Improvement of the resistance of Lactobacillus delbruekii spp bulgaricus to freezing by natural selection

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ABSTRACT

Lactic acid bacteria are often produced as frozen or freeze-dried cultures, that can be used for the direct inoculation of milk in cheese and fermented milk production processes. The objective of this study was to investigate whether the resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* to freezing could be improved by natural selection. Three parallel cultures of strain CFL1 were propagated for 30 cycles in which each cycle involved three serial transfers through milk, one freezing step and one thawing step. The concentration in viable cells after thawing as well as the acidifying activity of the thawed cultures increased dramatically throughout the experiment. This may be explained by the random appearance of better adapted mutants that can outcompete the other genotypes. However, after 30 cycles of subcultivation, freezing and thawing, all the cultures contained subpopulations having different survival rates to freezing. Our results show that serial transfer culture experiments may be used to improve technological properties of lactic acid bacteria. Furthermore, investigation of the mutations that are responsible for an increased cryotolerance may help to define new targets for improving the resistance of lactic acid bacteria to several stresses.

(**Key words:** lactic acid bacteria, freezing, acidifying activity, *Lactobacillus*)

Abbreviation key: RAPD = randomly amplified polymorphic DNA.

INTRODUCTION

Lactic acid bacteria belonging to the genus *Lactobacillus* are used in the manufacture of several kinds of cheeses and fermented milks. Their main function is the production of lactic acid. However, they may also be chosen for their contribution to aroma and texture, as well as for their probiotic role. They are generally used in mixed cultures containing other lactic acid bacteria (Hassan and Frank, 2001). In industrial processes, these cultures are often added to the milk as concentrated starter cultures that are in a frozen or freeze-dried form. Compared to the traditional inoculation procedure which consists of growing several successive cultures with a progressive increase in the culture volume, utilization of concentrated starter cultures has several advantages, such as increased flexibility in the fermentation process and standardization of biological activity (Lejard et al., 1994). However, lactic acid bacteria, and especially lactobacilli, often have a poor resistance to freezing or freeze-drying, which suggests why many strains cannot be easily produced in a frozen or freeze-dried form. Freezing has several deleterious effects on the cells. The formation of ice crystals induces mechanical damage on membranes and other cellular components (Mazur, 1965). Crystallization of water also leads to a cryoconcentration of the solutes, which induces some osmotic damage (Meryman, 1968). It has also been shown that an oxidative stress may occur during freeze-thaw treatments (Park et al., 1998; Stead and Park, 2000).

During the production of frozen concentrated starter cultures, several operating conditions have a great influence on the resistance of lactic acid bacteria to freezing (Fonseca et al., 2001) and numerous studies have been devoted to optimizing factors such as the composition of the culture medium or the addition of protective agents. Resistance to freezing may also be improved by applying moderate stress conditions before freezing (Béal et al., 2001; Broadbent and Lin,

1999; De Urraza and De Antoni, 1997; Kim and Dunn, 1997; Poirier et al., 1998; Teixeira et al., 1997). This is due to physiological changes such as synthesis of specific stress proteins (Broadbent and Lin, 1999; Panoff et al., 1994; Panoff et al., 1998; Whitaker and Batt, 1991) or modification of fatty acid composition of the membrane (Béal et al., 2001; Teixeira et al., 1996). Resistance of the bacterial cells to freezing may also be improved by genetic engineering. For example, overproduction of the cold shock proteins CspB and CspE increases the survival of *Lactococcus lactis* after four freeze-thaw cycles of a 10- and 5-fold factor, respectively (Wouters et al., 2000). Another way to generate strains with improved cryotolerance would be to select better adapted genetic variants by natural selection. This might be achieved by performing successive cycles of cultivation which include freezing and thawing of the culture. Theoretically, if better adapted spontaneous mutants appear, they will progressively outcompete the initial genotype (Dykhuizen, 1990). However, to our knowledge, such an approach has not yet been investigated.

