. thermophilus* in Peptide-containing Media—In addition to cell wall protease and the purine and branched-chain amino acid biosynthesis pathways, the oligopeptide transport system is one of the functions necessary for the optimum growth of *S. thermophilus* in milk (26, 24, 35). Amounts of free amino acids and peptides are limited in this medium and do not allow the optimum growth of lactic acid bacteria (36). Two elements are essential to ensure bacterial growth: cell wall protease, which hydrolyzes the caseins into oligopeptides and an oligopeptide transport system capable of internalizing the peptides. These two elements have been extensively studied in the reference lactic acid bacteria, *L. lactis* (37). The cell wall protease has

been only recently described in *S. thermophilus*, (26), and we report herewith the first characterization of the oligopeptide transport system. Both transport systems from *L. lactis* and *S. thermophilus* are ABC transporters, are of the same importance, and fulfill the same nutritional function. However, they have different compositions because the lactococcal system is, in most strains, encoded by an operon containing only one OBP-encoding gene (*oppA*) (37), whereas *S. thermophilus* expresses three homologous OBPs (*amiA1*, *amiA2*, and *amiA3*). Similar organizations, reported for *S. pneumoniae* and *S. gordonii* (*ami* and *hpp* systems, respectively), are also essential for the uptake of oligopeptides from media containing peptides as the nitrogen source (4, 38). The different reductions in the growth rates of AmiA mutants in a peptide-containing medium demonstrates that the three *S. thermophilus* OBPs are not of the same importance to the nutrition process. Inactivation of the *amiA3* gene had the most negative effect on growth rate in the peptide-containing medium. In *S. pyogenes*, the *opp* transport system is not essential for growth in complex media, which may contain sufficient di- and tripeptides and amino acids to ensure normal growth. This observation implies the existence of functional di- and tripeptide transport system(s) (29). Two di- and tripeptide transport systems have also been characterized in *L. lactis* (12, 15). At least one dipeptide transport system should be present and functional in *S. thermophilus* because both the wild type strain St18 and the triple mutant AmiA1/A2/A3⁻ grew in the presence of methionine-containing dipeptides.

An *S. thermophilus* Oligopeptide Transport System Involving Three Oligopeptide-binding Proteins—We sequenced a 7032-bp *S. thermophilus* DNA fragment comprising five genes in an operon structure and encoding a functional oligopeptide transport system. It belongs to the superfamily of ATP-binding cassette transporters, which are widespread in both Gram-negative and Gram-positive bacteria. The fragment encoding the Ami system was composed of AmiC and AmiD integral membrane proteins, AmiE and AmiF ATP-binding proteins, and a substrate binding protein, AmiA1.

In addition, we demonstrated the presence of two other oligopeptide-binding proteins, AmiA2 and AmiA3, encoded by isolated chromosome genes and working with the same permease system. This feature appears to be typical of streptococci, as the presence of three homologous oligopeptide-binding proteins has already been described in *S. pneumoniae* and *S. gordonii* (3, 4). Another example of multiple oligopeptide-binding proteins was reported for *Borrelia burgdorferi* containing three chromosome-encoded OBPs (39) and two plasmid-encoded OBPs (40). In other cases, the gene encoding an OBP and included in an operon is transcribed independently, *i.e.* at a higher level than the rest of the operon. In *S. pyogenes* and *L. monocytogenes*, the presence of a terminator downstream of *oppA* (the oligopeptide-binding protein-encoding gene) allows such an independent transcription (29, 30). The sole *oppA* in *L. lactis*, the last gene of the *opp* operon, is preceded by a promoter, which also permits its independent transcription (17).

