Appl Microbiol Biotechnol (2011) 92:441–447
DOI 10.1007/s00253-011-3546-2

MINI-REVIEW

Genomic variations of *Oenococcus oeni* strains and the potential to impact on malolactic fermentation and aroma compounds in wine

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Received: 1 June 2011 / Revised: 2 August 2011 / Accepted: 11 August 2011 / Published online: 26 August 2011

Abstract Malolactic fermentation (MLF) is the bacterially driven decarboxylation of L-malic acid to L-lactic acid and carbon dioxide, and brings about deacidification, flavour modification and microbial stability of wine. The main objective of MLF is to decrease wine sourness by a small increase in wine pH via the metabolism of L-malic acid. *Oenococcus oeni* is the main lactic acid bacterium to conduct MLF in virtually all red wine and an increasing number of white and sparkling wine bases. Over the last decade, it is becoming increasingly recognized that *O. oeni* exhibits a diverse array of secondary metabolic activities during MLF which can modify the sensory properties of wine. These secondary activities include the metabolism of organic acids, carbohydrates, polysaccharides and amino acids, and numerous enzymes such as glycosidases, esterases and proteases, which generate volatile compounds well above their odour detection threshold. Phenotypic variation between *O. oeni* strains is central for producing different wine styles. Recent studies using array-based comparative genome hybridization and genome sequencing of three *O. oeni* strains have revealed the large genomic diversity within this species. This review will explore the links between *O. oeni* metabolism, genomic diversity and wine sensory attributes.

Keywords *Oenococcus oeni* · Malolactic fermentation · Microarrays · Genomics · Wine aroma

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Introduction

In addition to the initial impact that wine colour has on the perception of wine quality, it is the aroma and flavour of a glass of red or white wine that has the greatest impact on the consumer. The formation of wine aroma is complex, with a combination of many factors contributing and interacting throughout grapegrowing, harvesting, winemaking and maturation. Grape variety and composition, viticultural practices, yeast and bacterial metabolism during fermentation, winemaking techniques, ageing in oak barrels and bottling with different closures (natural cork, synthetic closure or screw cap) all contribute to the sensory experience of wine.

Oenococcus oeni is the main lactic acid bacteria (LAB) involved in winemaking, and its major role is conducting malolactic fermentation (MLF), the decarboxylation of L-malic acid to L-lactic acid and CO_2 (Henick-Kling 1993). The consequence of MLF is an increase in wine pH 0.2 to 0.5 units and a decrease in titratable acidity, which translates into a decrease in wine sourness (Amerine and Roessler 1983). There are two other consequences of MLF: increased microbial stability of wine through the removal of a potential carbon source (malic acid) which can be utilised by spoilage yeast and bacteria, and the bacterial production of various secondary metabolites, which can improve the organoleptic properties of wine. Whilst other wine-associated LAB species, from the *Lactobacillus* and *Pediococcus* genera, are able to conduct MLF, species from these groups are also associated with wine spoilage and therefore are generally regarded as undesirable.

Malolactic bacteria have had an interesting history in microbiology. A summary of this is shown in Fig. 1. Evidence of winemaking dates back almost 7,000 years, and it has long been recognised that bacteria make a positive contribution (Chambers and Pretorius 2010; Swiegers et al. 2005).