The objective of the present work was to increase the cryotolerance of lactobacilli by natural selection. Such an increase should improve the acidifying activity of frozen cultures of lactobacilli. In this work, we studied the evolution of strain *Lb. delbrueckii* ssp. *bulgaricus* CFL1 during 30 successive cycles in which each cycle involved three serial transfers through milk, one freezing step and one thawing step. Like many lactobacilli (Tsvetkov and Shishkova, 1982), strain CFL1 has a low resistance to freezing. Three separate cultures were done in parallel in order to study the among-population diversity. The within-population diversity of the cultures was also investigated.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

After purification on MRS agar (de Man et al., 1960), *Lb. delbrueckii* ssp. *bulgaricus* CFL1 (INRA, Thiverval-Grignon, France) was cultivated for 24 h at 42°C in 5 ml of reconstituted skim milk (100 g/L; Elle et Vire, Condé-sur-Vire, France) that had been sterilized for 15 min at 110°C. Thirty cycles, in which each cycle involved three serial transfers through milk, one freezing step and one thawing step, were then performed. In each cycle, 250 ml of reconstituted skim milk that had been sterilized for 15 min at 110°C were inoculated at 0.4% (vol/vol) and incubated for 24 h at 42°C. After two other successive subcultures, 4 ml of the culture were poured in a 8 ml-volume glass tube, which was then placed in 50% $CaCl_2$ (wt/vol) at –16°C for 96 h. The frozen sample was then thawed for 10 min in a water bath at 30°C, and used for performing another subcultivation/freezing/thawing cycle. At each cycle, an aliquot of the culture was recovered before freezing at –16°C in order to preserve the cells for a long period. This was done by adding 5 ml of reconstituted skim milk and 0.75 ml glycerol to 1 ml of culture, and the resulting mixture was stored at –80°C.

Randomly Amplified Polymorphic DNA (RAPD) Analysis

Each culture to be analyzed was inoculated in MRS broth and incubated for 24 h at 42°C. This culture was then inoculated at 2% (vol/vol) in another tube containing MRS broth and the cells were recovered by centrifugation at the end of the exponential growth phase. Total DNA was extracted from bacterial cells (de los Reyes-Gavilan et al., 1992) and RAPD analysis was performed with the oligonucleotide 1254 (5'-CCGCAGCCAA-3') as described by Akopyanz and coworkers (1992).

Measurement of the Acidifying Activity

Acidifying activity of the cultures was measured before freezing at –16°C and after thawing. Two hundred fifty milliliters of reconstituted skim milk were inoculated at 0.4% and placed in a water bath at 42°C. The pH was continuously measured during 15 h using a CINAC apparatus (Ysebaert, Frépillon, France) and the time necessary to reach pH 5.5 ($t_{pH5.5}$ in min) was then determined (a low $t_{pH5.5}$ value corresponds to a high acidifying activity).

Measurement of Viable Cell Concentration

Viable cell concentration in the cultures was determined before freezing at –16°C and after thawing. The samples were diluted (1/100, vol/vol) in tryptone-salt solution (1 g tryptone and 8.5 g NaCl per liter) and vortexed for 1 min. After further dilution in tryptone-salt solution, 1 ml of the bacterial suspension was mixed with molten MRS agar and the colonies were enumerated after incubating the agar plates in anaerobiosis (Genboxanaer, Biomérieux, Marcy l'Etoile, France) for 2 days at 42°C.

RESULTS

Evolution of the Viability and the Acidifying Activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 During 30 Cycles of Subcultivation, Freezing and Thawing

A single colony of strain CFL1 was inoculated in three separate cultures, named A, B and C, which were then used for 30 successive cycles of subcultivation, freezing and thawing, as described in materials and methods. Evolution of the viable cell concentration in these cultures, before freezing and after thawing, as well as their acidifying activity, is shown in Figure 1. No significant increase of the viable cell concentration before freezing could be observed in culture A (Figure 1A). However, the acidifying activity before freezing increased slightly. Indeed, the time necessary to reach pH 5.5 ($t_{pH5.5}$) decreased from 288 min at the beginning of the experiment, to 221 min after 30 cycles. The viable cell concentration in culture A after thawing increased dramatically throughout the experiment. After the first thawing, only $5.9x10^{3}$ cfu/ml were enumerated, which corresponded to a survival rate of 10^{-5}. After the 30^{th} thawing, the viable cell concentration was $4.8x10^{7}$ cfu/ml, which corresponded to a survival rate of $4.3x10^{-2}$. This increase was not linear and at several stages, there was a stagnation or a temporary decrease in the viable cell concentration after thawing. There was also a strong increase in the acidifying activity of culture A after thawing, as the $t_{pH5.5}$ value decreased from 830 min at the beginning of the experiment, to 439 min after 30 cycles. As in culture A, culture B showed a slight decrease in the $t_{pH5.5}$ value before freezing, a dramatic decrease in the $t_{pH5.5}$ value after thawing, and a dramatic increase in the viable cell concentration after thawing (Figure 1B). However, the corresponding curves differed significantly from those of culture A. For example, in culture A the $t_{pH5.5}$ value after thawing started to decrease at the beginning of the experiment, whereas in culture B, it began to decrease only after the 8^{th} cycle. The overall evolution of the $t_{pH5.5}$ value

before freezing and after thawing, as well as of the viable cell concentration after freezing in culture C was similar to that in the two other cultures.