The homology between the three oligopeptide-binding proteins we identified in *S. thermophilus* is especially strong (97.6% between AmiA1 and AmiA2, 87.1% between AmiA1 and AmiA3) and much higher than those observed between homologous proteins in *S. pneumoniae* and *S. gordonii*, which exhibit identity reaching approximately 60% (3, 4). The strong identity found for the three AmiA proteins in *S. thermophilus* is probably a result of the recent and double duplication of the *amiA1* gene. The presence of IS upstream and downstream of *amiA2* and *amiA3* genes suggests the involvement of an IS-directed mobilization of *amiA*. The available sequenced part of the ge-

nome of the *S. thermophilus* LMG 18311 strain (data not shown; accessible at www.biol.ucl.ac.be/gene/blast/blast.html) reveals the presence of at least two ORFs encoding potential proteins homologous to AmiA1 and AmiA2 of the St18 strain. Similar to our observation in strain ST18, we found an IS in the neighborhood of these genes in the sequence of the LMG 18311 strain. No insertion sequences have been reported in the close vicinity of the OBP-encoding genes of other streptococcal species, suggesting that the origin of the multicopies differs in the case of *S. thermophilus*.

The duplication of AmiA genes is probably beneficial to *S. thermophilus*. The high number of OBP copies may facilitate the transport of oligopeptides by modifying the stoichiometry of the transporter. Using a mathematical model adapted to Gram-negative bacteria in which the binding proteins are generally free in the periplasm, Bohl *et al.* (41) demonstrated that the concentration of binding proteins influenced the kinetic parameters of transport. In this kind of model, binding proteins would facilitate the movement of substrates within the periplasm. In Gram-positive bacteria, binding proteins are generally linked to the membrane through their lipid moiety, as is probably the case for the OBPs from *S. thermophilus*. Their mobility is consequently reduced and restricted to the membrane. Their role may be to limit to two dimensions the diffusion of substrates in the close vicinity of the transporter, and thus becomes more important with their copy number (31). Another hypothesis has already been proposed for streptococcal OBPs (3, 4); the interaction of binding proteins with each other is necessary for substrate binding and uptake to occur. This suggestion is supported by the different phenotypes of two insertion mutations in *hppG* (one of the OBP-encoding genes in *S. gordonii*), leading to the absence or the production of a truncated protein and allowing or preventing growth on peptides, respectively. In this case, the formation of a multireceptor cell surface complex would be an efficient means of increasing permease affinity for peptides (4). This hypothesis was not confirmed by our findings because none of our single AmiA mutants, producing one in three truncated OBPs, totally lost its capacity for oligopeptides uptake.

Regulations between OBPs and between OBPs and PrtS are strongly suggested by growth experiments and amiA promoter sequences analysis and need to be further investigated.

Specificities of AmiA Proteins—We demonstrated in this work that the three AmiA of *S. thermophilus* exhibit different but overlapping specificities. AmiA3 is the most distinctive, which is in agreement with its markedly different protein sequence. The entire Ami system is capable of transporting peptides containing from 3 to 23 amino acids as well as aminopterin, as demonstrated in growth experiments. However, like other transport systems (42), peptide size is not the only parameter to be taken into account, as 17- and 24-amino acid fragments of adrenocorticotrophic hormone, which are relatively hydrophilic (average hydrophobicity: -1.62 and -1.23) were not internalized by the Ami system. Peptide hydrophobicity probably favors their transport, especially via AmiA3. The Opp system in *L. lactis* has been extensively studied and exhibits greater affinity for nonapeptides, although it is capable of binding up to 35-residue peptides and also preferentially transporting hydrophobic peptides. In the *Streptococcus* genus, the data available indicate that peptide hydrophobicity negatively influences the growth rate of oral streptococci, at least in the case of *S. mutans* and *S. sanguis* (43). No transport of peptides containing more than 10 amino acids has been reported in streptococci. In *S. pneumoniae*, peptides containing from 2 to 7 amino acids are transported by the Ami system, but longer peptides have not been tested. In *S. gordonii*, inactivation of

TABLE IV
Homology between Ami proteins in the Ami system of the *S. thermophilus* St18 strain and those in *S. pneumoniae* (Ami system; Ref. 3) and *L. lactis* (Opp system; Ref. 17)
Percentage identities are given as determined by the BestFit program (22).

