Gemo LAB Newsletter

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In this Issue

Demonstartion of improved dairy products through the application of starter strains of lactic acid bacteria with engineered fermentation pathways

"GEMOLAB"

Biotechnology (4th call)
Demonstration project - Microbial cell factories
Contract BIO-CT97-0118

Coordinator: Prof J. Delcour
Starting Date: 01.01.98
Duration: 24 months
Scientific Officer: Irene Norstedt

EDITORIAL

GEMOLAB is the acronym for "Demonstration of improved dairy products through the application of starter strains of lactic acid bacteria with engineered fermentation pathways". This Demonstration Project (BIO4-CT97-0118) is supported by the Biotechnology section of the European Community Framework Programme IV (Microbial Factories area). The project started on October 1st 1998 and will last for 2 years. The EC scientific officer in charge is Dr. Irene Norstedt.

An important task in the EC Demonstration Projects is communication to an extended audience. This GEMOLAB Newsletter is one of the media to be used for publicizing the project. In this period of consumers defiance, if not hostility, towards genetic engineering applied to food products, we feel it is important to convey the message to a large public that present-day molecular biology techniques, when properly implemented using food-grade methods, can lead to the development of new products meeting the consumers demand for safe and tasty food. This is especially true for recombinant lactic acid bacteria in the context of dairy food applications, an area where a tremendous research effort has been made during the last 15 years, in particular in the European Community with the continuous financial support of the Commission. The GEMOLAB partners believe that time has come to demonstrate that the results gained and the tools constructed in the laboratory are ready to be exploited for the development of new dairy starters in a true industrial perspective.

This first issue of the GEMOLAB Newsletter contains a description of the project and a presentation of the partners. Two more issues will follow. The second will appear next spring and report laboratory work dedicated to the construction and characterization of the prototype recombinant dairy starters. The last issue will be published in late autumn 2000 and will report the validation of these prototypes under real industrial conditions together with an evaluation of the organoleptic properties of the obtained dairy products.

Jean Delcour

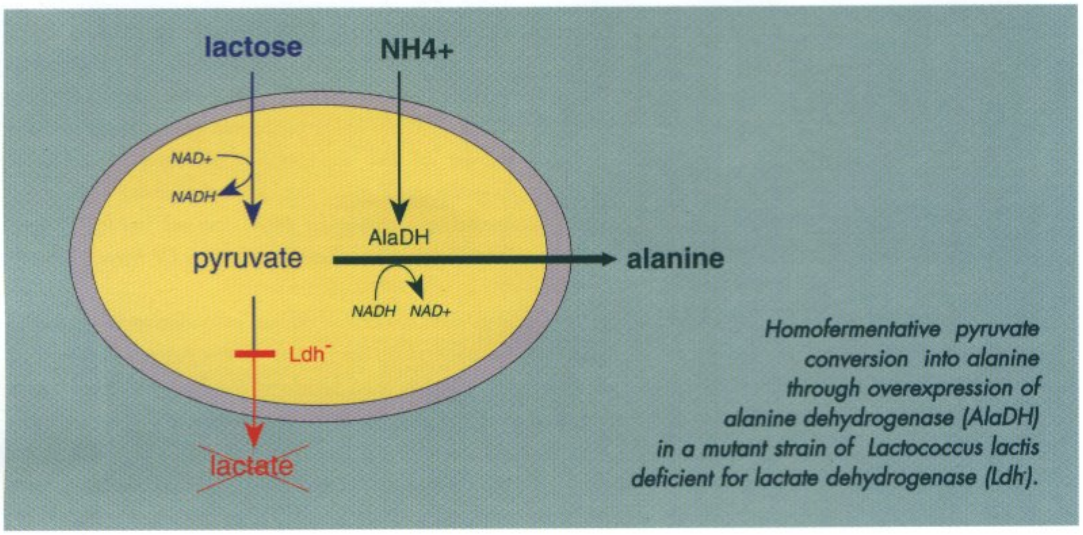
Introduction

Our knowledge of the physiology and molecular genetics of lactic acid bacteria has increased tremendously over the last 15 years as the result of continuous support from EC through the BAP, BEP, BRIDGE and BIOTECH I and II programmes. In particular, the cloning of key genes involved in various fermentation pathways and the exploitation of the homologous recombination process to perform gene disruption, deletion and substitution has opened the way to the metabolic engineering of these biotechnologically important microorganisms.

