



**bio**watch

SOUTH AFRICA biodiversity | food security | biosafety | social justice

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## Response to the objections of BioWatch SA

Hennie JJ van Vuuren (Ph.D.)

### ***Objection to application from Professor Hennie JJ van Vuuren for permission to apply genetically enhanced malolactic wine yeast ML01 for the commercial production of wine in South Africa***

Thursday, 30 November 2006

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#### ***Others supporting this objection***

*This objection, prepared by Biowatch South Africa, is supported by:*

1. Anthony Hamilton Russell, Hamilton Russell Vineyards
2. Anton Du Toit, Lourensford Lanzerac Wines
3. Etienne Le Roux, winemaker Winecorp Holdings Pty Ltd (Spier Vineyards)
4. Adian Fry, winemaker Tukulu Wines
5. Conrad Vlok, Agulhas Wines
6. Johnathan Grieve and Bertus Albertyn, Avondale Vineyards
7. Jan Klue, Wolvendrift
8. Lindi Broughton, Rusticus Vintage Cellar
9. Wayne Gabb, Lomond Wine Estate
10. Rob Armstrong, Haut Espoir
11. Hannarine Bester, Quality Manager, Waverley Hills Organic Wines

#### ***Introduction***

*The use of genetically modified yeast in the production of wine in South Africa is likely to have disastrous consequences for South Africa's wine industry. There is a ban on genetically modified wine and overwhelming rejection of all genetically modified food and drink by consumers in Europe – an important export market for South African wine.*

Yeasts are regarded as processing agents in the USA, Canada and also in Europe. According to European regulations, labeling is required only when the final product contains more than 0.9% of the GM product. Even in unfiltered wines, yeast will not comprise close to 0.9% of the wine. An analogous product that has been fully accepted by consumers around the world is the use of the genetically engineered enzyme, chymosin (rennet), which is used for the production of most of the world's cheeses. This enzyme is produced by a genetically engineered yeast strain; the enzyme remains in the GM cheese produced in Europe, South Africa and other countries around the world. The enzyme is

also seen as a processing aid and none of the GM cheeses produced with this enzyme requires labeling.

I fully understand the position of the South Africa wine industry, the Australian wine industry and the California Wine Institute. I was involved in a two-day workshop organized by the California Wine Institute on the use of genetically engineered yeasts and grape vines before they made a decision how to handle this situation. This workshop was attended by many prominent scientists (not only from the wine industry). The use of ML01 featured prominently. I believe it is prudent for the South African wine industry to state that they do not support the use of ML01 at this stage because of possible consumer resistance. However, the presence of bioamines and ethyl carbamate (a carcinogen in alcoholic beverages and food) is of great concern to consumers and the wine industry. Switzerland is the first country to ban the sale of wines that contain bioamines that exceed a certain concentration. I have had a delegation from Germany in my office that consulted with me on the use of ML01 to prevent the formation of bioamines. Many consumers get sick when they ingest food or alcoholic beverages contaminated with high levels of bioamines, which places an extra financial burden on health providers and governments in all countries making health care unsustainable. Scientists, food producers and wineries have a responsibility to ensure that foods and alcoholic beverages consumed by the public do not contain ANY compounds that could have a detrimental effect of the health of consumers. The ML01 yeast will provide significant benefits to consumers who are sensitive to bioamines and will prevent spoilage of wines that undergo sluggish or stuck bacterial malolactic fermentations; efficient malolactic fermentation by ML01 will prevent spoilage of wines by other microorganisms which will have significant financial benefits to wineries.

*There have been reports that the Australian wine industry has taken an official stand against the genetically modified yeast. Even in the United States of America, where the yeast has received a Generally Regarded as Safe status from the Food and Drug Agency, Californian winemakers are reported to have issued a statement saying that no genetically modified organisms would be used in their wine production.*

The California Wine Institute simply stated that they do not support the use of GMO's. The Australian wine industry stated that "in terms of health risks there should be none. It would seem from balancing some of the more obvious risks and benefits associated with the use of ML01, that having access to this yeast might be a good thing for Australian winemakers". <http://www2.awri.com.au/infoservice/media/releases/nogogmo.asp>

*Several surveys conducted in different countries to test consumer attitudes to genetically modified foods indicate that the more consumers know about genetically modified crops, the less accepting they are of these foods. This suggests that consumer acceptance of genetically modified food and drink is unlikely to change any time soon.*

Until recently the vast majority of genetically modified cells have only benefited the producer with no or little benefit to consumers. In addition, anti-GMO lobbyists have used scare tactics to frighten consumers. It is therefore not surprising that consumers up to now have not supported products obtained with gene technology. The bacterial malolactic fermentation is notoriously unreliable often resulting in sluggish or stuck malolactic fermentation that leads to the production of biogenic amines (neurotoxins) that cause headaches and other allergenic symptoms such as, migraines, hypotension, oedema,

palpitations, flushing, vomiting, diarrhea and hypertension (Wantle et al., 1994; Santos, 1996; Soufleros et al., 1998). Furthermore, alcohol, anti-depressive drugs and other biogenic amines such as cadaverine and putrescine enhance the toxic effect of histamine, tyramine and phenylethylamine (ten Brink et al., 1990; Straub et al., 1995). Biogenic amines are also linked to carcinogenesis and histamine, putrescine, spermidine and spermine can induce cell transformation and tumour pathogenesis (Medina et al., 1999; Pryme et al., 2001; Wallace et al., 2001). It can hardly be argued that these reactions are beneficial for consumers.

The use of the wine yeast ML01 to conduct the malolactic fermentation will have a direct benefit to consumers since the production of toxic biogenic amines produced by malolactic bacteria, can be prevented. In addition, ML01 will prevent spoilage and off-flavours caused by delayed sulphiting of wines to the benefit of wineries.

*So, it is not surprising that crucial organisations representing the South African wine industry have rejected the genetically modified wine yeast application in public statements.*

*The application to apply the genetically modified yeast ML01 to 20 wine-producing regions in the Southern and Western Cape is likely to engender general suspicion among consumers, especially in South Africa's key export markets, about the possible presence of genetically modified organisms in conventional wine originating from South Africa. It is also likely to jeopardise the organic wine sector.*

*There is a real possibility of the genetically modified yeast contaminating the microbial biodiversity of areas within wineries and beyond – through, for instance, waste water disposal. The effects of the genetically modified yeast on microbial biodiversity (which plays an important part in wine growing and making) are unknown.*

The ML01 yeast is identical to the parental strain (Prise de Mousse – S92), widely used by the industry in South Africa and around the world, except for its ability to conduct the malolactic fermentation. The ML01 strain contains the malolactic gene from *O. oeni* and the malate transport gene from *S. pombe*; both organisms are used for the deacidification of wine on a commercial scale. No genetic material or proteins foreign to the wine making process have therefore been inserted into the Prise de Mousse strain. Furthermore, the malolactic yeast ML01 does not contain any antibiotic resistance marker gene and ML01 does not have a selective growth advantage over the parental strain. The environmental impact of ML01 strain will therefore not be any greater than the environmental impact of the industrial S92 strain and *O. oeni*. Commercial yeasts such as the parental strain S92 are annually released in large quantities into the environment surrounding wineries. Recently, a large-scale three-year study of six different vineyards revealed that dissemination of commercial yeast in the vineyard is limited to short distances over short periods of time (Valero et al., 2005). Despite the annually intensive dissemination of commercial yeast into the local environment, 94% of the commercial yeast strains have only been found between 10 to 200m from the winery. This underscores the limited range of possible environmental impact beyond the winery. Moreover, analysis of population variations from year to year indicated that commercial strains do not settle in the vineyard or predominate over the indigenous flora (Valero et al., 2005). It has also been shown that colonisation of damaged grapes, where the modified ecology favours fermenting yeast species, by a selected *S. cerevisiae* wine strain is no different from colonisation of undamaged grapes (Comitini and Ciani, 2006). In both cases the inoculated wine strain could not out-compete the indigenous microflora resident on the grapes (Comitini and Ciani, 2006).

