Gourmet Genomics – Part 1

'Aromics' of Wine and Cheese

What's the molecular basis that makes gourmet food worth paying for? A question, now ripe for analysis, courtesy of the advances in high throughput genomics. The first part in this issue summarises the efforts on cheese and wine. The second part in the next issue will focus on a delicacy so expensive that many people have never even tried it: truffles.

The traditional image of gourmet food is one that is closer to the organic food movement than that of the laboratory – it is a celebration of the aromas, textures and flavours that exist "naturally" in food without the addition of chemical flavour enhancers and preservatives. When you pay more for a gourmet food item (ideally purchased from a market stall or specialised boutique, or eaten in a reputed restaurant), you expect a real gourmet experience with more taste and aromas and less of the afterthought that it might have been industrially sprayed and processed. Therefore, at least the image of genetically modified organisms is at odds with the elite status of gourmet food.

Nevertheless, luxury foods are increasingly being included in biotechnology research that aims at improving their quantity and quality. The reasons? High throughput genomics has since become a lot cheaper and there is a lot of commercial interest in the higher prices that consumers are prepared to pay for luxury foods. Take, for example, wine and cheese.

Wine

Wine is an alcoholic beverage made of fermented grape juice, a tradition from the Middle East and Southern Europe that goes back at least 7,000 years. It is produced by fermenting crushed grapes, using various types of yeast, which consume the sugars found in the grapes and convert them into alcohol. Until the 19th century, wine was regarded as the only storable, wholesome beverage.

Different varieties of grapes (*Vitis vinifera*) and strains of yeasts are used depending on the types of wine being produced. Red wine is made from the must (pulp) of red or black grapes that undergo fermentation together with the grape skins, whereas white wine is usually made by fermenting juice pressed from white grapes but can also be made from red grapes by keeping contact with the grapes' skins to a minimum. Rosé wines are made from red grapes where the juice is allowed to stay in contact with the dark skins just long enough to pick up a pinkish hue but little of the tannins contained in the grape skins.

During primary fermentation, which often takes between one and two weeks, yeast converts most of the sugars in the grape juice into ethanol. After the primary fermentation, the liquid is transferred to vessels for the secondary fermentation. Here, the remaining sugars are slowly converted into alcohol and the wine becomes clear. Some wines are bottled directly, others are aged in oak barrels before bottling – this adds extra aromas to the wine. The time from grape harvest to drinking can vary from a few months for Beaujolais nouveau wines to over twenty years for top wines. However, only about 10% of red and 5% of white wine will taste better after five years than they do after just one year. With sparkling wines such as Champagne, an additional fermentation takes place inside the bottle, trapping carbon dioxide and creating the characteristic bubbles.

The single most important factor in winemaking is the organoleptic quality (appearance, aroma and flavour) of the final product. The endless variety of flavours stems from a complex, completely non-linear system of interactions among many hundreds of compounds - terpenes in the aromatic grape varieties and alkoxypyrazines in the herbaceous cultivars, while the products of yeast fermentation (esters and alcohols) contribute to the generic background flavour and aroma, as well as to the complexity and intensity of the aroma and taste in the final product.

The elite tradition of fine wines dates from the mid-nineteenth century when French vineyards, particularly in the Bordeaux and Burgundy regions, institutionalised rules for the production of their best red wines, the 'grand crus'. Annual wine production is around 27 billion litres from eight million hectares of vineyards with France, Italy and Spain accounting for 60%. However, more wine is produced than drunk – up to five billion litres too much! Furthermore, the consumer market has increasingly turned towards better quality wines but is reluctant to spend large amounts on the traditional brand names. Hence, the arrival of 'New World' wines from countries like the US, Australia, Chile, Argentina and South Africa, who are proposing 'standardised' quality wines at a lower price than European producers.

However, planting the right grape variety in a particular area is of serious economic importance: a newly planted vine must grow for several years before producing grapes and can stay in service for up to 50 years, which makes it a life-time investment.

Grapevine Genomics

In 2007, the complete grapevine (*Vitis vinifera*) genome sequence was announced twice. Two different consortiums working independently of each other sequenced the genomic DNA of the Pinot Noir wine grape variety. It is the first fleshy fruit crop, the second woody species and one of only four flowering plants to be sequenced.

