Colony dimorphism associated with stress resistance in *Oenococcus oeni* VP01 cells during stationary growth phase

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Abstract

The resistance to stresses as starvation, the presence of ethanol, sulfite and low pH, is a fundamental prerequisite for starter cultures used to induce malolactic fermentation in wine. In order to evaluate stress resistance of cells undergone starvation, cells viability in laboratory cultures of *Oenococcus oeni* VP01 strain was monitored during prolonged stationary growth phase. Once entered the stationary phase, strain VP01 showed 99% reduction of cell viability within 4 days. The remaining cells population maintained viability over 70 days and, when plated on agar medium, generated small colonies. The occurrence of this phenomenon was associated to stress resistance, since 10-day-old cells resulted more resistant than 3-day-old cells to ethanol and low pH conditions. No genomic mutations were revealed by pulse-field gel electrophoresis (PFGE) analysis in aged cultures. Total protein analysis by bidimensional electrophoresis highlighted differential protein expression in cultures differentially aged. It was demonstrated that *O. oeni* starving cultures at the stationary phase are constituted by dynamic cell populations. These results offer interesting perspective for a better understanding of cells behavior when inoculated in wine.

1. Introduction

The wine represents a harsh environment for cell survival because of the nutrient limitation and the presence of ethanol, low pH, sulfite and some fatty acids [1]. *Oenococcus oeni* is the best adapted bacterial species to grow in wine and it is frequently associated to the spontaneous malolactic fermentation. In winemaking, the success of malolactic fermentation depends to the recovery capability of the starter cells inoculated in wine. *O. oeni* starter cells have to maintain high viability after the inoculum in wine to have high probability to overcome indigenous microflora [2].

It was demonstrated that long periods of starvation conferred competitive advantage in selected environments as well as adaptation to starvation enhances the potential for survival and growth of bacteria in harsh environments [3–6]. The entrance of bacteria cells into stationary phase, caused by starvation, induces the synthesis of protective proteins responsible for the resistance to chemical and physical challenged [7–9]. Moreover, the entrance into stationary phase is accompanied by structural and physiological changes that result in the increase of stress resistance. Evidences of change in colony morphology as bacteria cells become resistant to starvation were reported previously [10].

It has been reported that culture conditions influence *O. oeni* cells survival after exposure to stress and cells
harvested from stationary growth phase in preculture showed the best performance in wine [11,12]. Guzzo et al. [13,14] described the induction of LO18 protein associated to stress tolerance during stationary phase of O. oeni. Nevertheless, no investigations evaluated the O. oeni response to stresses with regard to the cell age after entering in the stationary phase as well as it was reported in the case of other bacteria species [4,5,15]. Due to the importance of survival capability of O. oeni when inoculated in wine, deeper investigations on the association between stationary phase and stress resistance are necessary.

In the present study cell viability of O. oeni VP01 strain during stationary phase and prolonged periods of starvation was determined. Thus, it was evaluated the resistance capability of O. oeni VP01 cells differentially aged to ethanol and low pH as main stress factors present in wine. Finally, proteome analysis of cells populations during extended stationary phase was carried out.

2. Materials and methods

2.1. Strain and growth conditions

The strain VP01 of O. oeni, isolated from Valpolicella wine (Verona, Italy), was used. This strain was grown at 28 °C in FT80 medium [16] added with meat extract instead of casamino acid. The initial pH values were adjusted to pH 5.3 or pH 3.5. In modified FT80 pH 5.3 agar, the strain was cultivated anaerobically using Anaerocult A kit (Merck, Germany) and plates were incubated at 28 °C for 5 days.

2.2. Stress resistance tests

Viability of cells differentially aged was tested after inoculation in FT80 with ethanol and low pH. An initial cell density of about 10^6 colony-forming units (CFU/ml), taken from culture of 3- (3D) and 10-day-old (10D) grown in FT80 pH 5.3, were inoculated in each 10 ml vial of FT80 pH 5.3 and pH 3.5 in presence or absence of 10% vol ethanol. Cells viability was estimated by plating serially diluting cell suspensions in FT80 pH 5.3 agar, in twice.

A spent medium of 30-day-old culture, growing in FT80 pH 5.3, was harvested after cells centrifugation, sterilized with 0.22 μm filter (Millipore, MA, USA), then transferred on pellets of 3D and 10D cells grown in 10 ml FT80 pH 5.3 and CFU was calculated. 3D and 10D cells maintained in FT80 pH 5.3 were used as control.