*The yeast may also have negative effects on human health, depending on the amount of yeast consumed – which in turn will depend on the sophistication of the wine filtering process. The novel, genetically modified, yeast could cause increased yeast infections in humans, could alter human intestinal bacteria, which could result in disturbances in digestive function. Allergic reactions could also arise if some of the proteins produced by the genetic modification are present in the wine.*

There is no scientific justification to propose that ML01 will cause allergic reactions if some of the proteins produced by the genetic modification are present in the wine. The modifications will produce the same proteins that are present in wine deacidified with malolactic bacteria or *S. pombe* (*mae1*) (Silva et al., 2003). However, there is scientific evidence to propose that the application of ML01 will prevent allergic reactions (that can result in disturbances in digestive function) by reducing biogenic amines produced by lactic acid bacteria.

***Detailed scientific objections to the release of transgenic ML01 Brewer’s yeast (Saccharyomyces cerevisiae).***

*The GMO under study, ML01, is a Saccharyomyces cerevisiae derived from the S92 isolate and engineered with two transgenes. The mae1 gene from the yeast Schizosaccharomyces pombe and the mleA gene from bacteria, Oenococcus oeni. Together the expression of these genes (under control of the Saccharyomyces cerevisiae PGK1 promoter) allows the transport and conversion of malate to lactate.*

*The aim is to engineer a yeast that can carry out the alcohol fermentation and malolactic fermentation in a single process. Traditionally, there is a two-stage process with an initial alcohol fermentation followed by a secondary bacterial malolactic fermentation that it leads to the deacidification and microbial stabilization of wines together with subtle changes in flavour and colour.*

*There are potential health benefits with this type of fermentation since some naturally occurring O. oeni starter cultures and other lactic acid bacterial strains produce compounds, such as biogenic amines that are the causative agent of head-aches and some other allergic symptoms (see Husnik et al., 2006, Lonvaud-Funel, 2001). However, there are certain biosafety risks to human health and the environment that have not been addressed:*

***Spread of ML01 transgenes to other yeasts in the environment***

*The elements of the transgenic cassette (PGK1, mae1 and mleA genes have homologous sequences in other bacteria and yeast) and therefore there is an increased probability of horizontal gene transfer to these other species. The effects on microbial biodiversity are unknown and there are no provisions of the applicant to monitor this effect on biodiversity (see e.g. Section 11.2 and 11.4).*

*The monitoring of these effects is important since numerous bacteria and yeast are found in vineyards where they form integral parts of the soil and plant ecosystem. These “wild” microorganisms may also significantly contribute to the wine-making process, particularly where traditional methods of wine-making are carried out. Despite the fact that evidence suggest contamination of the environment is limited to a few hundred (10-200m) of the winery (Valero et al 2006), the likelihood of escape and fitness in the field must be monitored, as required by the Convention on Biological Diversity and South Africa’s National Environmental Management Act.*

The PGK1 promoter and terminator sequences (present in wine yeast) as well as the malolactic and malate transport genes (*mae 1* is present in *S. pombe* and a malate permease gene *mleP* and the *mleA* gene are present in *O. oeni*) were isolated from microorganisms present in wine.

Due to the massive world-wide production and utilisation of millions of tonnes of baker's yeast, brewer's yeast and wine yeast annually, uncountable DNA fragments containing *PGK1* regulatory sequences have been dispersed into the environment over centuries. Furthermore, malolactic bacteria that have been employed by the wine industry for centuries, reach populations of  $10^9$  cells /ml in wine undergoing malolactic fermentation. Based on  $10^9$  cells /ml of wine, its impossible to calculate the large number of copies of the *O. oeni mleA* gene (malolactic enzyme) and the *mleP* gene (malate permease) that are present in the winery environment. The same is true for the *mae1* gene from *S. pombe*. If it is indeed true as stated by Biowatch that there is a probability of horizontal gene transfer to other species, this will have already occurred in all likely hood and the presence of the *PGK1* elements and the *mae1* and *mleA* genes in ML01 will have a negligible effect, if any, on a process that has been taking place over thousands of years. Furthermore, it is important to note that no antibiotic resistance marker gene is present in ML01.

*There is only limited data presented to determine if the ML01 transgenic has selective advantage- these experiments were carried out in the laboratory in soil microcosms. These are simulated soil experiments, which involve removing the top organic layer from the soil and, in the laboratory, sterilizing the soil and inoculating it with a few micro-organisms and monitoring growth of the different microorganisms over two weeks at 30degrees centigrade (page 88 of the application to the Registrar of Genetically Modified Organisms).*

*It is difficult to extend these results to the field where the abiotic and biotic factors may be very different and the microbial diversity is considerably larger (typically thousands of different species of microorganisms per gram of soil). Therefore, monitoring is perhaps the only way to accurately determine effects on the environmental microbial biodiversity and function at production scale.*

Apart from the experiments in soil that was carried out by an independent investigator, we have characterized the ML01 yeast strain at the level of the phenotype, genotype, transcriptome, proteome and metabolome; ML01 is identical to the parental strain except for its ability to decarboxylate malic acid to lactic acid during wine fermentations; the same property that malolactic bacteria has. Data from all these experiments have been provided in the Notification and in Husnik et al. (2006). No other genetically enhanced cell has been characterized to the same level. Apart from data presented in the Notification and in Husnik et al., 2006, we have studied and compared the growth rates of ML01 and its parental strain in laboratory media (YPD) and Chardonnay must. In YPD, there was a slight difference in  $\mu_{max}$  between ML01 ( $0.54 \pm 0.01 \text{ h}^{-1}$ ) and S92 ( $0.55 \pm 0.005 \text{ h}^{-1}$ ); the corresponding generation times were  $1.28 \pm 0.02 \text{ h}$  and  $1.26 \pm 0.01 \text{ h}$  for ML01 and S92, respectively ( $p < 0.05$ ,  $n=9$ ). In Chardonnay must no statistical difference was observed between the  $\mu_{max}$  for ML01 ( $0.37 \pm 0.03 \text{ h}^{-1}$ ) and the parental S92 ( $0.37 \pm 0.02 \text{ h}^{-1}$ ); the corresponding generation times were  $1.88 \pm 0.13 \text{ h}$  and  $1.86 \pm 0.08 \text{ h}$  for ML01 and S92, respectively ( $n=9$ ). These results taken together with all previously published results indicate that ML01 grows at the same rate as S92 (or slightly slower). Furthermore, ML01 and S92 cells die at the same rate (unpublished). Except for the production of equimolar amounts of lactate and carbon dioxide from malic acid, ML01 does not produce any other metabolite compared to the parental strain S92. ML01 does not contain an antibiotic resistance gene and has no growth advantage over the parental strain Prise de Mousse S92 that is used by the industry on large scale.

Other studies have been completed such as the survival of GM yeast strains in a confined wine cellar and greenhouse vineyard (Bauer et al. 2003 – see Schuller and Casal, 2005). In this study, four GM yeast containing resistance markers (*KanMX* or *SMR1-140*) and expressing the transgenes (with strong yeast promoters) for  $\alpha$ -amylase, endo- $\beta$ -1,4-

glucanase, xylanase or pectate lyase were sprayed onto vines in a confined greenhouse vineyard. Results showed that despite high initial cell counts, few *S. cerevisiae* cells were isolated from grapes, leaves, stems and soil during weekly monitoring. Furthermore, no significant difference between the occurrences of the modified strains compared to the parental strain was detected, including GM strains secreting glucanases and pectinases (modifications thought to provide a selective advantage). The total yeast population of treated vines was also very similar to the untreated control vines and spontaneous microvinifications resulted in no significant differences in the fermentations performances amongst the trials (Schuller and Casal, 2005).

*The effects on biodiversity may also extend far beyond the winery and vineyards since waste water from wineries are often discharged into local streams/rivers/wetlands. Several sensitive molecular methods can be used to assess the response of the environmental microbial biodiversity to the introduction of the ML01 transgenic (for example, review of by methods Kowalchuk et al 2003).*

Most of the wineries that I collaborated with in South Africa have their own effluent treatment plants or they rely on sewage treatment plants to treat winery effluent. It will be disastrous for local streams/rivers and wetlands if winery waste water is indeed discharged untreated in nature. Winery waste water can be acidic and well-buffered and it will undoubtedly have a detrimental effect on aquatic and terrestrial forms of life since it will acidify local streams/rivers and wetlands. Waste water could also contain wine yeasts and malolactic bacteria that have the same properties as ML01. Wineries should not be allowed to release waste water into local streams/rivers/wetlands.

