Improving dairy starter cultures

Gerard Venema and Jan Kok

It has long been recognized that various dairying-relevant properties of mesophilic lactic streptococci are unstable traits. During the past ten years it has become increasingly clear that these instabilities are due to loss of plasmids during growth of the bacteria. The plasmid-linkage of these properties has accelerated the identification and delineation of their genetic determinants and recently genes important for dairying have been cloned and expressed in a number of bacterial species, including plasmid-free lactic streptococci. The prospects for improving dairy starter cultures by enhancing and stabilizing the expression of dairying-relevant plasmid-borne genes look promising.

Lactic acid bacteria perform an essential role in the production and/or preservation of wholesome foods, ranging from a number of milk-derived products (cheeses, yoghurts, butter, butter milk, quark) to a variety of vegetables, cereal-derived products and meats.

In the manufacture of cheese, milk is inoculated with a mixture of lactic acid bacteria (starter culture) and a clotting enzyme (e.g. chymosin). After sufficient acid production by the bacteria and action of the clotting enzyme, the milk proteins coagulate to produce a curd. Having removed the whey the curds are subsequently salted, either by the addition of dry salt (e.g. Cheddar cheeses) or immersion in brine (e.g. Dutch cheeses), and finally stored to mature.

Until recently starter cultures with undefined composition were used for most cheeses. The dairying properties of these undefined mixed-strain starters are not completely predictable and so, in several countries, efforts have been made to reduce the number of strains and to compose starter cultures of defined composition and activity. The advent of computer controlled cheese manufacture has accentuated the demand for stable and predictable starter cultures.

The important dairying properties are lactic acid production, proteolysis of milk proteins (particularly, of casein), bacteriophage sensitivity, and miscellaneous properties which determine the ultimate quality of the final product such as the production of flavours, bacteriocins and nisin. All these properties appear to be plasmid-borne and while this means they are unstable traits in starter cultures, it also means that the relevant genes can be isolated relatively more easily. Genetic transformation systems and gene cloning systems have now been developed for the mesophilic lactic streptococci paving the way with these organisms.

In Europe, a network of collaboration has developed between the research groups of Daly (Cork, Eire), Gasson (Reading, UK), Venema (Groningen, The Netherlands), de Vos (Ede, The Netherlands) and Teuber (Kiel, FRG). The intermediate goals of these groups are the development of suitable gene-cloning systems, the cloning of genes important in dairying and the acquisition of knowledge leading to the construction of expression vectors for mesophilic lactic streptococci. The ultimate target, and one which might not ever be achieved, would be the stable, controlled expression of all the dairying-relevant genes achieved, perhaps, by inserting them into the chromosome of a bacterial single host. Progress towards this goal will require a much improved understanding of chromosomal genetics.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cloned genes of the lactose metabolic pathway of lactic streptococci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Source</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>phospho-β-D-galactosidase</td>
<td>S. lactis</td>
</tr>
<tr>
<td>phospho-β-D-galactosidase + Enzyme II&lt;sub&gt;ac&lt;/sub&gt; or Enzyme III&lt;sub&gt;ac&lt;/sub&gt;</td>
<td>S. cremoris H2</td>
</tr>
<tr>
<td>tagatose 1,6-biphosphate aldolase</td>
<td>S. lactis H1</td>
</tr>
<tr>
<td>phospho-β-D-galactosidase + Enzyme II&lt;sub&gt;ac&lt;/sub&gt; + Enzyme III&lt;sub&gt;ac&lt;/sub&gt;</td>
<td>S. lactis C2</td>
</tr>
</tbody>
</table>

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and the isolation and cloning of genes for each of the important dairying properties.

Lactate production

The main carbohydrate source in milk is lactose. Its very efficient conversion to lactic acid by lactic streptococci (almost quantitative under anaerobic conditions) is of great importance in dairying and in the preservation of a variety of fermented foods. Therefore, the lactose-catabolic system of lactic acid streptococci has been studied in considerable detail.9

There are two important groups of enzymes involved in the pathway for lactose-catabolism in Streptococcus lactis, a common component of dairy starter cultures (Fig. 1). The first is phosphoenol pyruvate (PEP) dependent lactose phosphotransferase system which is required for the uptake of lactose: it is composed of the membrane located Enzyme II^lac and Enzyme III^lac and the cytoplasmic proteins, Enzyme I and HPr. The second group of enzymes are those of the tagatose branch of the lactose catabolic pathway.