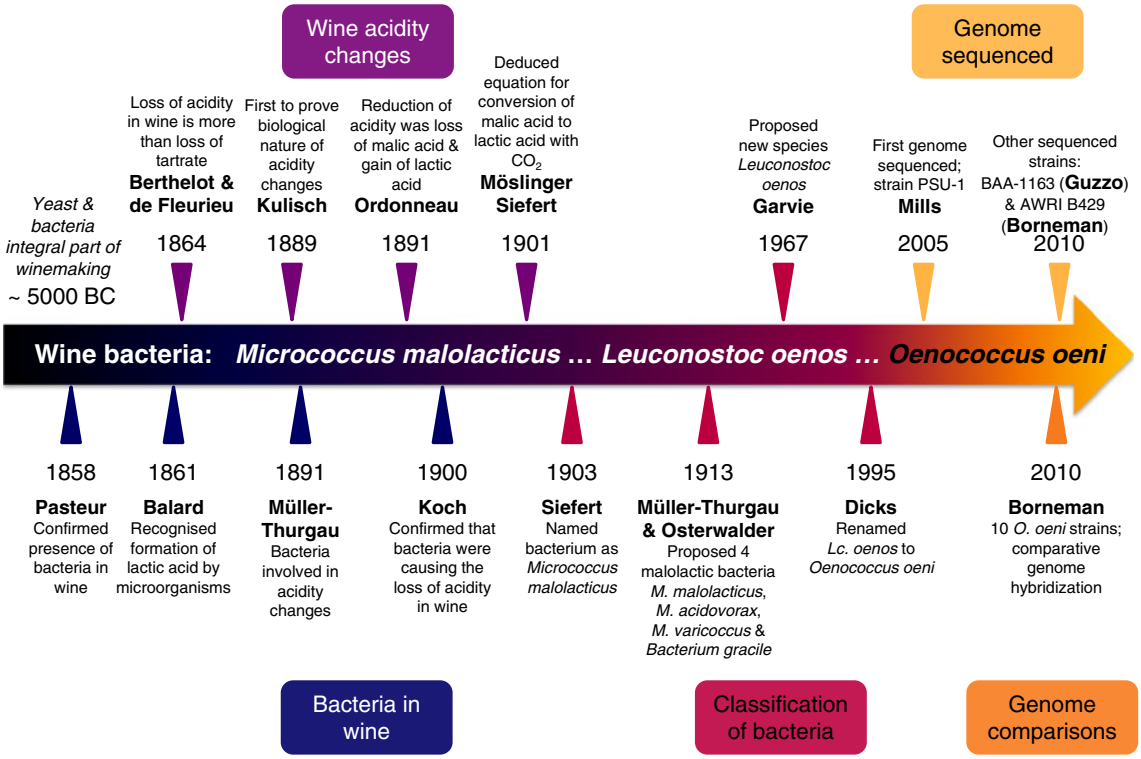

Fig. 1 Summary of the identification of bacteria in wine, elucidation of their role in winemaking, classification and genome analysis. Information was sourced from numerous articles (Garvie 1967; Kunkee 1967; Pasteur 1873; Möslinger 1901; Müller-Thurgau 1891; Müller-Thurgau and Osterwalder 1913)

Louis Pasteur first described the presence of bacteria in wine over 150 years ago, and despite their role in malic acid metabolism being elucidated only 50 years later, through the work of Müller-Thurgau, Berthelot, de Fleurieu, Ordonneau, Koch and Möslinger (Bartowsky 2005), the actual bacterial species responsible was not formally classified (originally as *Leuconostoc oenos*) until the mid-1960s by Garvie (1967). With the introduction of molecular biology techniques, a new genus, *Oenococcus*, was described, and *L. oenos* was reclassified as *O. oeni* (Dicks et al. 1995). *O. oeni* was the sole species in the genus until the mid-2000s when *Oenococcus kitaharae* was identified in composting distilled shochu residue (Endo and Okada 2006). The genome of *O. oeni* was mapped in the 1990s (Ze-Ze et al. 1998), and several strains have now been fully sequenced (Mills et al. 2005; Borneman et al. 2010; Guzzo, unpublished data).

This mini review aims to highlight how genomics is beginning to assist in connecting the sensory aspects of MLF and *O. oeni* metabolism with genetic diversity within the species.