The first to publish were the French-Italian Public Consortium for Grapevine Genome Characterization, coordinated by the French INRA (national agricultural research institute) station in Colmar, the Genoscope sequencing facility at Evry and the Italian Ministry of Agriculture's VIGNACRA initiative. Their assembled 487 Mb of DNA sequence was obtained from a Pinot Noir grapevine, PN40024 that had been bred to near homogeneity (around 93%).

They found 30,434 protein-coding genes (an average of 372 codons and 5 exons per gene), considerably less than the 45,555 protein-coding genes reported for the poplar tree (Populus trichocarpa) genome, which has a similar size, at 485Mb, and even lower than the 37,544 protein-coding genes identified in the 389Mb of the rice genome.

Grapevines, like the flowering plant, *Arabidopsis thaliana*, and the poplar tree, are dicotyledonous plants that diverged from monocotyledons about 130-240 million years ago. Comparative analysis of their genomes with the monocotyledon, rice, has led to the discovery of three ancestral genomes. It appears that grapevine, *Arabadopsis* and the poplar share a heritage with a "palaeo-hexaploid" organism, whose diploid content corresponds to the three full diploid contents of three ancestor plants. This "palaeo-hexaploid" plant evolved after the separation of the mono- and dicotyledons.

However, of more interest to wine-growers, the resulting grapevine genome sequence clearly carries the imprint of millennia of selective breeding. For example, there are 116 genes and pseudogenes for terpene synthases, almost three times the number in the other three plant genomes so far sequenced. These enzymes synthesize the terpenoids that contribute to the aroma and flavour of wines, and pathways associated with tannins are similarly amplified. This suggests that it may become possible to trace the diversity of wine flavours down to the genome level.

A less obvious target of selectivity are the genes that control the synthesis of resveratrol – this is the antioxidant that has been credited with the heavily advertised health benefits claimed for red wine following the discovery of the 'French paradox': the observation that the French suffer a relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats like foie gras, rich cheeses and other gastronomic excesses. The stilbene synthase genes associated with resveratrol synthesis are also moderately expanded in grapes, compared to other plants.

A key environmental/economic concern for grapes is the lack of resistance to the emergence of aggressive races of micro-

organisms that are currently controlled by massive use of agrochemicals (grapes are sprayed on average 12 times a season with pesticides and fungicides). A high number of genes related to disease–resistance have been identified. Many of them have been mapped to linkage groups and a large part of them are tagged with one or more single nucleotide polymorphisms (SNPs). However, these resistance genes did not co-evolve in the presence of the most important grape pathogens, a condition, which may not have sufficiently protected the species. The hope is that a deep knowledge of the grape genome may represent a new starting point for developing genetic strategies to counter pathogens. But the problem is not simple. How do you modify a complex and highly heterozygous genome without altering wine quality? Fortunately, the fertility of hybrids between wild and domesticated grape species, with 19 seemingly co-linear chromosomes, makes it feasible to introduce new resistance genes via traditional breeding but there are already many attempts at developing genetically modified (GM) grapevines.

Bacchus: His secrets revealed?

However, from the second grape genome project, Riccardo Velasco, of the Istituto Agrario di San Michele all'Adig in Trento, Italy, insists, "Our goals are on assisted breeding or more advanced breeding by design. Therefore, the huge amount of markers developed in the project and their link to genes, and, consequently, phenotypes, is fundamental." But he added, "The viticulture and oenology world is not ready to accept GM grapes."

GM Grapevines

Nonetheless, field trials on transgenic grapes have been going on for over a decade with some of the earliest in France, until fear of a backlash from the public caused the French champagne producer, Moët & Chandon, an early adopter of the technology, to abandon its programme in 1999. Transgenic grapes have undergone field testing in Italy (modified auxin production), Germany (fungal resistance), France (grapevine fanleaf virus resistance) and Australia (fruit quality and colour modifications). In the US, GM grapes are being developed for resistance to diseases including powdery mildew, Botrytis, Agrobacterium, Clostridium, Xylella, nepovirus and closterovirus.

In Chile, they have created transgenic table grapes with enhanced fungal resistance, targeting table grapes for the American market. "Wine producers use tradition as a marketing tool," says Patricio Hinrichsen, from the Chilean group. "With table grapes, it's a completely different world. There will be people who will not be worried about these issues."