For example, today one can routinely delete genes encoding L- or D-lactate dehydrogenases (LDH) in order to obtain strains either producing only the desired isomer of lactate or totally defective in lactic fermentation (as an example, see Ferain et al., J. Bacteriol., 1996, 178, 5431-5437). Such deleted strains are particularly suitable for food-grade applications since they belong to the "self-cloning" class of genetically modified microorganisms (GMM) exempted from heavy regulatory constraints. L- or D-LDH negative strains of *Lactobacillus helveticus* would be of particular interest for a better control of the process of emmental cheese production since the L/D ratio has an incidence on propionic and butyric fermentations. LDH-negative mutants also offer interesting perspectives of industrial applications as cell factories since their pyruvate pool is available for rerouting the carbon flux towards the production of metabolites of interest. For instance, recent work (Hols et al., Nature Biotechnology, 1999, 17, 588-592) has demonstrated that expression of the gene encoding alanine dehydrogenase in a LDH-negative strain of *Lactococcus lactis* allows overproduction of the natural sweetener alanine. Since reductive amination of pyruvate into alanine requires ammonium, an additional advantage of such "sweet" alanine starters will be to reduce the ammonium content of whey. It is known that

urease-positive *Streptococcus thermophilus* starters can yield whey with excessive ammonium content which reduces the suitability of this valuable byproduct as feed additive.

Genes involved in secondary fermentation pathways responsible for the production of flavour compounds in dairy products have also been cloned, overexpressed and knocked out. In particular, a strain defective for acetolactate decarboxylase has been shown to produce increased amounts of diacetyl, and recent work has shown that synthesis of diacetyl is greatly enhanced in such a mutant when an alternative route for NAD+ reoxidation is provided through transformation with a gene encoding NADH oxidase (for a recent review, see de Vos et al., Int. Dairy J., 1998, 8, 227-233).



Homofermentative pyruvate conversion into alanine through overexpression of alanine dehydrogenase (AlaDH) in a mutant strain of Lactococcus lactis deficient for lactate dehydrogenase (Ldh⁻).

Objectives

The GEMOLAB project aims at demonstrating that present day metabolic engineering technology can be implemented using food-grade tools and methods for the development of novel industrial dairy starters producing less acid, more flavour, and a natural sweet taste. Demonstration will also be made that the engineered strains allow a better control of the process of emmental cheese production and reduce the ammonium concentration of whey.

Work Content

- ***Workpackage 1 :*** construction of prototype dairy starters through metabolic engineering. The work will be targeted at three species of lactic acid bacteria: *Lactococcus lactis* (production of diacetyl or alanine), *Streptococcus thermophilus* and *Lactobacillus helveticus* (production of alanine).

- ***Workpackage 2 :*** industrial validation of prototype dairy starters. The engineered strains will be used for the production and conditioning of starters, and for the manufacture of dairy products, under real industrial conditions. The organoleptic properties of the dairy products obtained (buttermilk, yoghurt, emmental cheese) will be evaluated by expert tasting panels.

Related Patents

OA 1008054 "Werkwijze voor het produceren van alanine, alsmede hierbij toegepaste micro-organismen en recombinant DNA moleculen"

EP 0355036 "Werkwijze voor het selecteren en stabiel handhaven van recombinant DNA in melkzuur-bacterien"

EP 0712935 "Werkwijze voor het reguleren van de genexpressie in melkzuurbacterien"

Role of Partners

The work will be performed in an integrated way by five partners from four European countries: one University laboratory (Université catholique de Louvain, "UCL", Belgium), two Industry-funded Research Organizations (Nederlands Instituut voor Zuivel Onderzoek, "NIZO", The Netherlands, and Institut Technique Français des Fromages, "ITFF", France), one large multinational industry (Nestlé Research Center, "NRC", Switzerland) and one small-medium sized enterprise (CSK Food Enrichment, "CSK", The Netherlands).