In the three cultures, there was a good relationship between the logarithm of population after thawing and the time necessary to reach pH 5.5 (data not shown). Linear regression of the data for culture A resulted in the following equation:

$t_{pH5.5}$ = -86 x log(population) + 1114 (r^2 = 0.94)

For cultures B and C, the slope of the equation was –102 and -122, respectively (r^2 value was 0.90 and 0.85, respectively). This means that differences in the acidifying activity of the thawed cultures may be the result of differences in the concentration of viable cells after thawing.

Even if the three cultures reached more rapidly a low pH after 30 cycles of subcultivation, freezing and thawing, there was no significant evolution of the pH after 24 h of growth, which was always near 3.7-3.9 (results not shown). At this pH, strains of *Lb. delbrueckii* ssp. *bulgaricus* are not able to grow, which indicates that cells were always in the stationary growth phase when freezing treatment was applied.

RAPD Analysis

In order to verify absence of contamination, RAPD analysis was performed after the 30th cycle of subcultivation, freezing and thawing of the parallel cultures A, B and C. The percentage of similarity between the corresponding RAPD profiles and the profile of the initial culture was 96.3, 97.9 and 96.0%, respectively. The percentages of similarity that were obtained when the RAPD profile of the initial culture was compared to those of two other repetitions of the initial culture were 94.7 and 99.0%. It can thus be concluded that the RAPD profile did not evolve significantly during the 30 cycles of subcultivation, freezing and thawing. As the oligonucleotide that was used in the RAPD analysis provides highly polymorphic profiles (Torriani et al., 1999),

these results indicate that the evolution of the cultures cannot be explained by a possible contamination.

Within-Population Diversity of the Cultures After 30 Cycles of Subcultivation, Freezing and Thawing

After the 30 cycles of subcultivation, freezing and thawing, the cultures were inoculated onto MRS agar plates and 25 colonies were picked at random for each of the three parallel cultures. These clones were tested individually for their viability and acidifying activity during one cycle of subcultivation, freezing and thawing. For the clones arising from culture A, there was significant diversity in the viable cell concentration before freezing (Figure 2A). The standard deviation of the logarithms of viable cell concentration was 0.30 unit. This diversity cannot be explained by a low repeatability of the viable cell concentration measurements, as the standard deviation of repeated cultures of the same clone was always lower than 0.12 unit. The diversity in the viable cell concentration after thawing was higher than that before freezing, as the corresponding standard deviation (calculated from the logarithm of the viable cell concentration) was 0.48 unit. There was also diversity in the acidifying activity of the clones. Before freezing, the time necessary to reach pH 5.5 varied from 197 min for clone 9 to 307 min for clone 16, and after thawing, it varied from 352 min for clone 21 to 560 min for clone 1. There was also a within-culture diversity in culture B (Figure 2B), as the standard deviations of the logarithm of viable cell concentration before freezing and after thawing were 0.34 and 0.76 unit, respectively. The mean $t_{pH5.5}$ value after thawing in culture B was higher than in culture A (503 versus 411 min), whereas the mean viable cell concentration after thawing was lower ($8.7x10^6$ versus $9.2x10^7$ cfu/ml). Culture C (Figure 2C) showed a lower within-population diversity than the two

other cultures, as the standard deviation of the logarithm of viable cell concentration before freezing and after thawing was 0.22 and 0.30 unit, respectively.