Given these developments, it is hardly surprising that DNA fingerprinting of wine is being explored as a means of protecting areas of declared origin and for detecting potential transgenic events. Although degraded, it appears there is still enough DNA in wine to make an analysis.

GM Wine yeasts

However, there has also been extensive research on improving the performance of the yeast used to ferment wine. The emphasis is on the development of *Saccharomyces cerevisiae* strains with faster fermentation, processing and biopreservation abilities, and capacities for an increase in the "wholesomeness" and sensory quality of wine. Historically, winemakers have used spontaneous fermentation, allowing the yeasts and bacteria living on and in the grapes to do the work. However, as the phrase implies, spontaneous fermentation is an uncontrollable process; whole ecosystems of microorganisms live on the grapes and help to produce complex and interesting wines but can also spoil the process.

Purified yeast strains, which became available at the end of the nineteenth century, allow producers to have more control over what happens in the fermentation tank.

The US Food and Drug Administration has already approved two genetically modified yeasts for wine fermentation: GM yeast (ML01) is commercially distributed on the market. It contains a malolactic enzyme gene from the lactobacillus *Oenococcus oeni,* which, during fermentation, allows it to transform sour tasting malic acid into a milder lactic acid. It is said that wine made with ML01 yeast distinguishes itself not only through its better taste and colour stability but also through a lower concentration of histamines. The extent of actual use of ML01 yeast by US American vintners is unknown. Many of them, especially from California, have jointly declared that they are not using GM organisms in their wine. The other GM yeast contains a gene encoding urea amidolyase to prevent the formation in red wine of the suspected carcinogen, ethyl carbamate.

Cheese

In European and Middle Eastern history, cheese has a long tradition dating back to the domestication of sheep some 10,000 years ago, when its discovery represented a means of conserving milk. The earliest cheeses were probably sour, salty and had a crumbly texture similar to Greek feta. Egyptian tomb murals from 2000 BC show cheesemaking and in Homer's Odyssey (800 BC), the one-eyed Cyclops is busily making and storing sheep's and goat's cheese just before Odysseus pokes his eye out.

Several hundred different cheeses are produced in Europe but industrial cheese production, accounting for most of the world's annual 13 million tons, has reduced the choice. Although the US produces the largest quantity of cheese (about 30%), their qualitative approach to cheese manufacturing and consumption isn't quite that of countries like France and Italy. It is no surprise to learn that American cheese is not exported – just consider that a whole third of US cheese is a strange compacted form of mozzarella for pizzas! Much of the rest is "processed" cheese, made from "normal cheese" mixed with other unfermented dairy ingredients, emulsifiers (so that they melt more evenly on hamburgers!), extra salt, food colourings and/or whey. For European cheese fans and their long heritage, processed cheese is something of a culinary disaster!

Nevertheless, it is true that an appreciation of cheese flavours is very much a question of acquired taste – cheese is produced by a process of controlled spoilage such that many of the odour and flavour molecules in a mature cheese are the same as those found in rotten foods. Or, as an American food science writer put it, "An aversion to the odour of decay has the obvious biological value of steering us away from possible food poisoning, so it is no wonder that an animal food that gives off whiffs of shoes and soil and the stable takes some getting used to!"

Cheese basically consists of proteins and fat from milk, usually the milk of cows, buffalo, goats or sheep. But to understand the role of genetic and genomic research in identifying the origins of more complex cheeses, one needs to appreciate the astonishing complexity of microbial fermentation and enzymatic activity critical to the development of cheese's different flavours and textures.

The microbia

Cheese processing begins with curdling – milk is coagulated by the enzymatic action of rennet (principally the protease, chymosin) under acid conditions to give the solid curd and liquid whey. Rennet is a natural complex of enzymes produced in the mammalian stomach to digest mother's milk. Traditional cheese rennet comes from the inner mucosa of the fourth stomach of young calves. However, microbial rennet also exists (suitable for vegetarians) and chymosin from genetically modified organisms was the first artificially produced enzyme to be registered and allowed by the US Food and Drug Administration. Most US hard cheese contains genetically engineered chymosin, sometimes combined with rennet, to obtain cheese with more flavour, whereas traditional European cheese continues to include the more expensive (and complex) animal rennet.