- ***UCL*** coordinates the project and is responsible for the communication tasks (Website, Newletters and Workshops). UCL will contribute scientifically to the project by constructing prototype starter strains from two species. First, an emmental cheese strain of *L. helveticus* will be metabolically engineered so as to produce alanine instead of/in addition to lactate. Similarly, in collaboration with NRC, *S. thermophilus* starter strain of industrial interest for the production of yoghurt or emmental will be engineered so as to produce alanine instead of/in addition to lactacte.

- At ***NIZO***, novel lactococcal starter strains will be developed through metabolic engineering of the pyruvate metabolism of *L. lactis*, with a two-fold goal: first, to produce increased butterflavor (diacetyl) under aerobic conditions, and second, to produce the sweetener alanine either in combination with/instead of lactate.

- ***NRC*** will collaborate with UCL on the construction of *S. thermophilus* prototype strains producing alanine, and validate them for the production of yoghurt and related fresh dairy products. The effects of using starters engineered for the production of alanine instead of (or in conjunction with) lactate will be demonstrated in terms of the physico-chemical characteristics and organoleptic qualities of the products. Optimisation of starter composition, in particular with respect to the balance of adjunct "sweet" prototypes and conventional acidifying starters, will be achieved. The products will be submitted to expert tasting panels.

- At ***CSK***, the lactococcal starters with increased diacetyl producing properties as well as those that produce alanine will be validated in trial buttermilk fermentations as adjunct starters. The lactococcal strains that produce alanine will also be validated in a Gouda cheese model system. Furthermore, alanine producing starter will be combined with starter strains which are known to have high de-amination activity, in

order to create an endogenous ammonium source. Final cheese model and fermented dairy drink products will be evaluated by product analysis and taste evaluations. The most suited prototype starter strains will be tested at pilot scale, with specific attention for strain viability and stability.

- ***ITFF*** will be in charge of all the industrial tasks related to emmental cheese production and evaluation. Validation of starters (*L. helveticus*, *S. thermophilus*) will be on the following grounds: 1) control of the D/L lactate ratio during the lactic fermentation phase, with an impact on the rate and extent of the subsequent propionic fermentation step, through the use of *L. helveticus* starters producing a single isomer of lactate. 2) control of the production of alanine, with an impact on the taste of the product and the ammonium content of whey. The production tests will be performed at small and dairy scales, and the cheeses produced will be submitted to expert tasting panels.

Exploitation Plans and Target Groups for the Extended Audience

The partnership in this project is a combination of technology producers (molecular microbiologists: UCL, NIZO, and NRC) and users (dairy industrialists sensu lato: NRC, CSK, ITFF). There is a strong commitment on both sides to exploit and disseminate the results gained from demonstration. Patents have been taken to protect intellectual property rights on dairy starters producing diacetyl or alanine, respectively, and their industrial exploitation is ruled by a consortium agreement.

Marketing of the dairy products obtained with the engineered starters may be limited, at least in the beginning, by the consumers defiance towards GMMs. Again, there is a strong commitment in the consortium to address the public with the objective to better inform it about the issue. Thus, results of this demonstration programme will be disseminated through a newsletter, a website and on the occasion of targeted workshops, in addition to the usual channels: publications in scientific and technical journals, patents, communications at meetings etc.

Related Publications

Pascal Hols, Michiel Kleerebezem, André Schanck, Thierry Ferain, Jeroen Hugenholtz, Jean Delcour & Willem M. de Vos (1999). Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nature Biotechnology, **17**, 588-592.

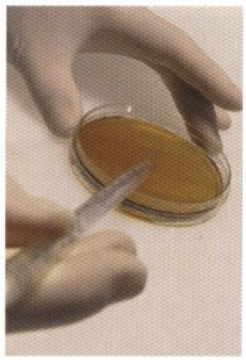
Willem M. de Vos, Pascal Hols, Richard van Kraenenburg, Evert Luesink, Oscar P. Kuipers, John van der Oost, Michiel Kleerebezem & Jeroen Hugenholtz (1998). Making more of milk sugar by engineering lactic acid bactertia. Int. Dairy J.,**8**, 227-233.