### **Possible effects on human health**

*The effects on human health largely depend on how much ML01 yeast (and transgene products) the human consumer ingests and the fate of this material.*

*The amount of yeast remaining in wine will vary from 1-1000 yeast cells per litre of wine. It depends on the wine (white wines are often filtered or clarified more than red wines) and the methods used by the winery for clarification and filtration. Only modern, expensive methods using ultra-fine filters will be able to assure wine free of the ML01 GMO and there is no surety that all wine makers will afford or choose this option since several methods exist (pages 18-21). Therefore, there are risks to the consumer with the ingestion of ML01. Although this GMO is derived from a microorganism (*Saccharyomyces cerevisiae*) which is generally regarded as safe, the intended and unintended genetic changes have created a novel *Saccharyomyces cerevisiae* genotype that may pose new risks. These include:*

- *Increased virulence of *Saccharyomyces cerevisiae* ML01. The escape of this GMO may result in increased infection of humans by *Saccharyomyces cerevisiae* (presently only occasionally causing human infections; approximately 1% of all yeast infections of hospitalized patients)*

There is no scientific justification to propose that ML01 will cause an increase in yeast infections in humans. Infection of humans by *S. cerevisiae* (or other saprophytic microorganisms) only occurs in immune compromised individuals (Sethi and Mandell, 1988). All of the wine strains currently used by the wine industry as well as bakers and brewers yeasts, can act as opportunistic pathogens in immune compromised individuals. The ML01 yeast is identical to the parental strain S92 except for the malolactic gene from *O. oeni* and the malate transport gene from *S. pombe*, both donor organisms are wine organisms. The US Environmental Protection Agency focuses primarily on the characteristics of the recipient organism (Anon, US EPA 1997

<http://www.epa.gov/opptintr/biotech/pubs/pdf/fd002.pdf> ;

<http://www.epa.gov/opptintr/biotech/pubs/pdf/fra002.pdf>) when evaluating the potential for unreasonable risk to human health or the environment. There is no reason to

conclude that the introduction of a malolactic cassette that has been constructed from wine microorganisms would increase the potential for adverse health risk in humans. On the contrary, the yeast's ability to significantly reduce bioamine and ethyl carbamate formation in wine could result in a significant health benefit for consumers.

The Australian wine industry does not see any health implications should ML01 be employed for wine making; they emphatically state "What are the risks associated with using ML01? In terms of health risks there should be none. The two foreign genes incorporated into the wine yeast to make it MLF-competent come from organisms that are typically associated with foods and/or beverages. One comes from the yeast *Schizosaccharomyces pombe*, which is found in many alcoholic beverages, and the other comes from *O. oeni*, which is used routinely in the wine industry for MLF. A great deal of work has been done to show that the two genes are stable in their new background and the U.S. Food and Drug Administration designated it a GRAS (generally recognised as safe) organism in their response to Lesaffre's submission to that office (although it should be pointed out that 'GRAS' is not recognised as a global standard). It would seem from balancing some of the more obvious risks and benefits associated with the use of ML01, that having access to this yeast might be a good thing for Australian winemakers".  
<http://www2.awri.com.au/infoservice/media/releases/nogogmo.asp>

- *Horizontal gene transfer of the transgenic cassette of ML01 into intestinal bacteria, thereby altering intestinal microbial flora. HGT to intestinal bacteria has been shown to occur (Netherwood et al 2004). The consequences may include disturbances in digestive function.*

Netherwood et al. (2004) detected an "indication of low-frequency gene transfer from GM soya to microflora of the small bowel in ileostomists" (individuals without a colon but with a colostomy bag). They further found that "The transgene did not survive passage through the intact gastrointestinal tract of human subjects fed with GM soya" (Netherwood et al., 2004). It is important to note that the *mleA* and *mae1* genes are present in all wines that undergo the bacterial malolactic fermentation. If indeed horizontal gene flow takes place to intestinal bacteria in the human gut, the *mleP* (malate permease) and *mleA* (malolactic enzyme) genes from malolactic bacteria stand a much bigger chance of being transferred to intestinal bacteria than the *mae1* and *mleA* genes from ML01 for the following reasons: Gene structure in prokaryotes (bacteria) and eukaryotes (yeast and other higher forms of life) are very different. Bacterial genes are polycistronic and yeast genes are monocistronic. Transcription and translation in bacteria and yeast are fundamentally different processes. Bacteria contain a single RNA polymerase and yeast contains three RNA polymerases. The short leader sequence that mediates transcription of genes in bacteria contains regulatory sequences that are very different from those found in much longer yeast promoters. Transacting factors that activate gene transcription or silence genes are unique for bacterial leaders and yeast promoters. The yeast *PGK1* promoter and terminator sequences will not be recognized by bacterial trans-acting factors and no transcription of these two genes in bacteria will occur. In fact, the *mae1* promoter from the yeast *S. pombe* was not functional in *S. cerevisiae* S92 and had to be replaced by the *S. cerevisiae* *PGK1* promoter and terminators sequences in ML01. Furthermore, bacterial mRNA's contain a Shine Dalgarno sequence required for binding of ribosomes that mediates translation of the mRNA into proteins. A Shine Dalgarno sequence is absent in yeast mRNA's and yeast mRNA's will not be translated in bacteria. In addition to the compelling reasons why yeast genes will not be expressed in bacteria, there are many other

differences that will make it impossible for the *mae1* and *mleA* genes to be functionally expressed in bacteria; the *mae 1* gene contains a leader peptide that directs integration of this protein into the yeast plasmamembrane. The leader peptide/*mae1* protein is produced in the nucleus of yeast cells and it is then directed to the plasmamembrane via a membrane system in the yeast cell. Bacteria don't have a nucleus or a membrane system to direct transport proteins to the plasmamembrane. In fact, we were unable to utilise the *O. oeni* malate transport gene (*mleP*) since the leader peptide on this protein was not recognized in the wine yeast S92. The concern that horizontal gene transfer of the malolactic cassette of ML01 into intestinal bacteria might occur, thereby altering intestinal microbial flora resulting in possible disturbances in digestive function, is unfounded. It is much more likely that the malolactic cassette from *O. oeni* that is present in high numbers in wine, might be transferred from this bacterium to intestinal bacteria if horizontal gene flow indeed takes place.

Deni et al., (2005) conclusively showed that DNA transfer from transgenic plants to native bacteria in the gut of the tobacco horn worm did not occur.

- *There are also risks from the ingestion of the transgene products, such as, the production of allergic reactions. The applicant provides no experimental evidence that the Mae1 and MleA proteins are absent from the final product (wine). A hypothesis is presented that much of the protein will be removed during the winemaking process (page 21 of application to Registrar Genetic Organisms). This hypothesis is put forward as scientific fact (on page 21) as follows: "Hence, the only suspected difference between a ML01 wine and a control wine, would be the possible presence of the ML01 recombinant yeast containing the malate permease and malolactic enzyme. These proteins, and most of all their hydrolysis products will essentially be found upon storage of the wines on lees. Moreover, the intact proteins and their larger polypeptides will be removed by obligatory clarification practices. Hence only the smaller peptides and amino acids will remain." This may be largely true but if yeasts do remain in the wine and can be detected then surely the transgene products will also be present. The applicant needs to provide experimental evidence to support the hypothesis (page 21) using a sensitive assay (e.g. ELISA) to detect the fate MleA and Mae1 proteins in wine. This is particularly important since the MleA protein from the ML01 is a mutant and therefore is different from the MleA found in the non-GM yeasts (see below).*

The argument that small amounts of DNA and proteins from ML01 may persist in wine is not relevant since no DNA that encodes for proteins foreign to the wine making process, was introduced into the malolactic wine yeast ML01. Wines produced by the bacterial malolactic fermentation may also contain small amounts of DNA including the *mleA* gene encoding the malolactic enzyme; the protein could also be present. The same argument can be made for the malate permease genes from *O. oeni* and *S. pombe* which are present in wine. It should be noted that is unlikely that the malate transport protein (from *O. oeni*, *S. pombe* or ML01) that contains many transmembrane domains, will be present in wine since this protein is hydrophobic and will not remain in solution.