Acidification of the milk is accomplished by the addition of starter bacteria (usually from the Lactococci, Lactobacilli or Streptococci families) that ferment lactose into L-lactic acid. The same bacteria (and the enzymes they produce) also play a large role in the eventual flavour of aged cheese. Lactic acid itself is also a component of cheese flavour and in Swiss-type cheese serves as a key nutrient for propionibacteria, such as *Propionibacterium shermani*, which convert lactic acid to propriionic acid producing the carbon dioxide gas bubbles during aging that give the holes in a particular Swiss cheese.

The microbia

Fresh cheese can be curdled only by acidity but most cheeses also use rennet. Rennet sets the cheese into a strong and rubbery gel compared to the fragile curds produced by acidic coagulation alone. It also allows curdling at a lower acidity, important because flavour-making bacteria are inhibited in high-acidity environments. Some soft cheeses are now essentially complete: they are drained, salted and packaged. For most of the rest, the curd is cut into small cubes.

Certain hard cheeses are then heated to temperatures of 35°C–55°C. This forces more whey from the cut curd and changes the taste of the finished cheese, affecting both the bacterial culture and the milk chemistry. Cheeses that are heated to the higher temperatures are usually made with thermophilic starter bacteria, because they survive this step, either lactobacilli or streptococci.

Salt has a number of roles in cheese besides adding a salty flavour. It preserves cheese from spoiling, draws moisture from the curd and firms up a cheese's texture in an interaction with its proteins. Some cheeses are salted from the outside with dry salt or brine washes.

Cheese gourmet or cheese researcher?

The curds are pressed into a mould to give the cheese its final shape. They are now left to rest under carefully controlled conditions, stored at low temperature for months or years to attain their characteristic flavour and body attributes. During storage, the microorganisms and enzymes that are trapped in the cheese matrix act on carbohydrates, citrate, proteins and lipids in a manner that is heavily influenced by the curd microenvironment and which ultimately yields distinct types of cheeses.

Some cheeses have additional bacteria or moulds intentionally introduced to them before or during aging. In traditional cheesemaking, these microbes might be already present in the air of the aging room where they are simply allowed to settle and grow on the stored cheese. More often, prepared cultures are used, giving more consistent results and putting fewer constraints on the environment where the cheese ages. These include soft-ripened cheeses, such as Brie and Camembert, which begin firm and rather chalky in texture but are aged from the exterior inwards by exposing them to mould - velvety blooms of *Penicillium candida* or P. camemberti. Blue cheeses such as Roquefort, Stilton and Gorgonzola are created by inoculating a cheese with *Penicillium roqueforti* or *P. glaucum*.

Cow genetics and transgenic milk

Although the cow's genome has just been determined, genetics has already been used to "improve" the quantity and quality of cow's milk for cheese production. K.F. Ng-Kwai-Hang, from Canada's McGill University, has spent the last 25 years studying the genetics of cows and how this affects the quality and type of cheese. By looking at the genetic profile of cows, he can predict, which one will produce the best cheese and has identified specific milk protein genes that affect cheese yield, composition and quality. There are about 50 known milk protein gene variants that have diverse effects on dairy product production, including two mutations in kappa-casein associated with a higher yield of cheese and "better quality".

Milk casein consists of a complex of alpha caseins 1 and 2, beta casein and kappa casein, which together form a colloidal complex of microscopic, bubble-like micelles.

Kappa casein is the protein that encapsulates the casein micelles; beta casein binds calcium. In 2003, Goetz Laible from AgResearch in New Zealand, published a paper in *Nature Biotechnology* announcing the first transgenic cows whose milk could "increase the speed and ease of cheesemaking". Laible's team inserted extra copies of the beta and kappa casein genes into fibroblast cells from the lung tissue of a female calf foetus, itself resulting from the cross between an elite, progeny-tested Friesian bull and a Friesian cow, selected for exceptional milk yield. They fused the transgenic fibroblasts with enucleated oocytes to produce transgenic calves that produced higher casein levels in their milk – up to 8%-20% more beta casein and twice as much kappa casein. Overall, their milk protein content rose to 5.65%, compared to the 5% normally found in cow's milk, leading to potential economic gains – more cheese from less milk! Laible said that his results showed that it is feasible to substantially alter a major component of milk in high-producing dairy cows by a transgenic approach and,