Description of Partners

Université catholique de Louvain (UCL)

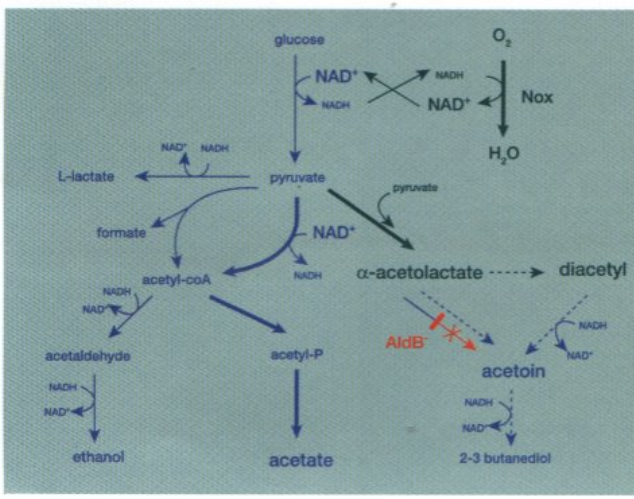
The Université catholique de Louvain is located in two different places in Belgium. The main campus (9 Faculties) is in Louvain-la-Neuve, a new city built from scratch in the mid-sixties in the countryside 30 km south of Brussels following a move from the old Flemish city of Leuven (Louvain in French) where it was established since its foundation in 1425 by a bill from Pope Martin V. The Faculty of Medicine, together with the 800-bed university hospital and the Schools of Pharmacy and of Physical Education, have their own settlement in Louvain-en-Woluwe, a southern suburb of Brussels. Altogether, the 10 UCL faculties host 50 departments holding more than 200 research units, which cover a full spectrum of disciplines ranging from theology to nuclear physics. More than 20,000 students are registered each year at UCL, including about 2,000 foreign citizens.

UCL participates to GEMOLAB through its Unit of Genetics, which has been active in the field of molecular genetics of lactic acid bacteria for the last 10 years with the support of several grants from the Framework III and IV EC Programmes. The main current themes of research are the following: 1) metabolic engineering in *L. plantarum*, *L. helveticus*, *L. lactis*, and more recently *S. thermophilus* (knockout of various genes involved in fermentation pathways and cell-wall synthesis; introduction of foreign genes providing new fermentation pathways); 2) heterologous expression and genetic containment of *L. plantarum* strains engineered to be used as live mucosal vaccines; 3) molecular genetic analysis of regulatory mechanisms induced by various stresses (cold-shock, exposure to oxygen). More recently, the complete genome sequencing of a yoghurt strain of *S. thermophilus* has been endeavoured in collaboration with the group of A. Goffeau.



Nedrlands Instituut voor Zuivel Onderzoek (NIZO)

NIZO food research is an international contract research organisation for food, feed and pharma. Within our company we are focusing on applicable and fundamental research in the field of food science. Three core areas of expertise are combined, namely knowledge of microbial systems, of biopolymer systems and of process systems. In the research area of microbial systems, NIZO food research aims at the production of natural ingredients, influencing product properties such as taste, shelf life and structure. Research towards molecular biology and physiology of lactic acid bacteria is one of the core competences of NIZO food research. Other important fields of activity are



Increased pyruvate conversion into diacetyl through overexpression of NADH oxidase (Nox) in a mutant strain of Lactococcus lactis deficient for acetolactate decarboxylase (AldB-)

optimisation of nutritional value and product quality. With biopolymers, emphasis is placed on the control and management of carbohydrate and protein properties during preparation, storage and consumption of food. Within process systems, research is focused on the development of new technologies and the improvement of existing processes in the field of separation technology and down-stream processing of fermentation liquids.