All the experiments were repeated at least twice and the results are expressed as averages with standard deviation.

2.3. Genomic and total proteins analysis

Genomic DNA extraction and subsequent PFGE analysis using Apal restriction enzyme were performed according to Zapparoli et al. [17].

Total proteins were extracted from 5 ml of 3D and 10D cultures, growing in FT80 pH 5.3. The cell pellets were lysed in 500 μl of solubilization buffer (7 M urea, 2 M thiourea, 20 mM Tris, 5 mM tributylphosphine, 1% ampholythes, pH 3–10 (Fluka, Germany), 3% Chaps), with 25 mg micro-glass beads by vortexing for 30 min at room temperature. After centrifugation the protein concentration on supernatant was measured by DC protein assay (Bio-Rad, CA, USA) and 0.2 mg of total proteins extract was analyzed by bidimensional PAGE according to Castagna et al. [18]. Five replicates for 3D and 10 D protein extracts were carried out. Protein bands were visualized by Sypro ruby staining (Bio-Rad) and gel analysis and statistical treatment of the data were carried out by PDQuest software (Bio-Rad).

3. Results

Growth kinetic of O. oeni VP01 on FT80 pH 5.3 was monitored by CFU count from the inoculum to over 70 days of incubation (Fig. 1). After cells had emerged from a lag phase, maximum cell density was reached in 45–50 h. At the entrance of stationary phase, cell population maintained a high cell density only for a short period (24–30 h), then a 99% decline of cell viability was registered. Therefore, a stable cell density (about 10^7 CFU/ml) was maintained for a period of over 6 days up to 70 days.

During viability determination of cells grown in broth culture, it was observed variation of colony morphology originated from culture samples taken at different time of stationary phase. Cultures aged over 6 days, after plating out onto the FT80 medium with agar and 5 days of incubation, generated colonies characterized by small size (<0.5 mm diameter) together normal size col-

![Fig. 1. Viable count of O. oeni VP01 cells in FT80 pH 5.3, expressed as log_{10} CFU/ml, during starvation for 70 days. Bars indicate standard deviations.](https://academic.oup.com/femsle/article-abstract/239/2/261/622530)
onies (1–2 mm diameter) (Fig. 2(a)). The colony morphology remained unchanged also after 10 days of further incubation on the same plates. The percentage of small colonies ranged from 10% to 60% of total colonies in relationship to the culture age. One-month-old cultures or older showed a higher percentage of colonies (40–60%) than younger cultures (10–30%). Ten colonies representative of each morphotype were randomly isolated, streaked out and inoculated in FT80 agar and broth, respectively. Isolates originated from small size colonies did not maintain the parental morphology and restored the growth kinetic of cultures derived from normal size colony (Fig. 1) when inoculated in broth medium (data not shown). In addition, by PFGE analysis using Apal enzyme, bands patterns of parental strain cells and cells obtained from different colony morphotypes resulted identical (Fig. 2(b)).

In order to study the variation of colony morphology associated with cell age at the stationary phase, resist-

ance tests to starvation of cells differentially aged were carried out. In Table 1 is reported viable cell counts of 3D and 10D O. oeni VP01 cells, before and after the

99% cells population mortality, respectively, inoculated in FT80 pH 5.3 and 3.5 with or without 10% vol ethanol. The survival rate of 10D was enhanced in the media with ethanol and at low pH with respect to 3D. A clear improvement in the survival ability of O. oeni in the most strictly condition tested in this study (FT80 pH 3.5 with 10% vol ethanol) was observed for 10D with respect to 3D.

To verify that old cells were more resistant than young cells to starvation condition it was evaluated the effects of spent medium on survival rate of cells 3-
day-old and 10-day-old cultures. The transfer of the spent medium to 3D culture caused about 99% cells mortality in advance with respect to the culture growing on FT80 pH 5.3 used as control (Fig. 3). On the contrary, 10D survived after being transferred in the same spent medium as it was for to the control culture. The 1% cell population viable in late stationary phase, as reported in Fig. 1, is the result of natural selection which favors only cells able to survive in starvation condition.