Which genes are plasmid-borne?

The supposition that in lactic acid streptococci genes for lactose catabolism are carried by plasmids was based mainly on two observations: (1) during normal growth cells spontaneously lost the ability to metabolize lactose (Lac^-) but this occurred at an increased frequency upon treatment of the cells with acridine dyes or when the cells were cultured at elevated temperatures; (2) the production of the Lac^- phenotypes was often correlated with the loss of a particular plasmid. Definite proof that genes for lactose-metabolism are plasmid-borne was obtained by introducing specific plasmids into Lac^- cells by conjugation, transduction or transformation and observing that the recipients acquired a Lac^+ phenotype.10

Enzyme complementation analysis showed that S. lactis C2 (which lacked the Lac plasmid) was deficient in Enzymes II^lac, Enzymes III^lac and phospho-β-D-galactosidase.11 In S. lactis H1, 133 and probably also in C10, plasmid-curing experiments strongly suggest that the majority of genes specifying the PEP-dependent transport system and those specifying the tagatose branch of the lactose catabolic pathway (Fig. 1) are plasmid-located, although the location of genes specifying Enzyme I and HPr has not been established. The 33 MDa plasmid of S. lactis H1^ 12 could certainly accommodate all these genes: their co-existence on a single plasmid would be an advantage for genetic engineers.

Several genes of the lactose catabolic pathway have recently been cloned from plasmid DNA (Table 1, Fig. 1), and successfully expressed in various hosts. It can be expected that in the very near future several additional genes of the lactose catabolism pathway will be isolated using established mechanisms (e.g. deletion formation during transduction) and/or recently developed gene cloning systems for lactic acid streptococci.6,7 (including a shuttle vector between lactic acid streptococci, Bacillus subtilis and E. coli). This, together with nucleotide sequence data, not yet available, will undoubtedly provide a molecular insight of how these important genes are regulated.

Proteolysis

The proteolytic system of lactic streptococci is required to release further amino acids from milk proteins to promote rapid growth and, consequently, adequate acidification of milk. In addition, the proteolytic system is essential for cheese matura-
ral uncertainties\textsuperscript{20}. Like lactose catabolism, proteolytic activity of lactic acid streptococci is an unstable trait. Bacteria without this activity grow slowly in milk, and consequently, are slow acid producers, resulting in slow coagulation of milk. By comparing the plasmid DNA profiles of slow and fast cultures, in several strains the proteolytic negative trait (Prt\textsuperscript{-}) could be correlated with the loss of particular plasmids\textsuperscript{10}. It has often been observed that loss of proteolytic activity occurs concomitantly with the appearance of the Lac\textsuperscript{-} phenotype and transduction studies\textsuperscript{22} and curing experiments\textsuperscript{23} have shown that genes for both lactose metabolism and proteolysis can be present on a single plasmid.

So far, only the structural genes encoding the cell wall bound proteinase of lactic acid streptococci have been (partially) cloned and expressed in three instances: the gene on plasmid pWVOS (26 kb) of \textit{S. cremoris} Wg2 has been expressed in \textit{B. subtilis} and \textit{S. lactis}\textsuperscript{24}; that of plasmid pSK11 (78 kb) of \textit{S. cremoris} SK11 has been expressed in \textit{E. coli} (as a phage \(\lambda\)-recombinant) and in \textit{S. lactis}\textsuperscript{7}; and the gene from pLP712 (56.5 kb) of \textit{S. lactis} 712 has been expressed in \textit{E. coli}\textsuperscript{25}.

The gene of the \textit{S. cremoris} Wg2 proteinase has now been sequenced completely (unpublished data). It carries a promoter similar to the \(\sigma^{54} B. subtilis\) and \textit{E. coli} consensus promoter, a strong ribosome binding site (\(5\textsuperscript{AG} of binding with 16S rRNA 14.4\textsuperscript{K}ca1\textsuperscript{Kcal mole\textsuperscript{-1}} typical for a variety of other Gram-positive promoters), and an open reading frame with a coding capacity of 1902 amino acids, followed by a rho-independent terminator. The protein is a bacterial serine-type protease; interestingly it contains three regions that constitute the active sites of a variety of subtilins (proteolytic enzymes from \textit{B. subtilis}) (unpublished data). Since the cell wall-bound proteinases have been implicated in the production of bitter peptides during cheese maturation, and since \textit{S. cremoris} SK11 does not seem to produce such peptides\textsuperscript{26} whereas \textit{S. cremoris} Wg2 does, it will be extremely interesting to see whether these differences can be correlated with possible amino acid sequence differences.