O. oeni and malolactic fermentation

One of the most important aspects of MLF is to ensure that the process is reliably completed in a timely manner so that the wine can be stabilised as soon as possible and spoilage microorganisms do not proliferate; a prolonged or delayed MLF augments the risk of spoilage by microorganisms including *Lactobacillus*, *Pediococcus*, *Acetobacter* and *Brettanomyces* species (Gerbaux et al. 2009; Bartowsky and Pretorius 2008; Renouf et al. 2007). Spoilage by the yeast *Brettanomyces bruxellensis* can, for example, lead to production of 4-ethyl phenol and related compounds giving rise to undesirable sensory qualities (e.g. sweaty, barnyard, horsey, medicinal characters) (Loureiro and Malfeito-Ferreira 2003; Curtin et al. 2007). Bacteria-related spoilage includes mousy off flavour, geranium taint, acrolein, ropy wines, elevated acetic acid production (volatile acidity) and the production of biogenic amines (Bartowsky and Henschke 2008; Bartowsky and Pretorius 2008; Sponholz 1993; Lonvaud-Funel 2001).

Metabolism of L-malic acid in *O. oeni* is via the malolactic enzyme (Kunkee 1991) and is a direct enzymatic decarboxylation, with NAD and Mn^{++} as cofactors and without free intermediates. The enzyme is composed of two identical subunits of 60 kDa and has been purified from several LAB species (Naouri et al. 1990; Lonvaud-Funel and Strasser de Saad 1982; Caspritz and Radler 1983; Spettoli et al. 1984). Genes encoding malolactic enzyme (*mleA*), malate permease (*mleP*) and a proposed regulatory protein (*mleR*) have been cloned, sequenced and mapped on

the *O. oeni* chromosome (Labarre et al. 1996a, b; Mills et al. 2005; Ze-Ze et al. 2008).

MLF can occur spontaneously via indigenous *O. oeni* populations, but better control over the time of onset of MLF can be achieved by inoculating wines with a selected bacterial culture (Nielsen et al. 1996). However, efficient MLF is not easily achieved as the often nutritionally poor and harsh chemical composition of wine (high ethanol concentration [can be >15% *v*/*v*], low pH [can be <3.5] and high SO_2 concentration [>50 mg/L]) are natural hindrances to the growth of *O. oeni*.

O. oeni strains vary in their ability to metabolise L-malic acid. However, strains selected for commercialisation are usually chosen for their ability to metabolise malic acid efficiently and confer desirable sensory properties on the wine. Recently there has been growing interest in characterising *O. oeni* strains that are unique to particular geographical wine regions in order to enhance regionality in the wines (Yanagida et al. 2008; Solieri et al. 2010; Ruiz et al. 2010; Capozzi et al. 2010; Vigentini et al. 2009; Canas et al. 2009; Sico et al. 2008; Li et al. 2006).

Aroma and flavour aspects of MLF

Wine is a highly complex mixture of compounds which largely define its appearance, aroma, flavour and mouthfeel properties. Grape-derived compounds provide varietal distinction and basic structure to wine, but it is largely the volatile metabolites that originate from yeast and bacterial metabolism of grape compounds that provide wine its individual character and shape wine style. *O. oeni* has an extensive suite of metabolic pathways and enzymes that generate volatile secondary compounds at concentrations well above their odour detection threshold, including ethyl and acetate esters, higher alcohols, carbonyls, volatile fatty acids and sulphur compounds (Siebert et al. 2005; Bartowsky 2005), and strain-to-strain variation in metabolic capabilities impact on the types and concentrations of compounds produced (Bartowsky 2005; Francis and Newton 2005; Matthews et al. 2004). For example, some *O. oeni* strains contribute neutral aroma–flavour to red and white wine (Bartowsky and Henschke 1995), whilst others enhance fruity (Laurent et al. 1994) or the buttery characters (Bartowsky and Henschke 2004; Martineau and Henick-Kling 1995).