DISCUSSION

Lactic acid bacteria are often used in a concentrated frozen or freeze-dried form, but freezing causes a decrease in cell viability (to varying degrees). In the present study, we show that the cryotolerance of lactobacilli can be improved by performing successive cycles of subcultivation, freezing and thawing. The survival rate of strain CFL1, which was 10^{-5}, increased to $4.3x10^{-2}$ after 30 cycles of subcultivation, freezing and thawing. The acidifying activity of the cells after thawing also increased during the experiment, and it is likely that this evolution is the consequence of the higher concentration of viable cells after thawing. Indeed, there was a good relationship between the viable cell concentration in the thawed culture and the time needed for the subsequently inoculated culture to reach pH 5.5. One characteristic feature of the curves of the viable cell concentration and of the acidifying activity after thawing is that their evolution was not regular throughout the experiment. In the three parallel cultures, there were phases where a rapid evolution took place, whereas in other phases, evolution was slow. This may be explained by the random appearance of new better-adapted spontaneous mutants that are able to outcompete the other genotypes. This phenomenon is called "periodic selection" (Dykhuizen, 1990). This may also explain why the evolution in the three parallel cultures was distinct, as there is no reason that these cultures contained the same better-adapted mutants and that these mutants had appeared at the same time. It is possible that the cultures would have continued to evolve if we had not stopped the experiment after 30 cycles of subcultivation, freezing and thawing. Experiments with evolving populations of *Escherichia coli* have shown that fitness of the cells may increase even after 20000 generations (Cooper and Lenski, 2000).

As *Lb. delbrueckii* ssp. *bulgaricus* CFL1 was very sensitive to the freezing conditions applied in the present study, there was a high pressure for the selection of spontaneous mutants

that are more resistant to freezing. This also resulted in an increase of the acidifying activity of the thawed cultures. During the serial transfer culture experiment, cells were repeatedly exposed to low pH (all cultures reached a pH near of 3.7–3.9). Furthermore, after 30 cycles of subcultivation, freezing and thawing, cultures were exposed to acidic conditions for a longer period than the initial cultures. Under these conditions, there may be a significant selection pressure for mutants that can survive better at low pH. This could explain the increase of cryotolerance, as it is well known that mutations that improve resistance to a specific stress may also improve the resistance to other stresses (van de Guchte et al., 2002). However, as the cell counts after 24 h of growth were always near $5x10^8$–10^9 ufc/ml, which is equivalent to the highest concentration of viable cells in culture of strain CFL1, there was no large decrease of viability during the exposure to low pH. It thus seems reasonable to consider that the main cause of evolution of the cultures is rather the high lethality of strain CFL1 during the freezing and thawing treatments. In order to confirm this hypothesis, it would be interesting to perform a serial transfer culture experiment in which cells are not subjected to freezing and thawing treatments.

The acidifying activity before freezing also increased in the three cultures. In contrast to what was observed for the thawed cultures, this evolution could not be linked to a higher concentration of viable cells in the culture that is used for inoculating milk. Several hypotheses may explain this result. For example, the lag phase of the cells may have decreased after 30 cycles of subcultivation, freezing and thawing. It is also possible that their growth rate had increased. An interesting issue to address is whether this evolution is the consequence of the selection of mutants having an improved cryotolerance or whether the two phenomena are independent. If the first hypothesis is true, it would mean that several mutations that improve the cryotolerance also improve the acidifying activity of cells. If not, it would mean that in these

experiments, there is also a significant selection pressure for mutants harboring mutations that increase the acidifying activity and that have no effect on cryotolerance.

After 30 cycles of subcultivation, freezing and thawing, the three cultures contained subpopulations having distinct cryotolerances and acidifying activities. This means that they were not composed of one single genotype that had previously outcompeted all the less adapted genotypes. One explanation for this within-population diversity is that the genotypes that have the highest cryotolerance had not appeared for long enough to outcompete all the other genotypes. This implies that the cryotolerance of the three parallel cultures would still have increased if the experiment had been carried on after 30 cycles of subcultivation, freezing and thawing. Another explanation for the presence of a significant within-population diversity in the cultures is that a polymorphism may persist, due to ecological interactions between genotypes, such as cooperation, competition etc. Rozen and Lenski (2000) observed that two distinct cell types of *Escherichia coli* coexisted for more than 14000 generations in a serial transfer culture experiment.

It would be interesting to investigate the mutations that have improved the cryotolerance of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 and to answer the following questions: (i) what are the genes that have been affected? (ii) are these genes different from one population to another? and (iii) are there important differences between the subpopulations? These results may help to define new targets for improving the resistance of lactic acid bacteria to several stresses.

In the present study, the acidifying activity of frozen cultures of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 could be improved by applying a high pressure for the selection of cells having an increased survival rate to freezing. However, it is likely that in the experiments that have been performed, there was only a limited selection pressure that concerns important technological properties such as the resistance of phages or the production of aroma compounds. These

properties should thus be carefully checked if the use of such types of cultures is considered in industrial processes.