Total protein analysis by 2D-PAGE of 3D and 10D was carried out and the maps were showed in Fig. 4. Highly significative differences between the two electrophoretic protein patterns were evaluated by spot intensity quantification and statistical analysis. Of the proteins detected in both samples 81 out of 186 varied significative more than 2-fold or less than half (p<0.05) in 10D respect to 3D: 13 and 10 proteins were expressed exclusively by 3D and 10D cells, respectively. A greater number of proteins appeared to be up- or down-regulated: 15 proteins were up-regulated and 43 proteins were down-regulated in 10D respect to 3D cells.

4. Discussion

O. oeni VP01 showed a biphasic kinetic of stationary phase survival as reported for other lactic acid bacteria [4,19]. The transition between the log and stationary phase has an effect on the cells survival during long-term starvation [20]. Similarly, the transition between early and late stationary phase has effects on induction of cross-protection against adverse growth conditions [5,6,15]. This study showed different survival rate

<table>
<thead>
<tr>
<th>Cells age in days</th>
<th>Hours of incubation</th>
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<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>3D</td>
</tr>
<tr>
<td>pH 5.3</td>
<td>8.2 ± 0.0</td>
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<tr>
<td>pH 5.3 with ethanol</td>
<td>6.0 ± 0.1</td>
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<tr>
<td>pH 3.5</td>
<td>5.6 ± 0.1</td>
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<tr>
<td>pH 3.5 with ethanol</td>
<td>2.0 ± 0.1</td>
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The data are averages with standard deviations of two independent trials.
between *O. oeni* VP01 cells taken at early and late stationary phase under stress as the presence of ethanol and/or low pH, and it is in agreement with the observation that stationary phase cells survive better in wine [12].

It was reported that the phenomenon of colony polymorphism, here observed only in late stationary phase of *O. oeni* VP01 and occurred in long term incubated cultures, is the result of the coexistence of different subpopulations originated by spontaneous mutations [4, 21, 22]. Nevertheless, the indistinctive PFGE profiles and the restoration of normal growth rate in fresh medium suggest that the presence of small size colonies could not be imputable to genomic mutations occurring during starvation. The reduction in cells size and the small colonies formation due to slow growth are the results of the general strategy bacterial cells used to conserve energy during starvation [23]. This phenomenon, occurring in *O. oeni*, could be related to the reduction in cell size associated with transformation to viable but unculturable state observed in other bacteria such as *Vibrio* and *Helicobacter* [10].

According to Finkel and Kolter [21] the behavior of bacteria populations during starvation in laboratory reflects the processes that act on natural populations and increase microbial diversity. Similarly, the existence of growth advantage in stationary phase (GASP) phenotype in *O. oeni* populations in prolonged starvation

![Graph showing viable count of cells (log$_{10}$ CFU/ml) after the transfer of spent FT80 pH 5.3 medium harvested from 30-day-old cultures into 3- (3D) and 10-day-old (10D) cells growing in FT80 pH 5.3. A parallel culture growing on FT80 pH 5.3 was used as control. Bars indicate standard deviations.](https://academic.oup.com/femsle/article-abstract/239/2/261/622530)

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![2D-PAGE of total proteins extracted from 3- (3D) and 10-day-old (10D) cells growing in FT80 pH 5.3. On the left side two bidimensional maps representative of 3D and 10D five replicates are displayed. The squares, on the right, show a detail of each map. The arrows indicate some spots taken as representative of the four possible expression trends in 10D vs. 3D comparison (ON, OFF, up- and down-regulated). Numbers refer to spots indicated by software used for gel analysis.](https://academic.oup.com/femsle/article-abstract/239/2/261/622530)
could be supposed. Because it is not infrequent to observe polymorphism among O. oeni colonies after plating a wine sample (unpublished data), additional studies are necessary to ascertain this attractive hypothesis. In this study the data obtained by proteome analysis of cells differently aged confirmed the dynamic state of cultures at stationary growth phase. The existence of different total protein profiles in cultures offers interesting perspectives for further investigations by bidimensional electrophoresis analysis, as reported here, to individuate in O. oeni starvation specific proteins induced in long term cell survival.

In conclusion, the present study highlighted that stationary phase cultures of O. oeni VP01 are constituted by different cell populations. The variation of colony morphology and the different proteome expression of cells differentially aged were associated to stress resistance during starvation conditions. These experiments applied on wine could have interesting applications on malolactic starters using in winemaking practices.

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References