Diacetyl is a major secondary metabolite associated with citric acid metabolism during *O. oeni*-driven MLF, and the kinetics of its production is well understood (Ramos et al. 1994, 1995; Bartowsky and Henschke 2004). Diacetyl imparts a buttery character which adds complexity to wine and is mostly present in concentrations well above its odour detection threshold (Bartowsky et al. 2002b; Martineau and Henick-Kling 1995; Martineau et al. 1995). The genetics of the diacetyl pathway are well described in the literature on dairy research (Cogan 1995; Smit et al. 2005). The two *O. oeni* genes (*alsS* and *alsD*) encode the main enzymes involved; α-acetolactate synthetase and α-acetolactate decarboxylase genes, respectively, have been cloned and characterised (Garmyn et al. 1996).

The operon carrying *alsS* and *alsD* has been shown to be constitutively expressed in *O. oeni* BAA-1163 (previously referred to as Lo84.13), but there is strain-to-strain variation in the final concentration of diacetyl synthesised during MLF of the same wine (Martineau and Henick-Kling 1995; Bartowsky et al. 2002a). *O. oeni* strains that produce high concentrations of diacetyl can be encouraged to do so, and winemaking techniques can be used to maintain the desired diacetyl concentration to accentuate the buttery character of wine (Bartowsky and Henschke 2004).

Latent aroma and flavour compounds are often glycosylated and can be enzymatically liberated by microbial glycosidases (Gunata et al. 1988, 1990). While numerous fungi and bacteria produce glycosidases, there is variation in the abilities of these microbes to function efficiently under the high alcohol and low pH present in winemaking conditions. *O. oeni* has numerous glycosidases (Grimaldi et al. 2005, 2000), and their activities contribute to the release of numerous aroma compounds, including monoterpenes, norisoprenoids and aliphatic compounds, all of which contribute to fruity and floral wine attributes (D'Incecco et al. 2004; Williams et al. 1989, 1982; Ugliano et al. 2003; Ugliano and Moio 2005, 2006). As for diacetyl production, there is large variation in *O. oeni* strain capacity to release aroma compounds from grape glycosides (Grimaldi et al. 2005; Ugliano et al. 2003; Ugliano and Moio 2006).

Most commercial glycosidase preparations are crude extracts prepared from fungi rather than bacteria. However, there has recently been renewed interest in using wine LAB as potential sources of these enzymes as these bacteria are well adapted to the high ethanol and acid conditions encountered during winemaking, and therefore, their enzymes might be expected to perform efficiently under these conditions. Several research teams have cloned and characterised β-glucosidases from *O. oeni* and *Lactobacillus brevis* strains (Michlmayr et al. 2010a, b; Capaldo et al. 2011) and α-arabinofuranosidase from *O. oeni* and *L. brevis* (Michlmayr et al. 2011).

Many wine aromas are attributable to interacting compounds, which together confer the sensory impact of the aroma descriptor. For example, the red and black berry aromas of a red wine are made up of at least six different esters and volatile fatty acids (Pineau et al. 2009). Other compounds play a more complex role. Dimethyl sulphide, for example, has a cooked corn aroma; however, it also enhances fruity characters in red wine (Segurel et al. 2004).

Enhancement of the fruity and berry characters of red wine is an important goal for winemakers, and one approach involves using selected *O. oeni* strains to attain sought-after wine styles (Iland et al. 2009). Using sensory descriptive wine analysis and wine composition–ethyl ester concentrations, we are gaining a better understanding of which metabolites contribute to the fruitness of red wine (Pineau et al. 2009, 2010; Guth 1997a, b). A recent study in Cabernet Sauvignon wines has identified relationships between enhanced red berry sensory attributes and ester concentration (Fig. 2) (Bartowsky et al. 2010).

