Challenges when transferring technology from *Lactococcus* laboratory strains to industrial strains

Eric Johansen

Applied Biotechnology, Chr Hansen A/S, 10-12 Bøge Allé, Hørsholm, Denmark
Corresponding author: E. Johansen
E-mail: Eric.Johansen@dk.chr-hansen.com

ABSTRACT. Many genetically modified *Lactococcus* strains have been constructed in research laboratories around the world. Most of these have originated from laboratory strains and therefore there are several barriers to using them in an industrial setting. Laboratory strains are often plasmid-free and consequently Lac⁻ and Prt⁻, rendering them unable to grow in milk. Many of the commonly used techniques have been optimised for laboratory strains and their application to industrial strains may require a great deal of effort. Often genetically modified organisms produced in the laboratory do not fit the published definition of 'food-grade' (Johansen, 1999, *Encyclopedia of Food Microbiology*, Academic Press, London, pp. 917-921) and a great deal of effort is required to eliminate undesirable DNA sequences. As a consequence, it is often necessary to recreate the strains in industrial backgrounds before the innovations described in the scientific literature can be applied to the real-world dairy industry.

Key words: *Lactococcus* strains, Cheese factory, Laboratory strain, Industrial strain, Food-grade genetically modified organism (GMO)

INTRODUCTION

Cheese making involves the acidification of milk by lactic acid bacteria, typically *Lactococcus lactis* and *Streptococcus thermophilus*. In order to get reproducible, reliable acidification, most dairies purchase starter cultures from a starter culture supplier. These cultures are carefully selected to ensure rapid acidification, bacteriophage resistance and proper flavour development in the cheese (Høier et al., 1999). Research on lactic acid bacteria is carried out by universities, research institutions and the starter culture companies themselves. Universities and research institutions typically work with laboratory strains, while the starter culture companies research their favorite industrial strains. The choice of strain is influenced by a number of factors, but ultimately work on industrial strains is most likely to lead to improved performance in the dairies.

DIFFERENCES BETWEEN LABORATORY STRAINS AND INDUSTRIAL STRAINS

Two laboratory *Lactococcus* strains dominate the research done in universities and at research institutions. These strains are designated IL1403 (Chopin et al., 1984) and MG1363 (Gasson, 1983), and are derived from the cheese starter culture strains IL594 and NCDO 712, respectively. While the parent strains were used commercially at the time these laboratory strains were isolated, they are not significant in industry today. Large starter culture companies have several hundred different *Lactococcus* strains and their product assortments typically contain several dozen different *Lactococcus* strains at any one time.

The total genome sequence of IL1403 is publicly available (Bolotin et al., 2001) and that of MG1363 will become available in the near future (Klaenhammer et al., 2002). No genome sequences of industrial strains are publicly available and it is unlikely that a starter culture company would publish the sequences of their important strains.

The laboratory strains IL1403 and MG1363 are plasmid-free due to loss of the plasmids present in the parent strains (Gasson, 1983; Chopin et al., 1984). Many traits of industrial importance are plasmid encoded in *Lactococcus* (Davies and Gasson, 1981). These include lactose metabolism and proteinase production, both of which are required for the acidification and coagulation of milk for cheese production, and both of which typically reside on the same plasmid. Thus, in order for a laboratory strain to be included in a cheese production trial, it is necessary to reintroduce at least one plasmid (Guldfeldt et al., 2001). In addition, many genes conferring resistance to bacteriophages are plasmid encoded. These are essential if the starter culture is to survive in the ecosystem present in cheese factory processing facilities.

A prophage present in NCDO 712 was also cured (removed) during the isolation of MG1363 (Gasson, 1983). Prophages in lactic acid bacteria can have two characteristics that are potentially relevant for the dairy industry. The presence of a prophage may give immunity to infection by other bacteriophages, thereby enhancing the bacteriophage resistance of the strain. The presence of prophages has also been shown to positively correlate with the autolytic properties of a starter strain (O'Sullivan et al., 2000). Autolysis is generally believed to be important for flavor development in cheese.

In order to genetically modify a *Lactococcus* strain, it is necessary to introduce DNA. This is routinely done using electroporation of glycine-grown competent cells (Holo and Nes, 1989). For laboratory strains, the conditions are well established and transformation efficiencies

above 10^6 transformants per microgram of DNA can be routinely attained. These high transformation efficiencies are only attained following a great deal of optimization effort. Transformation of industrial strains is possible (Sørensen et al., 2000), but it is typically much less efficient.

In order for a genetically modified organism (GMO) to be used in food, it must fulfill a number of safety criteria and must be able to attain 'generally recognized as safe' (GRAS) status in the United States. While there is no official definition of what constitutes a 'food-grade' GMO, a working definition has been elaborated (Johansen, 1999) and at least one *Lactococcus* strain fulfilling this definition has been affirmed as GRAS and brought to the market in the United States (Kondo and Johansen, 2002). An important element of this definition is that a food-grade GMO can only contain DNA from the same species. In a broader definition of food-grade, genes from other GRAS food microorganisms would be considered acceptable (Johansen, 1999). In either case, the use of antibiotic resistance genes as selectable markers is not allowed. Many of the strains constructed at universities and in research institutions are for 'proof of concept' and as such there is often no need for them to be food-grade. Thus, antibiotic resistance markers have been used due to the ease of working with them. If these strains are to be used in the industry, it is necessary to eliminate all foreign DNA or to reconstruct the strains in a more appropriate manner.