- *The insertion of this transgenic cassette may also have induced subtle changes in the metabolism of Saccharomyces cerevisiae ML01 with the production of different organic metabolic products that may carry health risks. The applicant does present data (GC/MS- appendix11) to observe any possible changes in the metabolism (that is, Metabolomics). However, there are no clear conclusions drawn from the data and without statistical tools, the data presented as is cannot easily be interpreted. It is requested that the applicant provide scatter plots (incorporating standard deviations) so that the metabolic profiles can accurately be overlaid and compared.*

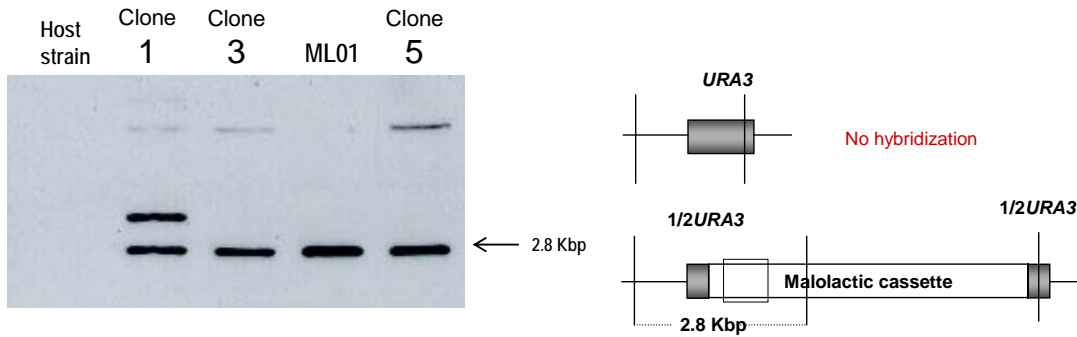
The ML01 yeast has been fully characterized at the level of the phenotype, genotype, transcriptome and metabolome (see Notification and Husnik et al., 2006). ML01 is identical to the parental Prise de Mousse strain S92, except for the ability of ML01 to conduct an efficient malolactic fermentation. We have indeed used statistical tools to analyze metabolome data obtained and a manuscript entitled "Functional analyses of the malolactic wine yeast ML01" has been accepted for publication in the American Journal of Enology and Viticulture (Husnik et al., 2007). This manuscript will be published in the first issue of Am. J. Enol. Vitic. in 2007 and I will forward an electronic copy of the manuscript to the Registrar as soon as it becomes available. No other genetically enhanced cell has been studied and characterized to the same extent as ML01.

It is also important to note that one finds a great deal of variation in metabolism between different wine yeast strains. What is important is that ML01 does not produce any novel proteins.

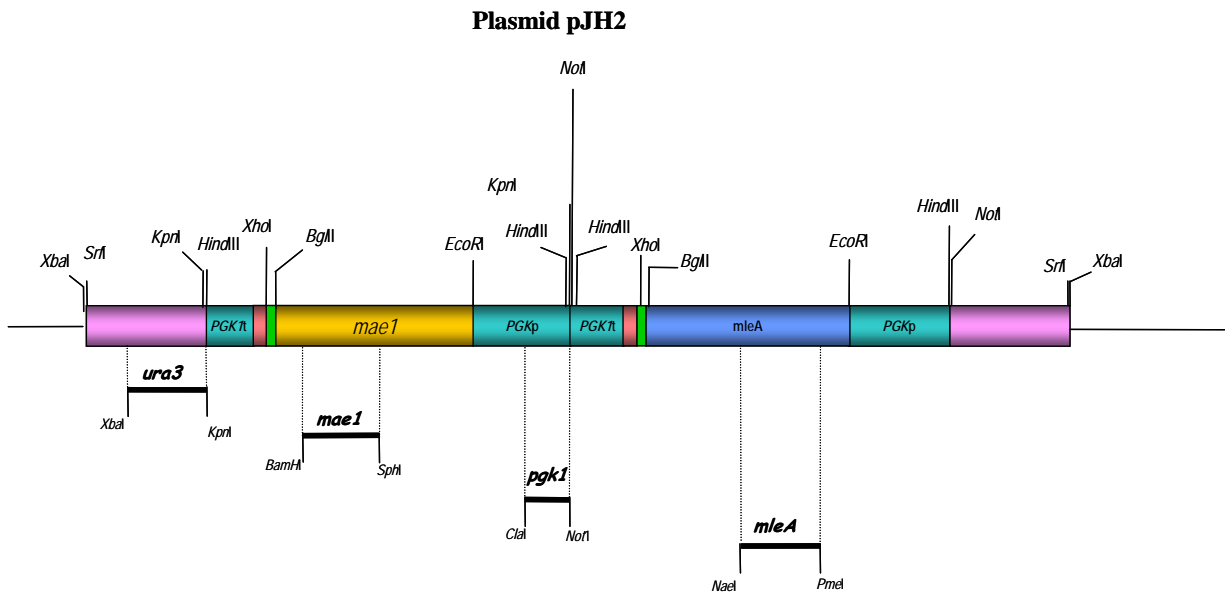
#### ***Unintended genetic changes- uncertain effects***

*This transgenic cassette (PGK1 promoter, mae1 and mleA genes) was inserted into the ura3 gene of the Saccharomyces cerevisiae genome and hence the ML01 transgenic is auxotrophic for uracil (requires uracil for growth). The site of insertion and integrity of the construct was determined by DNA sequencing. This data is presented in Table 5. There are a total of 15 differences; these unintended genetic changes are:*

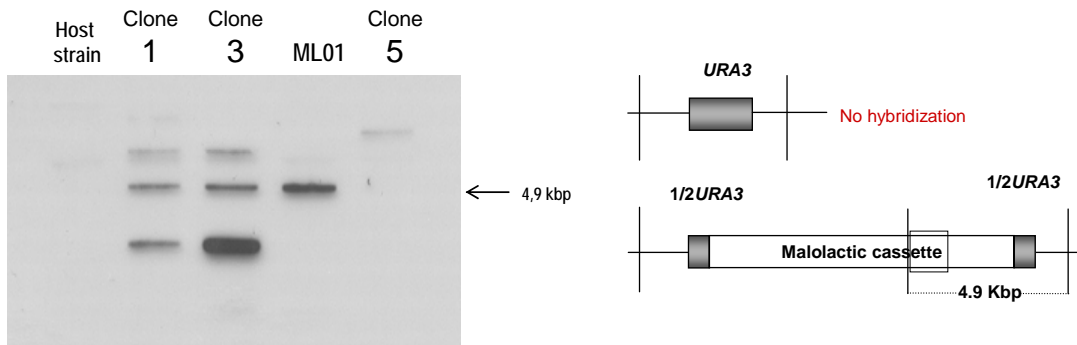
The statement that the ML01 yeast auxotrophic for uracil is not correct since the yeast is diploid and only one copy of the targeted *URA3* gene was disrupted (Figure 3, Husnik et al., 2006). The correct integration of the malolactic cassette into the yeast genome is an important issue that needs to be verified since it can have adverse consequences on yeast metabolism if the cassette is split up during integration or if it is inserted into the wrong locus. We originally found four transformants containing the malolactic cassette. Southern blotting revealed that three clones had inserts into more than one locus (Figure 1). Only ML01 had single insertion. We further verified that no section of the malolactic cassette was inserted elsewhere in the genome by probing with all elements comprising the malolactic cassette (Figure 2). Experimental procedures are fully described in Husnik et al., 2006 and a Southern blot with the *URA3* probe is shown in Figure 3 (Husnik et al., 2006). Southern blots in which the *mae1*, *mleA* and *PGK1* probes were used are shown in Figures 1, 3 and 4, respectively (see below). All blots indicate that the malolactic cassette was integrated correctly into the *URA3* locus of ML01. The integrity of the site of insertion and integrity of the single copy was determined by DNA sequencing (See Notification).