Clearly, MLF is important for winemaking, and *O. oeni* strains make a significant contribution to the aroma and flavour of wine. The next phase to unravelling how malolactic bacteria enhance wine sensory characters is to link phenotypic with genotype characteristics. However, *O. oeni* is not very amenable to the uptake of DNA; even though there are transformation and conjugation methods cited, they are not very efficient (Dicks 1994; Beltramo et al. 2004; Assad-Garcia et al. 2008; Eom et al. 2010). Nonetheless, with the aid of modern 'genomic' technologies, we can now use comparative genomic approaches to enable the identification of genetic elements in *O. oeni* that shape wine flavour during MLF.

Connecting the *O. oeni* genome with wine

O. oeni has a compact genome of approximately 1.8 Mb which appears to be highly streamlined as a consequence of adaptation to its ecologically restricted niche, i.e. grape juice and wine. Prior to several strains having their genome fully sequenced, detailed genome mapping had been conducted on several strains, providing insight into the genetic organisation of the bacterium (Ze-Ze et al. 1998, 2000). Important phenotypes, such as malate degradation (MLF), citrate metabolism and diacetyl production, were mapped. Many of the stress response genes have been studied, and metabolic pathways relating to amino acid metabolism have also been well characterised (summarised in Bartowsky 2005). However, the genetics underpinning the generation of metabolites contributing to wine aroma have been less thoroughly investigated.

Comparison of the three available *O. oeni* genome sequences suggests the three strains share a group of conserved ORFs (52% of total ORFs), with up to 10% of the coding potential of any one strain being specific to that strain (Borneman et al. 2010). It is presumably this variation in the *O. oeni* species-wide (pan) protein coding capacity that will ultimately be the likely key to phenotypic differences between *O. oeni* strains.