**Bacteriophage resistance**

The main cause of slow acid production in lactic streptococci is bacteriophage infections. Although this is generally recognized, the biochemistry of phage infection in lactic streptococci is poorly understood. Numerous virulent and temperate (latent) bacteriophages have been isolated from lactic streptococci but most characterizations have been limited to their morphology, and other general properties such as latency, burst-size and host-range\textsuperscript{27}. On the basis of DNA homology, serology and genome size, four morphological groups can be distinguished\textsuperscript{28}, one of which, comprising the prolate-headed phages, is capable of attacking several strains of one lactic streptococcal species and often strains of different species\textsuperscript{29}. In a few cases phage receptor sites have been characterized to a limited extent\textsuperscript{30,31} but virtually nothing is known concerning the molecular details of phage adsorption, penetration and DNA-replication.

Although, in principle, mutation in temperate bacteriophages might produce lytic bacteriophages, this does not seem likely because there is little genetic homology between the two phage types\textsuperscript{32} and susceptible indicator bacteria for induced phages are lacking\textsuperscript{27}. (This is discussed in more detail in Ref. 33.)

To obtain phage-resistant starter components, in several laboratories and institutions\textsuperscript{34} commercial starters have been challenged with a variety of lytic phages and phage insensitive survivors have been used to compose new, defined mesophilic starter cultures which can be used repeatedly in dairy fermentations without the need for starter rotation (see Glossary). By challenging hundreds of bacterial strains from a variety of commercial mixed-strain starters with a cocktail of virulent phages, many \textit{S. cremoris} strains have been obtained showing long-term resistance to more than 400 virulent phages. The same procedure, applied to single phage-sensitive strains, yielded a variety of phage resistant mutants. Some of the phage resistant isolates, as well as some phage resistant mutants could be used in dairy starter cultures for a better control of bacteriophage attack in commercial cheese vats\textsuperscript{35}.

In lactic streptococci, several phage defence mechanisms including DNA restriction and modification, prevention of phage adsorption and other undefined yet powerful mechanisms appear to be correlated with the presence of particular plasmids (Table 2). In only one case has a restriction endonuclease been isolated and the restriction site been determined (SsrF1 from \textit{S. cremoris} F, recognizing 5\textsuperscript{GCG} of binding with 16S rRNA 14.4\textsuperscript{K}ca1\textsuperscript{Kcal mole\textsuperscript{-1} typical for a variety of lytic phages and phage insensitive survivors have been used to compose new, defined mesophilic starter cultures which can be used repeatedly in dairy fermentations without the need for starter rotation (see Glossary). By challenging hundreds of bacterial strains from a variety of commercial mixed-strain starters with a cocktail of virulent phages, many \textit{S. cremoris} strains have been obtained showing long-term resistance to more than 400 virulent phages. The same procedure, applied to single phage-sensitive strains, yielded a variety of phage resistant mutants. Some of the phage resistant isolates, as well as some phage resistant mutants could be used in dairy starter cultures for a better control of bacteriophage attack in commercial cheese vats\textsuperscript{35}.

An important question is whether other properties relevant for dairying are impaired when strains acquire plasmids specifying phage resistance. The only evidence available so far suggests that no such complications are likely: acid production, milk-coagulation and proteolytic activities of transconjugants carrying the phage resistance plasmid, pTR2030 (Table 2) were similar to those of the parental strain\textsuperscript{37}.

Plasmid-linked phage-resistance provides an excellent basis for the cloning of genes involved in defence against phage attack and will undoubtedly contribute to the construction of improved starter cultures.

**Miscellaneous properties**

**Production of diacetyl**

Perhaps the most important of the flavour and aroma compounds produced by components of mesophilic starter cultures is diacetyl, produced by \textit{S. lactis} subspecies \textit{diacetylactis} and several Leuconostic-species. The compound is formed from citrate, which is internalized by a membrane-located citrate-permease. Citrate catalyses the formation of oxaloacetic acid which, via pyruvate and various other intermediates, is converted to diacetyl\textsuperscript{37}.

Like so many other dairying-relevant properties, the production of diacetyl is an unstable trait; at least one component of citrate metabolism, the citrate-permease system is plasmid-linked\textsuperscript{36,39}. Since plasmid-cured Cit\textsuperscript{+} variants retained citritase activity, the gene(s) specifying citritase appears to be chromosomally located.

