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 *AmiAs* 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), 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 *Streptococcus* strains was 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 bacitracyn (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...
inoculate each well. The optical density was measured at 600 nm every 20 min. 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 amiA1 genes were cloned into the pGEMT easy vector (Promega) according to the manufacturer’s instructions. amiA2 deletion was obtained by double digestions with PstI and BsiNI followed by ligation step. The partially deleted amiA2 gene fragment was then cloned into pGEMT Vector (Invitrogen), according to the manufacturer’s instructions. The pStS gene fragment was cloned in pGEMT, and a deletion was obtained by HpaI and NruI digestion followed by ligation. The procedures for S. thermophilus electroporation, pGEMT 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 μg of aminopterin. The inhibitory zone was measured as the diameter in millimeters of the clear zone established in growing S. thermophilus cultures.

### Results

Characterization of One Mutant with Slower Growth in Milk—The insertional mutagenesis 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 Differentiating 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 pGEMT-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 amiA1 genes were cloned into pGEMT easy vector (Promega) according to the manufacturer’s instructions. amiA2 deletion was obtained by double digestions with PstI and BsiNI followed by a ligation step. The partially deleted amiA2 gene fragment was then cloned into pGEMT Vector (Invitrogen), according to the manufacturer’s instructions. The pStS gene fragment was cloned in pGEMT, and a deletion was obtained by HpaI and NruI digestion followed by ligation. The procedures for S. thermophilus electroporation, pGEMT-h9 integration, and excision were similar to those used for insertional mutagenesis, as described previously (24, 27).

**Results**

**Characterization of One Mutant with Slower Growth in Milk**—The insertional mutagenesis in S. thermophilus St18 produced 1.183 × 10^4 Em^2^ integrants. Based on their phenotype on Fast Strain Differentiating 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 bacitracin (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 *HindIII* and EcoRI-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 (**A**D**T**Y**I**R**K**G**I**K**W**) (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 *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 *amiA*s, 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 EcoRI-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 *amiA1* 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
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 mutant. We used CDM containing a trypsic hydrolysate of s2-casein as the nitrogen source to compare the effects of AmiA mutations on AmiA/PrtS' mutants. The growth of all AmiA/PrtS' mutants was lower 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 trypsic hydrolysate of s2-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,
presented in Table II, the 92–114 \(\alpha_s\)-casein fragment (FPQYLQYLYQGPIVLNPWDQVKR) was still present in the culture supernatants of AmiA3/PrtS\(^{-}\)/H11002 and AmiA1/A3/PrtS\(^{-}\)/H11002 mutants, but not in that of the PrtS\(^{-}\)/H11002 strain or AmiA1/PrtS\(^{-}\)/H11002 and AmiA2/PrtS\(^{-}\)/H11002 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, AmiA/PrtS, and AmiA1/A3 negative mutants were cultured in CDM containing a single peptide as a source of methionine or glutamate (the St18 strain is auxotrophic for methionine).

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

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

### TABLE II

| Peptide mass | Sequence and average hydrophobicity (in parentheses) of peptides | Presence in culture supernatant of |
|--------------|-------------------------------------------------|----------------------------------|
| 1098         | AMPKPWIQPK (-1.15)                               | PrtS\(^{-}\)                      |
| 746          | VIPYVR (0.14)                                    | AmiA1/PrtS\(^{-}\)               |
| 979          | FALPQYLR (-0.22)                                 | AmiA2/PrtS\(^{-}\)               |
| 2709         | FPQYLGQYGGPVLPNPDQV (0.51)                      | AmiA3/PrtS\(^{-}\)               |
| 2885         | FPQYLGQYGGPVLPNPDQV (0.8)                       | AmiA1/A3/PrtS\(^{-}\)            |
| 1367         | ALNEINQYQK (-1.14)                               |                                  |
| 1633         | LTEUEKNRNLK (-1.64)                              |                                  |
methionine and glutamate). None of the strains was able to grow with EA and ED as the sole source of glutamate or with EA and ED as the source of glutamate.

**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 bio-synthesis 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, 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 systems (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 Amy system was composed of AmyC and AmyD integral membrane proteins, AmyE and AmyF ATP-binding proteins, and a substrate binding protein, AmyA1.

In addition, we demonstrated the presence of two other oligopeptide-binding proteins, AmyA2 and AmyA3, 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 AmyA1 and AmyA2, 87.1% between AmyA1 and AmyA3) 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 Amy 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-

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**TABLE III**

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

| Mutant                  | Radius |
|------------------------|--------|
| St18                   | 2      |
| PrtS~                   | 2      |
| AmiA1~                  | 3      |
| AmiA2~                  | 4      |
| AmiA3~                  | 2.5    |
| AmiA1/AmiA2~            | 4      |
| AmiA1/AmiA2/AmiA3~      | 0      |
| AmiA1/AmiA2/AmiA3~PrtS~ | 1.75   |

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.
name 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 *hpg* (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 amip promoter sequences analysis and need to be further investigated.

**Specificities of Ami Proteins**—We demonstrated in this work that the three AmiA of *S. thermophilus* exhibit different but overlapping specificities. AmiA3 is the most distinctive, its capacity for oligopeptides uptake.

| Protein    | *S. thermophilus* | *S. pneumoniae* | *L. lactis* |
|------------|-------------------|-----------------|-------------|
|            | % 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 hereewith 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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