CONCLUSIONS

In the present study, we show that the cryotolerance of lactobacilli can be improved by performing successive cycles of subcultivation, freezing and thawing. This approach may be used for improving the activity of concentrated frozen starter cultures. Furthermore, it is possible that other properties of lactic acid bacteria, such as the resistance to freeze-drying or spray-drying, may also be improved by natural selection.

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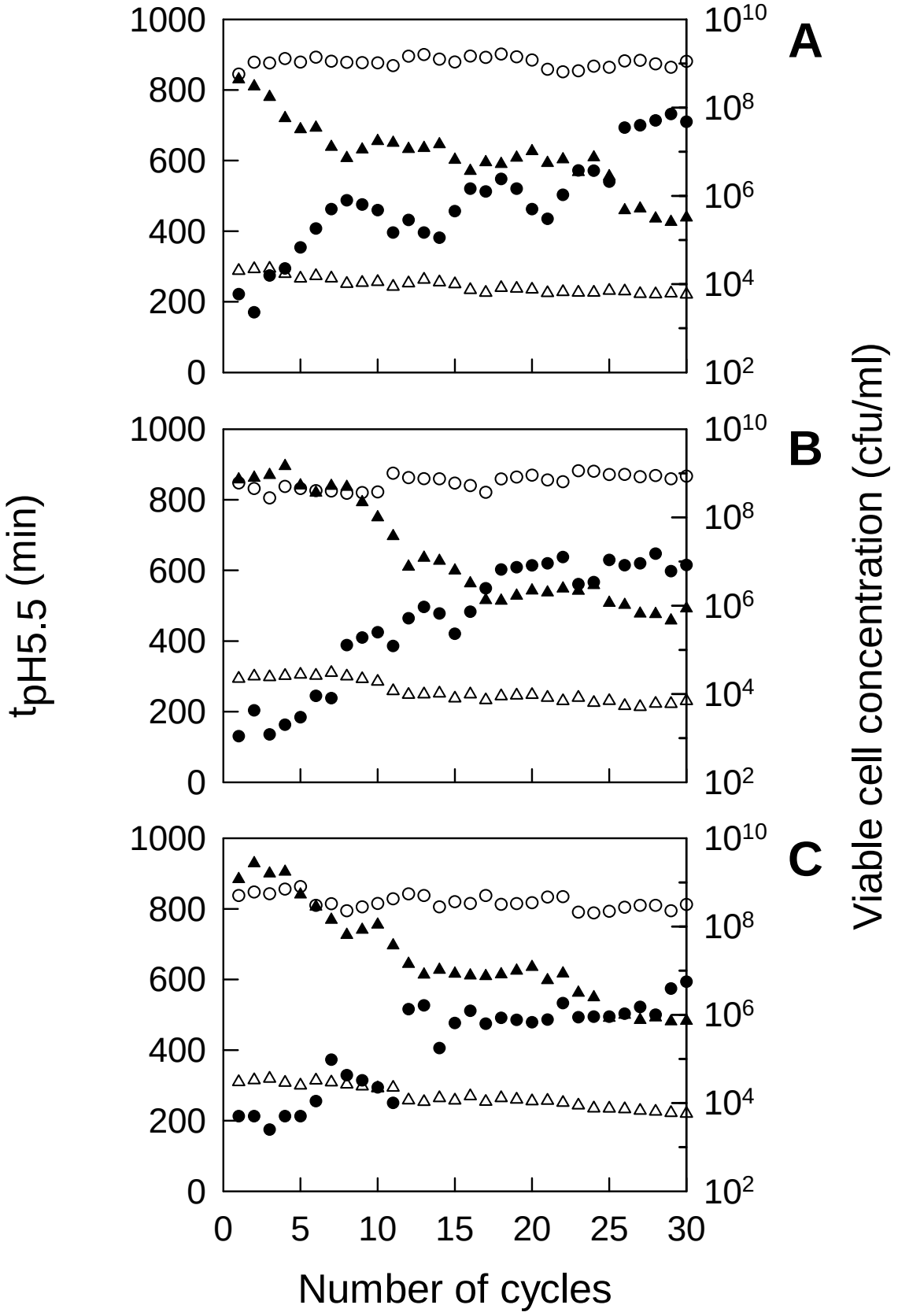
Figure 1. Evolution of the viable cell concentration (❍,●) and of the time necessary to reach pH5.5 (Δ,▲) before freezing (open symbols) and after thawing (closed symbols) during 30 cycles of subcultivation, freezing and thawing of cultures A (A), B (B) and C (C).