Nestlé Research Center (NRC)

Nestlé Research is as old as the Company itself, that is 130 years. The very first product which was at the birth of the corporate history in 1867, "Farine lactée Nestlé" (Nestlé's milk food), was the result of research. It was due to the spirit of innovation and entrepreneurship of Henri Nesté, a pharmacist from Germany, who was both scientist and businessman. Thus, from the first industrially manufactured infant formula in 1867 and the first soluble coffee "Nescafé" (1938) to the first probiotic yoghurt of our time "LC1", there is a recurrent series of breakthrough inventions resulting from Nestlé research. However, research does not only consists of highlights. The constant improvement of existing products is just as important as the invention of new ones. Innovation as renovation go hand by hand.

Henri Nestlé's one-man laboratory has developed into today's world-wide network of 18 research and development center, with a total staff of 3,500 people in eleven countries on four continents. About a quarter of this staff works at Vers-chez-les-Blanc, the heart of this network. The Nestlé Research Center is primarily devoted to basic research, while applied research and the development of new products and technologies is the responsibility of the 17 decentralized centers, each of which concentrates on one or more special research fields.

A research effort which is unique in the food industry – with an investment of some 700 million Swiss francs per year – allows Nestlé to be constantly on the cutting edge of development and to offer consumers in the more than hundred countries where its products are marketed, healthy, tasty and varied high-quality foods which also suits the different traditions and tastes in each of these countries – another core objective of Nestlé research.

CSK Food Enrichment (CSK)

CSK food enrichment B.V. is a Dutch-based company involved in the production and marketing of ingredients and processing aids for the food industry, in particular the dairy industry. For fermented milkproducts like cheese, yoghurt and buttermilk CSK food enrichment B.V. produces rennet, cheese coatings, starter cultures and lactic acid permeate (for butter production). For the latter two products CSK food enrichment B.V. is strongly involved in fermentation processes to obtain biomass and ingredients. The company has a lot of experience in the application of the obtained products (application of starters, yoghurt- and butter-making processes, etc.) and technological know-how (e.g. down-stream processing) enables the company to develop new production processes.

Research involving lactic acid bacteria is mainly focused on the development of new starter- and adjunct cultures to be applied in fermented milkproducts. Functional properties of the cultures are investigated, like flavour formation and growth profiles, and tested for industrial application. Because of CSK food enrichment B.V. strong interest in products obtained from fermentation processes it has the intention to develop and produce new ingredients.

Institut Technique Français des Fromages (ITFF)

Institut Technique Français des Fromages (ITFF) [previously Institut Technique du Gruyère (ITG)] was founded in 1967 by hard-cooked-cheese professionals as a technical centre for cheese factories. Only cheesemakers form its Board of Directors. Since 1998, delegates of Centre National Interprofessionnel de l'Economie Laitière (CNIEL-F) and other Cheese Unions have joined this Board. The Institute, whose President is M. Patrick RAMET - a milk producer - has 60 people among whom 15 experts, 13 engineers and 6 Ph.D. scientists. In the field of research, ITFF leads studies on the abilities of milk to cheese making, cheese processes, starters and microbial ecosystem of cheeses, mechanisms of ripening. Basic research at ITFF is done on the influence of lactic acid bacteria (LAB) and propionibacteria (PAB) on the cheese quality, to evaluate their technological properties and to select available strains. LAB and PAB research laboratories are located in La-Roche-sur-Foron and Rennes where, respectively, a cheese pilot plant (140- to 3,000-l cheese vats) and mini-cheese equipment (12-l vats) are used in starters studies. For this purpose, ITFF develops many partnerships with French and European research teams. Thermophilic LAB and PAB cultures selected by ITFF for the emmental cheese industry are marketed by Standa Industry (Caen - France). Daily, ITFF experts carry out technical audits at the cheese-makers' request to improve technological processes and quality. "Les Maisons du Goût" are the network of five ITFF's laboratories, which make sensorial analyses of cheese and food products. ITFF is also in charge of training. Courses in dairy or food schools alternate with inter- and intra-company sessions, as well as individual courses. Finally, ITFF publishes "La Documentation Fromagère Résumée" (100 abstracts per year) where the international cheese literature is reviewed.

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