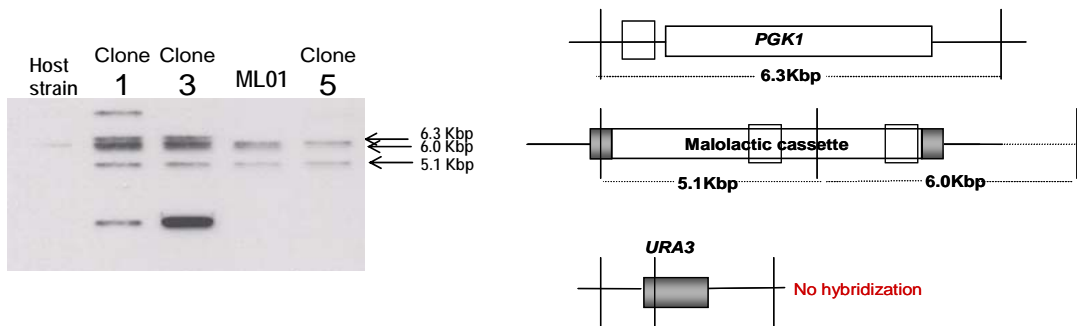
**Figure 1: Southern blots representing hybridization of genomic DNA with the *mae1* probe and schematic representation of the integrated and non integrated *URA3* loci.** Genomic DNA from either the host strain or the malolactic clones was totally digested with restriction enzyme *NsiI*. *NsiI* restriction sites are represented by vertical lines. The *mae1* probe hybridization sites are schematized as hatched boxes and the restriction fragment detected by the probe are represented as dotted lines. Clones 1, 3 and 5 had multiple inserts in the genome and were discarded. ML01 had only a single insert in the *URA3* locus.



**Figure 2: Schematic representation of probes originating from plasmid pJH2 used in hybridization experiments.** All probe templates were isolated from plasmid pJH2 by digestion with appropriate restriction enzymes. Template of probe *ura3* was retrieved as a *XbaI-KpnI* fragment, template of probe *mae1* as a *BamHI-SphI* fragment, template of probe *pgk1* (promoter) as a *ClaI-NoI* fragment, and template of probe *mleA* as a *XhoI-PmeI* fragment.



**Figure 3: Southern blot representing hybridization of genomic DNA with the *mleA* probe and schematic representation of the integrated and non-integrated *URA3* loci.** Genomic DNA from either the host strain or the ML01 strain was totally digested with restriction enzyme *PvuII*. *PvuII* restriction sites are represented by vertical lines. The *mleA* probe hybridization sites are schematized as hatched boxes and restriction fragments detected by the probe are represented as dotted lines. Only ML01 had a single insert.



**Figure 4: Southern blots representing hybridization of genomic DNA with the *PGK1* probe and schematic representation of the *PGK1* locus and the integrated and non integrated *URA3* loci.** Genomic DNA from either the host strain or the ML01 strain was totally digested with restriction enzyme *EcoRV*. *EcoRV* restriction sites are represented by

double arrows. *PGK1* probe hybridization sites are schematized as full red lines and restriction fragments detected by the probe are represented as dotted lines.

- *Small DNA regions (polylinker) either side of the cassette remain. These small regions may contain ORFs coding for protein, they may effect DNA stability (particularly if they are repetitive DNA sequence) and may act as cis-acting factors to alter the transcriptional profile (transcriptome). There appears to be minimal effects on the transcriptome (Husnik et al. 2006) but little data is presented to demonstrate the genetic stability of the construct following sexual recombination (stable inheritance analyzed from Saccharyomyces cerevisiae ascospores over several generations). This accounts for seven of the 15 unintended changes.*

The two longest linkers in the cassette are 19 nucleotides in length and neither contains a start or a stop codon and cannot therefore code for a protein. Genetic stability was indeed assessed; after large-scale active dry yeast production (1000 kgs) and inoculation into Chardonnay grape must, 401/404 randomly chosen ML01 colonies tested positive for the MLF phenotype ( $99.3 \pm 1.0\%$   $p < 0.05$ ). The malolactic phenotype segregated 2:2 in ascospores. The two spores that were positive for malolactic fermentation were auxotrophic for uracil since the malolactic cassette was integrated into one of the two *URA3* loci in S92; if uracil is not provided, they were unable to grow. Ascospores originating from ML01 will thus have a definite disadvantage in nature and they will not be able to reproduce sexually or asexually unless uracil is provided. For this reason, we did not follow stable inheritance of the malolactic phenotype in ascospores originating from ML01.

- *Four differences in the site of insertion (ura3 sequence). These may be unintended changes or genetic polymorphism of the original strain. This could easily have been determined (sequence the ura3 from the parent isolate) and should be carried out by the applicant.*

The *ura3* flanking sequences were used only for targeted integration of the malolactic cassette, the four differences were not found in the site of insertion as claimed by BioWatch SA. The *ura3* flanking sequences are non-coding and are therefore not important since they do not code for any proteins. There is therefore no need to sequence *URA3* sequence of the original strain.

- *There are two mutations in one copy of the PGK1 promoter. The effects of this mutation on promoter activity have not been determined. It is recommended that this unwanted mutation be repaired.*

The only function of the *PGK1* promoter is to activate transcription of the *mae1* and *mleA* genes. Both copies of the *PGK1* promoter in ML01 are fully functional, they do not encode for proteins, and there is no need to “repair” the one copy of the *PGK1* promoter.

- *There are two unintended genetic changes (mutations) that are present in the mleA gene. One of these changes is of particular concern since it changes the protein primary sequence (aspartate to glutamate). The effects of even single amino acid sequence on protein folding, activity and stability cannot be predicted. It would be unwise to work with this uncharacterized mutant. The*

uncertainty of the applicant if these unintended changes are due to the creation of errors during cloning, errors in the published sequence or errors in the original strain can easily be determined by sequencing *mleA* from the original isolate (ATCC) the parent strain used in the cloning experiment and the ML01 transgenic. This must be carried out. Either experimental evidence must be presented that the mutant *mleA* is no different from the published *mleA* (same protein activity, stability, turnover/degradation and human allergenicity) or the applicant should repair these mutations in the final transgenic cassette.