**Production of antagonistic compounds**

Some strains of lactic streptococci are capable of producing proteins or proteinaceous substances either
## Table 2

Plasmid-encoded bacteriophage defence systems in lactic streptococci

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Md</th>
<th>Host</th>
<th>Plasmid identified by</th>
<th>Defence system</th>
<th>Remarks</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>unnamed 10</td>
<td>S. cremoris KH</td>
<td>curing</td>
<td>restriction</td>
<td>restriction of ϕc2</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>pIL6 28</td>
<td>S. lactis IL594</td>
<td>curing and conjugation (pIL6) and co-transformation (pIL7)</td>
<td>restriction</td>
<td>restriction of ϕpIL6 5.4 kb</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>?</td>
<td>S. cremoris F1</td>
<td>curing</td>
<td>restriction</td>
<td>resistance of ϕpME0030 30</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>pME0030 30</td>
<td>S. lactis ME2</td>
<td>curing</td>
<td>adsorption, restriction and suppression of phage development at 30°C</td>
<td>resistance of ϕME2 curing and restriction of ϕpME0030 30</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>pSK112 34</td>
<td>S. cremoris SK11</td>
<td>curing</td>
<td>adsorption</td>
<td>resistance to ϕpSK112 34</td>
<td>e</td>
<td></td>
</tr>
<tr>
<td>pTR2030 30</td>
<td>S. lactis ME2</td>
<td>conjugation</td>
<td>? (adsorption normal, early viral gene expression not affected)</td>
<td>resistance to ϕpTR2030 30</td>
<td>f</td>
<td></td>
</tr>
<tr>
<td>unnamed 40</td>
<td>S. lactis, subsp. diacetylactis</td>
<td>conjugation</td>
<td>? (adsorption normal)</td>
<td>resistance to a variety of phages; probably also contains Tra functions</td>
<td>g</td>
<td></td>
</tr>
<tr>
<td>pNP40 40</td>
<td>S. lactis, subsp. diacetylactis</td>
<td>curing + conjugation</td>
<td>? (adsorption normal)</td>
<td>resistance to ϕpNP40 40</td>
<td>h</td>
<td></td>
</tr>
<tr>
<td>pC1750 50</td>
<td>S. cremoris UC653</td>
<td>conjugation</td>
<td>? (adsorption normal)</td>
<td>resistance to ϕpC1750 50</td>
<td>i</td>
<td></td>
</tr>
<tr>
<td>pBU1-8 39</td>
<td>S. lactis, subsp. diacetylactis</td>
<td>conjugation</td>
<td>? (adsorption normal)</td>
<td>resistance to several phages. No breakdown of phage defence at elevated temperatures</td>
<td>j</td>
<td></td>
</tr>
<tr>
<td>pC1829 29</td>
<td>S. cremoris BA2</td>
<td>conjugation</td>
<td>? (adsorption normal)</td>
<td>resistance to several phages. No breakdown of phage defence at elevated temperatures</td>
<td>k</td>
<td></td>
</tr>
</tbody>
</table>

Supplementary information:

Inhibiting the growth of taxonomically related members (bacteriocins) or a variety of unrelated Gram-positive organisms (nisin). Under conditions known to lead to loss of plasmid DNAs the potential for producing these substances may be lost. This, and the observation that the properties may be conjugally transferred suggests that they are plasmid-linked, although it has not always been possible to demonstrate the association of the property with plasmid DNA in the transconjugants. However, with the advent of improved plasmid DNA-isolation procedures, some of the initial uncertainties concerning plasmid-linkage...
now seem to be resolved: it has now been shown by conjugal transfer, that bacteriocin production in S. cremoris 9B4 and 4G6 is linked to a 39.6 Md plasmid in S. lactis subspecies diacetylactis WM4 to an 88 Md conjugative plasmid and in S. cremoris 346 to a 54 Md plasmid.

Similarly, at least in a number of strains, the production of and resistance to nisin (a peptide antibiotic active against Gram-positive bacteria deleterious to dairy products, and potentially useful as a preservative for certain meats and some other products), seems to be linked to the presence of certain plasmids. However, for both bacteriocin and nisin production, demonstration of plasmid-linkage does not prove that the structural genes rather than a regulatory element for these proteins are plasmid-located. This will require the isolation of the gene and the characterization of the gene product.