Another significant difference between industrial strains and laboratory strains is the research effort devoted to each strain. Laboratories all over the world work with MG1363 and IL1403, while only the research teams at the individual starter culture companies work with specific industrial strains. Thus, more knowledge will be generated using laboratory strains, though there are a number of important challenges to using this knowledge in an industrial setting.

CHALLENGES TO TRANSFERRING KNOWLEDGE FROM LABORATORY STRAINS TO INDUSTRIAL STRAINS

A number of food-grade genetic modifications can be made in laboratory or industrial strains. These include: deletion of a gene from a strain (Johansen et al., 1995); replacing a gene in a strain with a similar gene from another strain (Johansen et al., 1995); replacing a gene in a strain with the same gene that has been slightly modified *in vitro* (Sørensen et al., 2000); increasing the number of copies of a gene in a strain (Dickely et al., 1995); introducing a new gene into a strain (Joutsjoki et al., 2002), and using the presence of recombinant DNA as a selection principle for the isolation of mutants with interesting properties (Curic et al., 1999).

All of these modifications require the introduction of plasmid DNA, and some of them require the subsequent elimination of these plasmids. A major challenge in working with industrial strains is the difficulty encountered in the introduction of plasmid DNA. This is in part due to the lack of optimization and to strain differences. Industrial strains have been selected for their superior resistance to bacteriophages. One mechanism of bacteriophage resistance commonly found in *Lactococcus* is restriction/modification (R/M) systems (Forde and Fitzgerald, 1999). These systems recognize incoming DNA and degrade it regardless of whether it is plasmid DNA or bacteriophage DNA. Strategies for overcoming R/M systems include the use of DNA modified so it is not recognized as incoming, or brief heat treatment of recipient cells to transiently inactivate the restriction function. The cell walls of industrial strains are often more robust than the cell walls of laboratory strains. This may be related to the high level of bacteriophage resistance in the industrial strains and may contribute to the difficulties in transforming them.

Industrial strains normally contain a large number of plasmids. Plasmid incompatibility in *Lactococcus* has been described (Gravesen et al., 1997) and prevents the co-existence of some plasmids. Plasmid incompatibility usually results in greatly reduced transformation efficiencies and the transformants that are obtained typically have lost one or more of the indigenous plasmids. Since many genes of technological importance are plasmid encoded, plasmid loss from an industrial strain is usually not acceptable.

Another problem is that many of the food-grade selection schemes derived for laboratory genes do not work in industrial strains. Several selection systems based on resistance to the bacteriocin nisin have been described (e.g., von Wright et al., 1990). Many industrial strains have been isolated from undefined mixed strain starter cultures (Høier et al., 1999), a complex mixture of strains competing and interacting with each other. A nisin-producing strain would have an advantage if the other members of the culture were nisin sensitive. As a consequence, many industrial strains are nisin resistant and selection systems based on nisin resistance cannot be used. Other selection systems require the presence of specific mutations in the recipient strain. These can be introduced in a food grade manner as described previously. One big surprise was that the selection scheme used successfully in laboratory strains with a plasmid called pFG1 (Dickely et al., 1995) resulted in an unacceptable reduction in the acidification rate when introduced into industrial strains (Sørensen et al., 2000). This reduction was a consequence of the specific allele chosen as the selectable marker in pFG1 (*supB*). Replacing *supB* with a different selectable marker (*supD*) resulted in a new vector, designated pFG200, which did not reduce the acidification rate when introduced into industrial strains (Sørensen et al., 2000).

While there is a total genome sequence available for *Lactococcus*, it has limited usefulness when working with industrial strains. Comparison of the DNA sequence of the *pepN* gene from *L. lactis* subsp. *lactis* IL1403 with the *pepN* gene of the industrial strain *L. lactis* subsp. *lactis* CHCC377 reveals 99.5% identity at the DNA sequence level. When the *pepC* genes of these two strains are compared, the similarity is reduced to 91.4% identity, while for *pepA* the similarity is 85.4% identity (P. Strøman, personal communication). The greater the divergence between two genes the more likely problems will arise in designing primers for making precise genetic alterations *in vitro*, and for molecular biological techniques, such as the polymerase chain reaction, which is used for rapid cloning.

CONCLUSIONS

Laboratory strains of *Lactococcus* are great for proof of concept because they are easy to work with, there are a large number of mutant and other derivative strains available, and there are many scientists involved in research of these strains. This research is typically published in the scientific literature and results are discussed at scientific meetings. Additionally, the complete genome sequence of several of the most important laboratory strains will soon be available. However, laboratory strains are not useful for the development of new industrial starter cultures. They must be further modified, for example by reintroduction of the lactose fermentation pathway and the proteolytic system, so that they can grow in milk. In addition, undesirable antibiotic resistance genes need to be eliminated. Finally, if the proposed definition of food-grade (Johansen, 1999) is used, all DNA from outside the genus being modified must be eliminated. This includes any remnants of *Escherichia coli* cloning vectors that might have been used in early steps in the construction process.

One very relevant question is "Why not make the strains food-grade in the first place?". The tools to do so exist and can be established in any laboratory capable of making non-food-grade GMOs. It simply requires a little more effort, but the ability to use the same strains for application trials and ultimately in industrial settings should compensate for this extra effort.

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