There are two base pair changes present in the *mleA* gene in ML01 compared to the sequence of one published *mleA* gene. Several *mleA* gene sequences are now available (NCBI) and an alignment of all seven published sequences reveals that the “two unintended genetic changes” according to BioWatch SA, are in fact natural single nucleotide polymorphisms in the *mleA* gene. We have aligned the sequences of seven *mleA* genes from *O. oeni*. The genetic polymorphisms in question are highlighted in yellow (nucleotide 1614). Other polymorphisms in the *mleA* gene in seven *O. oeni* strains are highlighted in red. Both glutamate and aspartate are thus present at this position in natural strains of *O. oeni* used by the wine industry.

```
FILE: Multiple_Sequence_Alignment
PROJECT:mleA alignment
NUMBER: 8
MAXLENGTH: 1626
NAMES: mleA from ML01, ATCC 23279, ATCC 39401, strain 8413, strain CH,
       strain M1, strain SD-2a, strain Y5
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ORIGIN
mleA from ML01 ATGACAGATCCAGTAAGTATTTTAAATGATCCTTTTATTA      40
ATCC 23279     ATGACAGATCCAGTAAGTATTTTAAATGATCCTTTTATTA      40
ATCC 39401     ATGACAGATCCAGTAAGTATTTTAAATGATCCTTTTATTA      40
strain 8413    ATGACAGATCCAGTAAGTATTTTAAATGATCCTTTTATTA      40
strain CH      ATGACAGATCCAGTAAGTATTTTAAATGATCCTTTTATTA      40
strain M1      ATGACAGATCCAGTAAGTATTTTAAATGATCCTTTTATTA      40
strain SD-2a   ATGACAGATCCAGTAAGTATTTTAAATGATCCTTTTATTA      40
strain Y5      ATGACAGATCAGTAAGTATTTTAAATGATCCTTTTATTA      40
Consensus     atgacagatc agtaagtattttaaatgatccttttatta

mleA from ML01 ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGGAGCT      80
ATCC 23279     ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGAGGAGCT      80
ATCC 39401     ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGAGGAGCT      80
strain 8413    ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGAGGAGCT      80
strain CH      ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGAGGAGCT      80
strain M1      ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGAGGAGCT      80
strain SD-2a   ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGAGGAGCT      80
strain Y5      ACAAAGGAACTGCTTTTACGGAAGCGGAGAGAGAGAGGAGCT      80
Consensus     acaaaggaactgcttttacggaagcgagagagaggagct

mleA from ML01 TGGTTTAAACGGTTTATTACCGCCAAGGTTTCAGGCTTTA      120
ATCC 23279     TGGTTTAAACGGTTTATTACCGCCAAGGTTTCAGGCTTTA      120
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ATCC 39401	TGGTTTAAACGGTTTATTACCGGCCAAGGTTTCAGGCTTTA	120
strain 8413	TGGTTTAAACGGTTTATTACCGGCCAAGGTTTCAGGCTTTA	120
strain CH	TGGTTTAAACGGTTTATTACCGGCCAAGGTTTCAGGCTTTA	120
strain M1	TGGTTTAAACGGTTTATTACCGGCCAAGGTTTCAGGCTTTA	120
strain SD-2a	TGGTTTAAACGGTTTATTACCGGCCAAGGTTTCAGGCTTTA	120
strain Y5	TGGTTTAAACGGTTTATTACCGGCCAAGGTTTCAGGCTTTA	120
Consensus	tggtttaaacggtttattaccggccaaggttcaggcttta	
mleA from ML01	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
ATCC 23279	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
ATCC 39401	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
strain 8413	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
strain CH	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
strain M1	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
strain SD-2a	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
strain Y5	CAAGAGCAAGTTGATCAGACTTATGCTCAATTTCAAAGCA	160
Consensus	caagagcaagttgatcag cttatgctcaatttcaaagca	
mleA from ML01	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
ATCC 23279	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
ATCC 39401	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
strain 8413	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
strain CH	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
strain M1	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
strain SD-2a	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
strain Y5	AGGTTTCAAATCTCGAAAAACGATTGTTTTTAATGGAAAT	200
Consensus	aggtttcaaatctcgaaaaacgattgTTTTTAATGGAAAT	
mleA from ML01	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
ATCC 23279	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
ATCC 39401	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
strain 8413	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
strain CH	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
strain M1	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
strain SD-2a	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
strain Y5	ATTCAATACGAATCACGTGTTGTTTTATAAGCTTTTTTCT	240
Consensus	attcaatacga tcacgtgttgTTTTATAAGCTTTTTTCT	
mleA from ML01	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
ATCC 23279	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
ATCC 39401	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
strain 8413	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
strain CH	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
strain M1	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
strain SD-2a	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
strain Y5	CAACATGTTGTTGAATTTATGCCAATTGTTTATGACCCGA	280
Consensus	caacatgttgttgaatTTATGCCAATTGTTTATGACCCGA	
mleA from ML01	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
ATCC 23279	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
ATCC 39401	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
strain 8413	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
strain CH	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
strain M1	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
strain SD-2a	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
strain Y5	CAATTGCTGATACAATTGAAAATTATTCGGAATTATTTGT	320
Consensus	caattgctgatacaattgaaaattatTCGGAATTATTTGT	
mleA from ML01	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360
ATCC 23279	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360

ATCC 39401	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360
strain 8413	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360
strain CH	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360
strain M1	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360
strain SD-2a	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360
strain Y5	TGAACCGCAAGGTGCCGCTTTTTTGGATATTAATCATCCG	360
Consensus	tgaaccgcaaggtgccgcttttttggatattaatcatccg	
mleA from ML01	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
ATCC 23279	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
ATCC 39401	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
strain 8413	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
strain CH	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
strain M1	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
strain SD-2a	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
strain Y5	GAAAACATTCAATCGACTCTGAAAAATGCTGCTAATGGCC	400
Consensus	gaaaacattcaatc actctgaaaaatgctgctaattggcc	
mleA from ML01	GCGATATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
ATCC 23279	GCGATATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
ATCC 39401	GCGATATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
strain 8413	GCGATATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
strain CH	GCGATATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
strain M1	GCGATATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
strain SD-2a	GCGATATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
strain Y5	GCGTATCAAGCTGCTGGTCGTTTCTGATGCCGAAGGTAT	440
Consensus	gcg tatcaagctgctggctcgtttctgatg cgaaggtat	
mleA from ML01	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
ATCC 23279	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
ATCC 39401	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
strain 8413	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
strain CH	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
strain M1	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
strain SD-2a	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
strain Y5	TCTTGGGATTGGAGACTGGGGTGTCAGGGTGTTGATATT	480
Consensus	tcttgggattggagactgggggtgtccaggggtgttgatatt	
mleA from ML01	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
ATCC 23279	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
ATCC 39401	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
strain 8413	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
strain CH	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
strain M1	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
strain SD-2a	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
strain Y5	GCTGTCGGCAAACCTGATGGTTTATACAGTTGCGGCCGGAA	520
Consensus	gctgtcggcaaacctgatggtttatacagttgcgggccggaa	
mleA from ML01	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
ATCC 23279	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
ATCC 39401	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
strain 8413	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
strain CH	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
strain M1	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
strain SD-2a	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
strain Y5	TCGATCCATCAACAGTTCTTGCAGTTGTTATTGATGCTGG	560
Consensus	tcgatccatcaacagttcttgcagttgttattgatgctgg	
mleA from ML01	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTG	600
ATCC 23279	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTG	600

ATCC 39401	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTG	600
strain 8413	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTG	600
strain CH	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTG	600
strain M1	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTG	600
strain SD-2a	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTG	600
strain Y5	AACAAATAACGAAAAGCTTTTGAAAGATCCTATGTATTTa	600
Consensus	aacaaataacgaaaagcttttgaaagatcctatgtattt	
mleA from ML01	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
ATCC 23279	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
ATCC 39401	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
strain 8413	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
strain CH	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
strain M1	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
strain SD-2a	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
strain Y5	GGAAATAAATTTAATCGTGTTCTGGCGATAAGTACTATG	640
Consensus	gaaataaatttaacgctggttcggtggcgataagtactatg	
mleA from ML01	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
ATCC 23279	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
ATCC 39401	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
strain 8413	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
strain CH	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
strain M1	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
strain SD-2a	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
strain Y5	ATTTTATCGACAAATTTGTTAATCATGCCGAATCGCTTTT	680
Consensus	attttatcgacaaatttgtt aatcatgccgaatcgctttt	
mleA from ML01	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
ATCC 23279	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
ATCC 39401	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
strain 8413	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
strain CH	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
strain M1	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
strain SD-2a	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
strain Y5	TCCTAATTTATATTTGCATTGGGAAGATTTTGGCCGTTTCG	720
Consensus	tcct atttatatttgcattggga gattttggccgtttcg	
mleA from ML01	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
ATCC 23279	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
ATCC 39401	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
strain 8413	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
strain CH	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
strain M1	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
strain SD-2a	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
strain Y5	AATGCTTCTAATATCTTAAACAGCTATAAAGATAAAAATTG	760
Consensus	aatgcttctaataatctttaaacagctat aagataaaaattg	
mleA from ML01	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
ATCC 23279	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
ATCC 39401	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
strain 8413	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
strain CH	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
strain M1	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
strain SD-2a	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
strain Y5	CTACTTTTAATGATGATATTC AAGGA ACTGGAATCGTCGT	800
Consensus	ctacttttaatgatgatattcaaggaactggaatcgtcgt	
mleA from ML01	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCGGTCAG	840
ATCC 23279	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCGGTCAG	840

ATCC 39401	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCG <b>a</b> TCAG	840
strain 8413	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCGGTCAG	840
strain CH	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCGGTCAG	840
strain M1	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCGGTCAG	840
strain SD-2a	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCGGTCAG	840
strain Y5	TCTTGCCGGCGTTCTTGAGCGTTGAAGATTTCCGGTCAG	840
Consensus	tcttgccggcgttcttggagcgttgaagatttccg tcag	
mleA from ML01	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
ATCC 23279	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
ATCC 39401	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
strain 8413	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
strain CH	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
strain M1	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
strain SD-2a	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
strain Y5	AAATTAACTGATCAAACCTTACATGAGCTTCGGTGCCGGAA	880
Consensus	aaattaactgatcaaacttacatgagcttcggtgccggaa	