<i>S. thermophilus</i> St18	<i>S. pneumoniae</i>	<i>L. lactis</i>
	% identity	% identity
AmiA1	64.9	24.2
AmiC	76.7	30.7
AmiD	80.5	30.4
AmiE	86.2	46.6
AmiF	84.9	43.4

the three OBP-encoding genes results in a loss of ability to utilize specific 5–7-amino acid peptides for growth, whereas the utilization of peptides containing from 2 to 5 and 8 or 9 amino acids remains possible, probably because of the activity of an extracellular protease (44).

An Atypical Oligopeptide Transport System in *S. thermophilus*—*S. thermophilus* is atypical of both the lactic acid bacteria family, where it is the only streptococcus *sensu stricto*, and the *Streptococcus* genus, where it is nonpathogenic and used in food processing. The only natural medium known for the development of *S. thermophilus* is milk, a medium in which oligopeptide transport is essential for growth. The oligopeptide transport system of *S. thermophilus* we have described in this work is also atypical. We have demonstrated the ability of *S. thermophilus* to transport oligopeptides containing from 3 to 23 amino acids, with a preference for hydrophobic oligopeptides similar to those found in *L. lactis*. However, although an ABC transporter is involved in both cases, the genetic organization of the systems clearly differs. We describe herewith three highly homologous copies of oligopeptide-binding proteins, which exhibit only 24.2% identity with the only OBP in the Opp system of *L. lactis* (Table IV). The organization of the system with three OBPs copies is clearly of a streptococcal type. However, the specificity of the oligopeptide transport we describe for *S. thermophilus* is considerably broader than that already reported for other streptococci.

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REFERENCES

- Tam, R., and Saier, M. H. (1993) *Microbiol. Rev.* **57**, 320–346
- Payne, J. W., and Smith, M. W. (1994) *Adv. Microb. Physiol.* **36**, 1–80
- Alloing, G., de Philip, P., and Claverys, J.-P. (1994) *J. Mol. Biol.* **241**, 44–58
- Jenkinson, H. F., Baker, R. A., and Tannock, G. W. (1996) *J. Bacteriol.* **178**, 68–77
- Lanfermeijer, F. C., Picon, A., Konings, W. N., and Poolman, B. (1999) *Biochemistry* **38**, 14440–14450
- Verheul, A., Rombouts, F. M., and Abee, T. (1998) *Appl. Environ. Microbiol.* **64**, 1059–1065
- Desmazeaud, M., and Hermier, J. H. (1972) *Eur. J. Biochem.* **28**, 190–198
- Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T., and Yura, T. (1981) *J. Bacteriol.* **148**, 64–71
- Deguchi, Y., and Morishita, T. (1992) *Biosci. Biotech. Biochem.* **56**, 913–918
- Cogain-Bousquet, M., Garrigues, C., Novak, L., Lindley, N. D., and Loubiere, P. (1995) *J. Appl. Bacteriol.* **79**, 108–116
- Thomas, T. D., and Mills, O. E. (1981) *Neth. Milk Dairy J.* **35**, 255–273
- Smid, E. J., Plapp, R., and Konings, W. N. (1989) *J. Bacteriol.* **171**, 292–298
- Kunji, E. R. S., Smid, E. J., Plapp, R., Poolman, B., and Konings, W. N. (1993) *J. Bacteriol.* **175**, 2052–2059
- Hagting, A., Kunji, E. R. S., Leenhouts, K. J., Poolman, B., and Konings, W. N. (1994) *J. Biol. Chem.* **269**, 11391–11399
- Foucaud, C., Kunji, E. R. S., Hagting, A., Richard, J., Konings, W. N., Desmazeaud, M., and Poolman, B. (1995) *J. Bacteriol.* **177**, 4652–4657
- Sanz, Y., Lanfermeijer, F. C., Konings, W. N., and Poolman, B. (2001) *Biochem.* **39**, 4855–4862
- Tynkkynen, S., Buist, G., Kunji, E., Kok, J., Poolman, B., Venema, G., and Haandrikman, A. (1993) *J. Bacteriol.* **175**, 7523–7532
- Sambrook, J., and Russel, D. W. (2001) *Molecular Cloning: A Laboratory*

- Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19. Thomas, T. D., and Turner, K. W. (1977) *N. Z. J. Dairy Sci. Technol.* **12**, 15–21
 20. Terzhaghi, B. T., and Sandine, W. E. (1975) *Appl. Microbiol.* **29**, 807–813
 21. Letort, C., and Juillard, V. (2001) *J. Appl. Microbiol.* **91**, 1023–1029
 22. Devereux, J., Haerberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395
 23. Maguin, E., Prévost, H., Ehrlich, S. D., and Gruss, A. (1996) *J. Bacteriol.* **178**, 931–935
 24. Garault, P., Letort, C., Juillard, V., and Monnet, V. (2000) *Appl. Environ. Microbiol.* **66**, 5128–5133
 25. Huggins, A. M., and Sandine, W. E. (1984) *J. Dairy Sci.* **67**, 1674–1679
 26. Fernandez-Espla, M.-D., Garault, P., Monnet, V., and Rul, F. (1999) *Eur. J. Biochem.* **66**, 4772–4778
 27. Biswas, I., Gruss, A., Ehrlich, S. D., and Maguin, E. (1993) *J. Bacteriol.* **175**, 3628–3635
 28. Juillard, V., Guillot, A., Le Bars, D., and Gripon, J.-C. (1998) *Appl. Environ. Microbiol.* **64**, 1230–1236
 29. Podbielski, A., Pohl, B., Woischnik, M., Körner, C., Schmidt, K. H., Rozdzinski, E., and Leonard, B. A. B. (1996) *Mol. Microbiol.* **21**, 1087–1099
 30. Borezee, E., Pellegrini, E., and Berche, P. (2000) *Infect. Immun.* **68**, 7069–7077
 31. Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8**, 67–113
 32. Sutcliffe, I. C., and Russel, R. R. B. (1995) *J. Bacteriol.* **177**, 1123–1128
 33. Rauch, P. J., Beerthuyzen, M. M., de Vos, W. M. (1990) *Nucleic Acids Res.* **18**, 4253–4254
 34. Rul, F., and Monnet, V. (1997) *J. Appl. Microbiol.* **82**, 695–704
 35. Garault, P., Letort, C., Juillard, V., and Monnet, V. (2000) *Lait* **81**, 83–90
 36. Mills, O. E., and Thomas, T. D. (1981) *N. Z. J. Dairy Sci. Technol.* **15**, 43–55
 37. Kunji, E. R., Fang, G., Jeronimus-Stratingh, C. M., Bruins, A. P., Poolman, B., and Konings, W. N. (1998) *Mol. Microbiol.* **27**, 1107–1118
 38. Alloing, G., Granadel, C., Morrison, D. A., and Claverys, J. P. (1996) *Mol. Microbiol.* **21**, 471–478
 39. Kornacki, J. A., and Oliver, D. B. (1998) *Infect. Immun.* **66**, 4115–4122
 40. Bono, J. L., Tilly, K., Stevenson, B., Hogan, D., and Ross, P. (1998) *Microbiol.* **144**, 1033–1044
 41. Bohl, E., Shuman, H. A., and Boos, W. (1995) *J. Theor. Biol.* **172**, 83–94
 42. Detmers, F. J. M., Lanfermeijer, F. C., Abele, R., Jack, R. W., Tampé, R., Konings, W. N., and Poolman, B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 12487–12492
 43. Cowman, R. A., and Baron, S. S. (1990) *J. Dent. Res.* **69**, 1847–1851
 44. Juarez, Z. E., and Stinson, M. W. (1999) *Infect. Immun.* **67**, 271–278
 45. Kyte, J and Doolite, R. F. (1982) *J. Mol. Biol.* **157**, 105–132

**Three Oligopeptide-binding Proteins Are Involved in the Oligopeptide Transport of
*Streptococcus thermophilus***

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