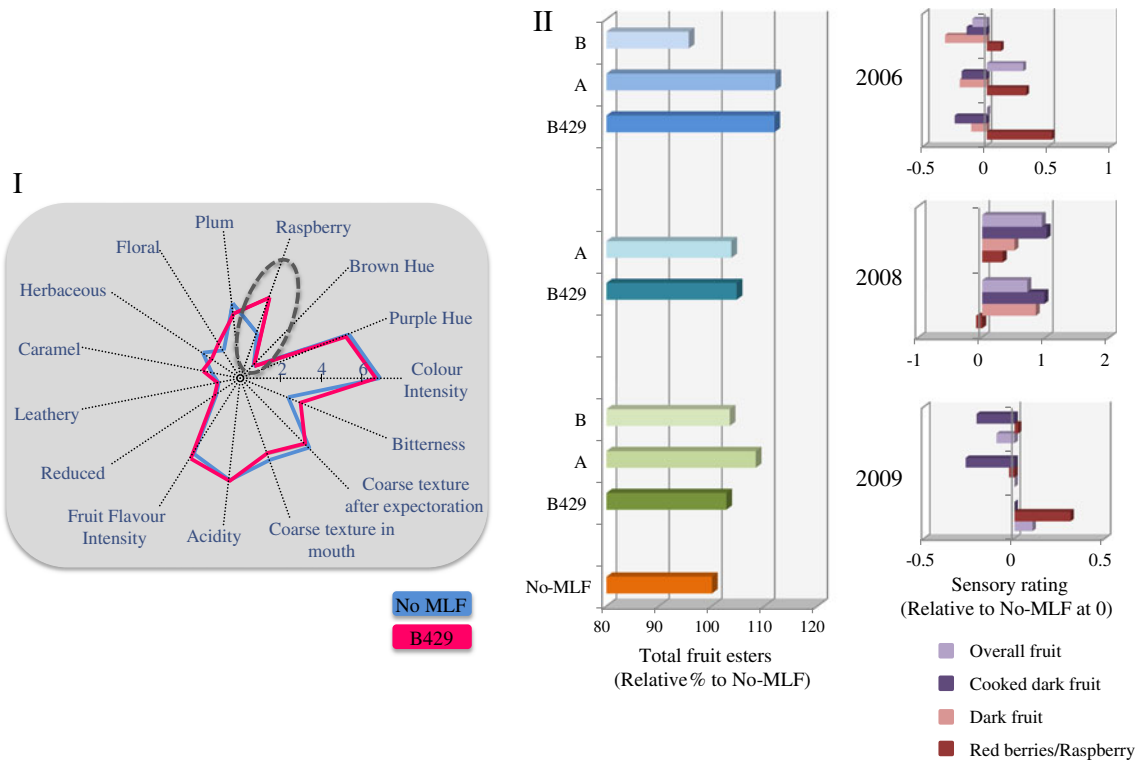

Fig. 2 Example of sensory changes following MLF in Cabernet Sauvignon wines with different strains of *O. oeni*. *I* Radial plot of aroma and flavour sensory descriptors of Cabernet Sauvignon wine (Berri, Australia) following MLF with *O. oeni* strain AWRI B429 (adapted from Bartowsky et al. 2008). *II* Relative changes in sensory descriptor rating for Cabernet Sauvignon wines (sourced over three vintage from Clare Valley, Australia) and the concentration of fruity esters following MLF by three *O. oeni* strains (P. Costello and colleagues, 2009, unpublished). Total fruity esters are based on a combination of ethyl ester concentrations (Pineau et al. 2009)

In our recent array-based comparative genomics research, we analysed 11 *O. oeni* strains, all of which were either commercial or natural isolates that are routinely used in Australian winemaking. One of the most interesting observations from this work was that there was significant intra-specific genomic variation due to substantial insertions and deletions throughout the 11 *O. oeni* strains, including two large deletions in regions 45–65 and 1,400–1,450 kb (Borneman et al. 2010). However, as all of these strains are used in the wine industry, these rearrangements clearly do not have a significant impact on the MLF capability of the *O. oeni* strains, but may well contribute to the phenotypic differences between strains, resulting in wines with different sensory profiles.

A relationship between genome variation and efficient malate metabolism has been suggested by studying the MLF capacity of over 70 *O. oeni* strains in three wines and using comparative genome subtractive hybridization to propose that the presence of eight stress-responsive genes could be associated with high MLF performance (Bon et al. 2009).

The winemaking capabilities of *O. oeni* strain AWRIB429 have been well studied with observations that red wines produced with this *O. oeni* strain consistently lift red fruit–red berry aroma attributes (Schmid et al. 2007; Borneman et al. 2010; Bartowsky et al. 2010, 2008) (Fig. 2). Within the *O. oeni* AWRIB429 genome, numerous novel ORFs were identified, and amongst the annotated ORFs were several potential glycosidases (Borneman et al. 2010). Thus, in addition to other aroma-forming pathways, the acquisition of additional glycosidases by AWRIB429 might provide a genomic link to the consistent ability of this strain to enhance red fruit–red berry aroma attributes to red wines.

Conclusions and future outlook

O. oeni conducts malolactic fermentation, often under difficult environmental circumstances, to impart important sensory attributes to wine. It has a compact genome of approximately 1.8 Mb resulting from a high degree of genomic streamlining which occurred during its adaptation to the complex wine environment. Recent analysis of several *O. oeni* genomes has demonstrated a shared core genome with up to 10% variation in coding capacity between strains. This variation in coding capacity is likely to be responsible for observed differences in winemaking phenotypes. A link between the presence of several stress-related genes and MLF capability has been proposed, and the presence of additional glycosidases appears to enhance the fruity sensory attributes of an *O. oeni* strain. Even though *O. oeni* is not easily genetically manipulated, due to poor transformability, the inter-strain genetic variation of the *O. oeni* genome holds the secret to exploiting its potential to influence wine aroma and flavour.

Acknowledgements The authors thank Dr. Paul Chambers for discussions during the preparation of this mini review and Lallemand SA for allowing us to use data presented in Fig. 2 from a project for which they provided financial support. The Australian Wine Research Institute (AWRI) is supported by Australian grapegrowers and winemakers through their investment agency, the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster in Adelaide.

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