Transposition

A surprisingly large number of properties important for dairying, therefore, are plasmid-encoded, or at least plasmid-linked. It looks as if nature has been kind to lactic streptococcal geneticists by having put the genes on plasmids or in having prevented them from being added to the chromosome, thus facilitating their delineation and isolation. The chromosome, however, is almost entirely uncharted. Recently, prospects for the genetic analysis of lactic streptococcal chromosomes have improved following the observation that the 15–17 kb tetracycline-resistance transposon Tn919 from S. sanguis FC1 can be conjugally transferred to S. lactis. Subsequent work demonstrated that this precisely excising transposon can be conjugally transferred to a variety of lactic streptococci, and that high-frequency transfer occurred when either the recipient, or the transposon-donating cells contain the conjugally highly active Lacplasmid pMG600 (Lax cells display a tendency to agglomerate). Since transposition was random in at least some strains, the targeting and cloning of chromosomal genes now seems feasible.

Gene expression signals

To express foreign genes in lactic streptococci and to enhance expression of dairying-relevant genes properly characterized gene expression signals are essential. Based on the broad-range shuttle vector pGK12, promoter and transcription termination signal screening vectors have been developed. S. cremoris-specific promoters of varying strength, which also function in B. subtilis and E. coli, and conform to the E. coli and B. subtilis consensus promoter, have been obtained. Typically, the -35 hexanucleotide sequences of these promoters are preceded by regions with high A-T content, a feature also seen in other Gram-positive organisms. Ribosome binding sites with high Shine-Dalgarno complementarity were identified, preceding open reading frames, and the sites of transcription initiation have been mapped (pers. commun.). Thus it appears that all conditions to construct efficient vectors for the expression of both homologous and heterologous genetic information are fulfilled.

Future developments

It seems unlikely that the gene-cloning vectors currently available and expression vectors based on these vectors will satisfy the current strict dairy regulations. Therefore, there is a need to develop vectors composed either entirely of DNA from lactic streptococci and/or their phages, or vectors from which non-lactic streptococcal sequences can be conveniently removed, before the genetically engineered bacteria can be put to work. Now that several genes conferring a readily distinguishable phenotype to the host have been isolated (phospho β-d-galactosidase, proteinase and bacteriophage or nisin resistance will follow soon) the development of such vectors of the former type in the near future can be confidently expected.

A second readily foreseeable development, through necessity perhaps, will be the stabilization of dairying-relevant genes. These genes could be stabilized by incorporation into the chromosome. This might have the additional advantage (as for B. subtilis) that the frequency of deletion may be lower in chromosomally-integrated DNA than in recombinant plasmid DNA. Gene-amplification strategies worked out in B. subtilis might also be applied to the lactic streptococci expression of chromosomally-integrated DNA expression.

By definition lactic acid streptococci are Generally Regarded As Safe (GRAS) organisms. It will be interesting to see whether this property can be exploited in the commercial production of heterologous proteins.

Acknowledgements

The support from the Biomolecular Engineering Program (BEP) and the Biotechnology Action Program (BAP) of the Commission of the European Communities is gratefully acknowledged. We thank Dr. Colin Hill (University College, Cork, Ireland) for careful reading of the manuscript.

Glossary

Starter rotation — several cultures with susceptibility to different bacteriophage strains are used in rotation as inocula. This reduces the likelihood that a particular bacteriophage will become established in a cheese manufacturing plant and halt production.

References

Microbial energetics should be considered in manipulating metabolism for biotechnological purposes

A.H. Stouthamer and H.W. van Verseveld

The conversion of substrates into products and biomass in a microbial culture is a chemical reaction, albeit a complicated one. The stoichiometry and kinetics of this reaction provide information which can be useful in demonstrating how the intrinsic properties of microorganisms or the conditions imposed on them influence productivity in bioreactors. Microbial energetics can be used to guide the selection of production strains both by culture screening and recombinant DNA techniques, to predict the maximum yield of a product from a particular organism, and to explain the influence of culture conditions on productivity.

Studies on the energetics of microbial growth play a central role in microbial physiology. Their purpose is to determine the relationship between substrate utilization, ATP generation, the formation of new cell material and the formation of products. During growth, fixed relationships exist between the yield of biomass and product and the consumption of substrate, oxygen and nitrogen source and between oxygen consumption and heat production. These relationships are obtained by elemental balance equations. In these equations, empirical formulae (e.g., CH$_n$O$_x$N$_y$) are used to represent...