mleA from ML01	CTGCTGGAATGGGAATTGTTAAACAGTTGCATGAAGAAAT	920
ATCC 23279	CTGCTGGAATGGGAATTGTTAAACAGTTGCATGAAGAAAT	920
ATCC 39401	CTGCTGGAATGGGAATTGTTAAACAGTTGCATGAAGAAAT	920
strain 8413	CTGCTGGAATGGGAATTGTTAAACAGTTGCATGAAGAAAT	920
strain CH	CTGCTGGAATGGGAATTGTTAAACA <b>c</b> TTGCATGAAGAAAT	920
strain M1	CTGCTGGAATGGGAATTGTTAAACAGTTGCATGAAGAAAT	920
strain SD-2a	CTGCTGGAATGGGAATTGTTAAACAGTTGCATGAAGAAAT	920
strain Y5	CTGCTGGAATGGGAATTGTTAAACAGTTGCATGAAG <b>a</b> AT	920
Consensus	ctgctggaatgggaattgttaaaca ttgcatgaaga at	
mleA from ML01	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
ATCC 23279	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
ATCC 39401	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
strain 8413	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
strain CH	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
strain M1	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
strain SD-2a	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
strain Y5	GGTTGAACAGGGTCTTTCCGACGAAGAGGCTAAAAAGCAT	960
Consensus	ggttgaacagggctttccgacgaagaggctaaaaagcat	
mleA from ML01	TTCTTCCTTGTTGACAAACAAGGCCTCTTGTTTG <b>a</b> TGATG	1000
ATCC 23279	TTCTTCCTTGTTGACAA <b>a</b> CAAGGCCTCTTGTTTG <b>a</b> TGATG	1000
ATCC 39401	TTCTTCCTTGTTGACAAACAAGGCCTCTTGTTTG <b>a</b> cGATG	1000
strain 8413	TTCTTCCTTGTTGACAAACAAGGCCTCTTGTTTG <b>a</b> cGATG	1000
strain CH	TTCTTCCTTGTTGACAAACAAGGCCTCTTGTTTG <b>a</b> cGATG	1000
strain M1	TTCTTCCTTGTTGACAAACAAGGCCTCTTGTTTG <b>a</b> cGATG	1000
strain SD-2a	TTCTTCCTTGTTGACAAACAAGGCCTCTTGTTTG <b>a</b> cG <b>a</b> c <b>G</b>	1000
strain Y5	TTCTTCCTTGTTGACAAACAAGGCCTCTTGTTTG <b>a</b> cGATG	1000
Consensus	ttcttccttgttgacaaa caaggcctcttgtttga ga g	
mleA from ML01	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
ATCC 23279	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
ATCC 39401	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
strain 8413	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
strain CH	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
strain M1	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
strain SD-2a	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
strain Y5	ATCCGGATTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	1040
Consensus	atccggatTTAACTCCAGAGCAAAGCCTTTCGCTGCTAA	
mleA from ML01	ACGAAGTGATTTCAAAAATGCTAATCAATTGACCAATCTC	1080
ATCC 23279	ACGAAGTGATTTCAAAAATGCTAATCAATTGACC <b>a</b> ATCTC	1080

ATCC 39401	ACGAAGTGATTTCAAACAAATGCTAATCAATTGACCAATCTC	1080
strain 8413	ACGAAGTGATTTCAAAAAATGCTAATCAATTGACCAATCTC	1080
strain CH	ACGAAGTGATTTCAAACAAATGCTAATCAATTGACCAATCTC	1080
strain M1	ACGAAGTGATTTCAAAAAATGCTAATCAATTGACCAATCTC	1080
strain SD-2a	ACGAAGTGATTTCAAAAAATGCTAATCAATTGACCAATCTC	1080
strain Y5	ACGAAGTGATTTCAAAAAATGCTAATCAATTGACCAATCTC	1080
Consensus	acgaagtgatttcaa aatgctaataatgacc atctc	
mleA from ML01	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
ATCC 23279	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
ATCC 39401	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
strain 8413	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
strain CH	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
strain M1	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
strain SD-2a	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
strain Y5	CAAGCAGCTGTTGAAGCTGTCCACCCGACCATTTTGGTTCG	1120
Consensus	caagcagctgttgaagctgtccacccgaccatTTTGGTTCG	
mleA from ML01	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
ATCC 23279	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
ATCC 39401	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
strain 8413	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
strain CH	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
strain M1	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
strain SD-2a	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
strain Y5	GAACCTCGACACATCCAAATTCCTTTACTGAAGAAATTGT	1160
Consensus	gaa ctcgacacatccaaattcctTTTactgaagaaattgt	
mleA from ML01	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
ATCC 23279	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
ATCC 39401	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
strain 8413	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
strain CH	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
strain M1	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
strain SD-2a	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
strain Y5	TAAAGATATGTCTGGTTATACTGAAAGACCAATCATTFFFF	1200
Consensus	taaagatatgtctggttatactgaaagaccaatcattffff	
mleA from ML01	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
ATCC 23279	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
ATCC 39401	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
strain 8413	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
strain CH	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
strain M1	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
strain SD-2a	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
strain Y5	CCAATTTCCAATCCAACAAAAATTAGCCGAAGCAAAAGCCG	1240
Consensus	ccaatttccaatccaacaaaaattagccgaagcaaaagccg	
mleA from ML01	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
ATCC 23279	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
ATCC 39401	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
strain 8413	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
strain CH	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
strain M1	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
strain SD-2a	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
strain Y5	AAGATGTTTTGAAATGGTCTAATGGAAAAGCCTTGATCGG	1280
Consensus	aagatgTTTTgaaatggtctaataaggaaaagccttgatcgg	
mleA from ML01	TACTGGTGTTCAGTTGACGATATTGAATATGAGGGCAAC	1320
ATCC 23279	TACTGGTGTTCAGTTGACGATATTGAATATGAGGGCAAC	1320

ATCC 39401	TACTGGTGTTCCAGTTGACGATATTGAATATGAGGGCAAC	1320
strain 8413	TACTGGTGTTCCAGTTGACGATATTGAATATGAGGGCAAC	1320
strain CH	TACTGGTGTTCCAGTTGACGATATTGAATATGAGGGCAAC	1320
strain M1	TACTGGTGTTCCAGTTGACGATATTGAATATGAGGGCAAC	1320
strain SD-2a	TACTGGTGTTCCAGTTGACGATATTGAATATGAGGGCAAC	1320
strain Y5	TACTGGTGTTCCAGTTGACGATATTGAATATGAGGGCAAC	1320
Consensus	tactggtgttccagttgacgatattgaatatgagggcaac	
mleA from ML01	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
ATCC 23279	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
ATCC 39401	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
strain 8413	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
strain CH	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
strain M1	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
strain SD-2a	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
strain Y5	GCTTACCAAATCGGTCAGGCCAACAAATGCCTTGATCTATC	1360
Consensus	gcttaccaaatcggtcaggccaacaatgccttgatctatc	
mleA from ML01	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
ATCC 23279	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
ATCC 39401	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
strain 8413	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
strain CH	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
strain M1	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
strain SD-2a	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
strain Y5	CAGGTCTTGGCTTTGGTGCCATTGCCGCTCAATCAAAGCT	1400
Consensus	caggtcttggctttggtgccattgccgctcaatcaaagct	
mleA from ML01	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
ATCC 23279	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
ATCC 39401	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
strain 8413	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
strain CH	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
strain M1	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
strain SD-2a	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
strain Y5	GCTTACGCCTGAAATGATTTCTGCTGCTGCCCATAGTCTT	1440
Consensus	gcttacgcctgaaatgatttctgctgctgcccatagcttt	
mleA from ML01	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
ATCC 23279	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
ATCC 39401	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
strain 8413	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
strain CH	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
strain M1	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
strain SD-2a	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
strain Y5	GGAGGAATCGTTGATACAACAAAAGTTGGTGCTGCTGTTT	1480
Consensus	ggaggaatcgttgataacaacaaaagttggtgctgctgttt	
mleA from ML01	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
ATCC 23279	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
ATCC 39401	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
strain 8413	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
strain CH	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
strain M1	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
strain SD-2a	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
strain Y5	TGCCACCAGTTTCAAAATTAGCCGACTTTTCGCGTACAGT	1520
Consensus	tgccaccagtttcaaaattagccgacttttcgcgtagagt	
mleA from ML01	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560
ATCC 23279	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560

ATCC 39401	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560
strain 8413	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560
strain CH	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560
strain M1	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560
strain SD-2a	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560
strain Y5	CGCTGTCGCTGTCGCTAAAAAAGCTGTTGAACAAGGTCTT	1560
Consensus	cgctgtcgcgtgctcgtataaaaaagctgttgaacaaggtctt	
mleA from ML01	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
ATCC 23279	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
ATCC 39401	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
strain 8413	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
strain CH	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
strain M1	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
strain SD-2a	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
strain Y5	AATCGCCAGCCGATTGATGATGTTGAAAAGGCCGTCGACG	1600
Consensus	aatcgccagccgattgatgatgttgaaaaggccgctcgacg	
mleA from ML01	ATTTGAAGTGGGAGCCGAAATACTAA	1626
ATCC 23279	ATTTGAAGTGGGAGCCGAAATACTAA	1626
ATCC 39401	ATTTGAAGTGGGAGCCGAAATACTAA	1626
strain 8413	ATTTGAAGTGGGAGCCGAAATACTAA	1626
strain CH	ATTTGAAGTGGGAGCCGAAATACTAA	1626
strain M1	ATTTGAAGTGGGAGCCGAAATACTAA	1626
strain SD-2a	ATTTGAAGTGGGAGCCGAAATACTAA	1626
strain Y5	ATTTGAAGTGGGAGCCGAAATACTAA	1626
Consensus	atttgaagtggga ccgaaatactaa	

***Additional specific criticisms:***

*Section 8.5 of the application to the Registrar requests, “protocols for the detection of foreign genes in the environment including sensitivity, reliability and specificity of the techniques”. This section is insufficient. No evidence is presented for the sensitivity, reliability and specificity of the PCR detection technique. The protocols for the quantitative detection of the transgene need to be stated together with the limits of detection (sensitivity, and reliability of the technique. Methods, such as, quantitative PCR (real time PCR) would be appropriate.*

PCR technology is used by law enforcement agencies to convict criminals of offences. This technique is highly sensitive and a single DNA molecule can be detected by PCR. We will provide data obtained by Real-Time PCR if required by the Registrar.

*Section 8.3 describes hybridization methods that have been used to determine the correct integration of the cassette into the genome. PCR with several flanking primers should be use to establish the integrity of the integration site. Together with quantitative PCR, these experiments are required in order to establish that there are no other fragments of the cassette in the genome and that there is a single integration event.*

The correct integration of the malolactic cassette was confirmed by multiple Southern analyses using DNA probes from all elements in the malolactic cassette (see data provided in Figures 1-4). Furthermore, the site of insertion and the integrity of the single copy insert were determined by DNA sequencing. Both strands of the region upstream and downstream of the malolactic cassette were sequenced (Figure 5). Our approach to detect unexpected DNA inserts in the genomes of the other three malolactic transformants that

were discarded, worked well (Figures 1 – 4). We have thus provided ample evidence there are no other fragments of the cassette in the genome and that there has been a single integration event.

Alignment of DNA sequences: ML01 and Native *URA3* locus

Upper line: ML01, from 1 to 8901

Lower line: Native *URA3* locus, from 1 to 2081

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1      CAGCAATTAATACTTGATAAGAAGAGTATTGAGAAGGGCAACGGTTCATCATCTCATGGA
      |||
1      CAGCAATTAATACTTGATAAGAAGAGTATTGAGAAGGGCAACGGTTCATCATCTCATGGA

61     TCTGCACATGAACAAACACCAGAGTCAAACGACGTTGAAATTGAGGCTACTGCGCCAATT
      |||
61     TCTGCACATGAACAAACACCAGAGTCAAACGACGTTGAAATTGAGGCTACTGCGCCAATT

      .....
      .....continuation of malolactic cassette.....
      .....

8641   CGCTGCCTTGGGACAAGGCTTGGGCCGATAAGGTGTACTGGCGTATATATATCTAATTAT
      |||
1821   CGCTGCCTTGGGACAAGGCTTGGGCCGATAAGGTGTACTGGCGTATATATATCTAATTAT

8701   GTATCTCTGGTGTAGCCCAATTTTGTAGCATGTAATATAAAGAGAAACCATATCTAATCTA
      |||
1881   GTATCTCTGGTGTAGCCCAATTTTGTAGCATGTAATATAAAGAGAAACCATATCTAATCTA

8761   ACCAAATCCAAACAAAATTCAATAGTTACTATCGCTTTTTTCTTTCTGTATCGCAAATAA
      |||
1941   ACCAAATCCAAACAAAATTCAATAGTTACTATCGCTTTTTTCTTTCTGTATCGCAAATAA

8821   GTGAAAATTAATAAAGAAAGATTAAATTGGAAGTTGGATATGGGCTGGAACAGCAGCAGT
      |||
2001   GTGAAAATTAATAAAGAAAGATTAAATTGGAAGTTGGATATGGGCTGGAACAGCAGCAGT

8881   AATCGGTATCGGGTTCGCCAC
      |||
2061   AATCGGTATCGGGTTCGCCAC
  