thus, to improve the functional properties of dairy milk. Notably, he claimed that his transgenic milk could improve the cheesemaking process because raising beta casein levels might reduce the clotting time of the rennet and increase expulsion of the whey, while higher levels of kappa casein could increase the overall heat stability of the cheesemaking process. A moratorium on GMOs in New Zealand still holds so we have not yet seen any of Laible's theoretically "improved" cheese. Nevertheless, research continues worldwide on modifying the milk of transgenic cows but this is primarily to use cows as large bio-reactors for the generation of pharmaceutically interesting recombinant proteins in their milk.

The microbial complexity of cheese

Lactic acid bacteria (LAB) are essential for flavour development in cheddar and other cheeses. They may include deliberately added starters, adjunct bacteria and adventitious species, called non-starter lactic acid bacteria (NSLAB), that enter curd from the processing environment. Research in this area has mostly focussed on *Lactococcus lactis*, which serves as the starter bacterium for Cheddar, Gouda and many other cheeses, and on dairy-related species of *Lactobacillus*.

The number of starter bacteria commonly exceeds 100 per gramme of cheese when ripening begins, but the microenvironment of ripening cheese is harsh - it is typified by an absence of residual lactose, high levels of NaCl, low pH and low temperature. These conditions extract a toll on starter viability and a sizable fraction of the starter cells undergo autolysis, which releases intracellular enzymes and other cellular components into the cheese matrix, where they too can influence ripening. Meanwhile, NSLAB populations begin to grow and eventually plateau after three to nine months of aging.

Proteolysis and its secondary reactions also play a major role in bacterially ripened cheese, making casein hydrolysis and its relationship to flavour development an area of intense research interest. The hydrolysis of intact caseins is almost exclusively catalysed by the coagulant and endogenous milk proteinases, while microbial proteinases and peptidases produce water-soluble peptides and free amino acids. A growing body of evidence indicates that LAB's conversion of free amino acids into aroma compounds is the rate-limiting step in the development of mature cheese aromas.

Genomes of cheese microbes

Genome information for the first of several industrially important LAB starter species appeared in 2001, when the genome sequence of *Lactococcus lactis subsp. lactis* was released by a French INRA/Genoscope team. The genome is 2.4Mb and contains 2,310 predicted genes, of which 16% appear to be unique to this bacterium. Twelve aminotransferases were identified, some of which break down complex, branched, ring-shaped and sulphur-containing amino acids. Another 29 genes were identified that build up the bacterium's cell wall.

At least 30 genome sequence projects for other important dairy-related bacteria have since been announced or completed, including sequence information for more than one strain of a particular species. For example, INRA in France has also sequenced *L. casei*, *L. delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, while Nestlé in Switzerland have worked on *Bifidobacterium longum* and *L. johnsonii*. There are also projects in Belgium (*S. thermophilus*), Ireland (*L. helveticus*) and the Netherlands (*Propionibacterium freudenreichii*, *L. plantarum*).

They're looking for the molecular basis of commercially significant, strain-dependent properties, such as the ability to produce specific flavours, propensity for autolysis, acidification rates and cell vitality in frozen or lyophilized starter concentrates. For example, the genome sequence of L. helveticus has quadrupled the number of known enzymes in the proteolytic system with the identification of ten new proteases, five endopeptidases, eight aminopeptidases, six new Di-Tripeptidases and nine enzymes in oligo- and di-tripeptide transport systems.

New bacteria in Reblochon

However, the search for all of the microbes involved in cheese manufacture isn't finished yet. In December, 2008, Michael Goodfellow from the UK's University of Newcastle announced the discovery, by DNA fingerprinting, of eight previously unknown bacteria on the French mountain cheese, Reblochon, at the late stage of ripening. Reblochon is 'smear-ripened', that is, the surface of the cheese is washed with a salt solution containing bacteria that ripen the cheese from outside-in. The new gram-positive, aerobic, non-sporulating, rod-shaped bacteria were named *Mycetocola reblochoni*, and it appears that they are responsible for much of the cheese's characteristic flavour acquired during the ripening process.

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