Figure 2. Viable cell concentration (❍,●) and time necessary to reach pH5.5 (Δ,▲) before freezing (open symbols) and after thawing (closed symbols) in cultures of 25 clones isolated from cultures A (A), B (B) and C (C) after 30 cycles of subcultivation, freezing and thawing.


A
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400
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10^{10}
10^{8}
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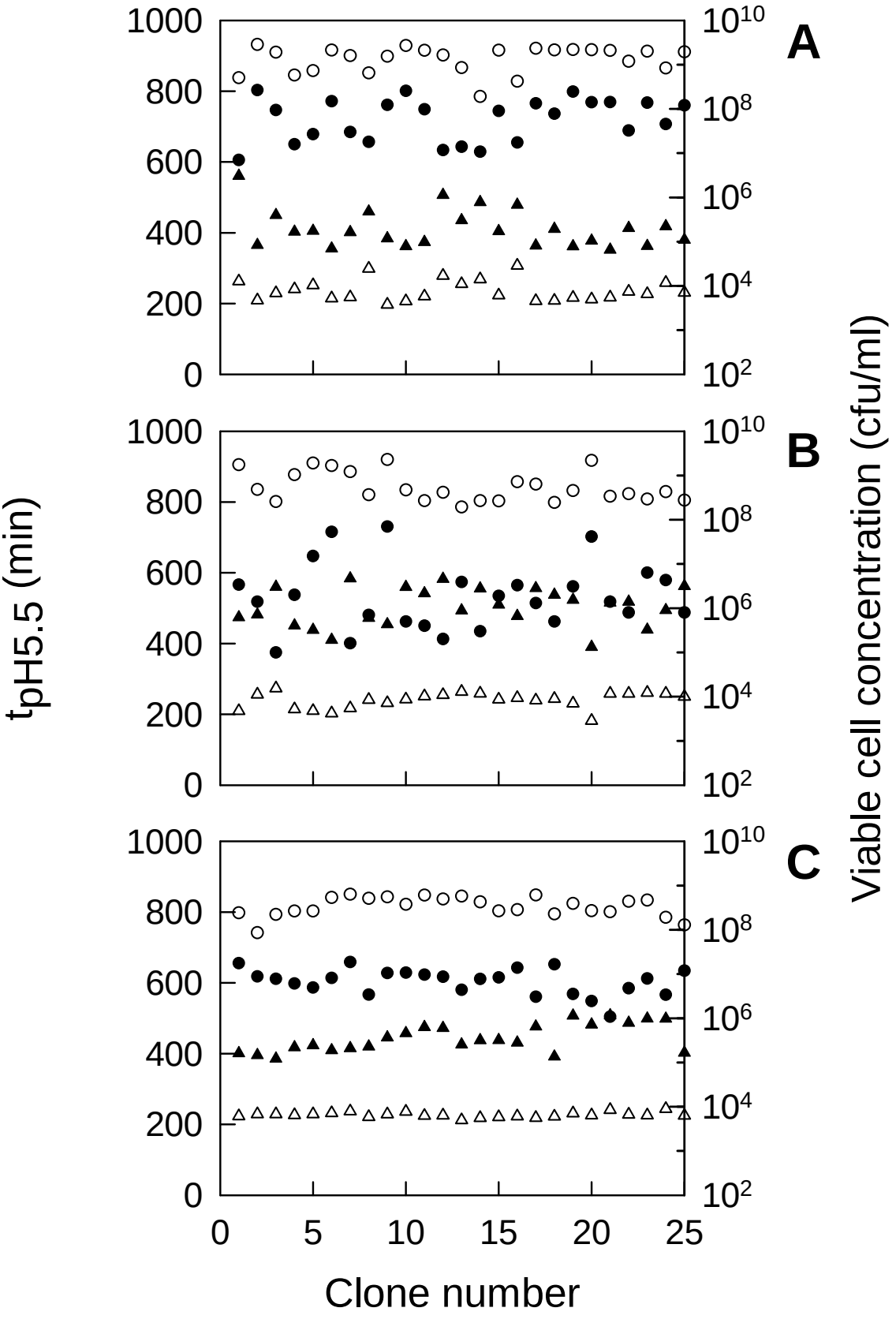
25
30
Number of cycles
$t_{pH5.5}$ (min)
Viable cell concentration (cfu/ml)

Fig.1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Clone number
$t_{pH5.5}$ (min)
Viable cell concentration (cfu/ml)

Fig.2