```

Figure 5: The upstream and downstream sequences flanking the malolactic cassette in *S. cerevisiae* ML01 are 100% identical. The sequence obtained from ML01 is shown in the upper line and the native sequences are shown in the lower line. Only a partial sequence of the malolactic cassette is displayed. The *Srfl* half sites (integration sites) are indicated in bold and underlined. The full sequence of the malolactic cassette has previously been provided to the Registrar.

*It is also somewhat unclear how many genetic changes have been made to Saccharyomyces cerevisiae ML01. Although detailed proteome and transcriptome analysis has been performed (Husnik et al 2006) it is unclear from these studies what the genetic changes are.*

No other genetically enhanced cell has been tested to the same extent as the malolactic wine yeast ML01. We have analyzed the phenotype, genotype, transcriptome, proteome and metabolome of ML01. Data were provided in the Notification to the Registrar, Husnik et al. (2006) and this document. Only two genetic changes were made; the introduction of the *mleA* and the *mae1* genes resulting in an efficient malolactic fermentation by ML01. In the process one of the two *URA3* loci in the parental strain S92 were disrupted. ML01 is identical to the parental strain S92 except for its ability to conduct an efficient malolactic fermentation.

The applicant states, “ ... there is a vast amount of sequence similarity between the genomes of *S. cerevisiae* S288c , ML01 an dS92”. However, the evidence needs to be presented and quantified (the term “vast” is imprecise) if the transgenic cassette is the only change to the genome of the parent strain then the genome similarity should be >99.9%. The methods used to determine genome similarity (PCR targeting *Ty1* retrotransposon) will only detect gross genetic changes and more sensitive methods such as RAPD or AFLP should be used.

Conclusive data have been provided that ML01 is identical to S92 except that ML01 can conduct the malolactic fermentation. There is no need to provide evidence how similar ML01 (and S92) is to S288c. This comment was made only with respect to the fact that Affymetrix DNA chips based on the sequence of S288c, were used successfully in microarray tests indicating that a “vast amount of sequence similarity exists between S288c, ML01 and S92”. We have used Affymetrix S288c gene chips successfully to analyze global gene transcripts in different wine yeast strains (Erasmus et al., 2003; Husnik et al., 2006; Marks et al., 2003). It is important to note that there is substantial variability in metabolism between different wine yeasts.

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