Chapter 7

Evolution Assays for the Isolation of Mutant Bacteria Resistant to Natural Antimicrobials

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Abstract

Natural antimicrobial compounds have been proposed as a promising alternative to current preservation treatments for minimally processed foods. However, the currently required doses are too high, which leads to sensory alteration due to their strong organoleptic properties. For this reason, further research is still needed regarding their mode of action in order to optimize their antibacterial properties. In this regard, it has become useful to deliberately obtain resistant mutant strains in order to study the underlying mechanisms of antimicrobial resistance. Two different evolution assay protocols have been designed for the obtention of mutant strains with increased resistance against natural antimicrobials: cyclic exposure to prolonged sub-inhibitory doses and cyclic exposure to short lethal treatments. The phenotypic and genotypic characterization of the evolved strains will provide knowledge about cellular response and resistance mechanisms against antimicrobial compounds, which will help to optimize their use as preservatives in the food industry or as cleaning and disinfection treatments.

Key words Antimicrobial resistance, Mutagenesis, Evolution assay, Natural antimicrobial compounds, Essential oils, Individual constituents, Bacteria, Whole-genome sequencing

1 Introduction

Thermal inactivation is the main technology used in the industry as a preservation method to ensure food safety and stability. However, new consumer trends have encouraged the search for preservation methods that manage to maintain the nutritional and sensory properties of food while ensuring microbial safety and stability. In this regard, natural antimicrobial compounds such as essential oils (EOs) and their individual constituents (ICs) have been proposed as a promising alternative to current preservation treatments for minimally processed foods [1, 2]. These natural compounds have been extensively studied and have been shown to possess excellent antimicrobial properties against food-related pathogens

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[3, 4]. Moreover, most of them are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. However, the doses required to use them as a single method of food preservation are too high, since their strong organoleptic properties might cause sensory alteration [5, 6]. One of the devised solutions is to apply them in combination with other antimicrobial compounds or food preservation technologies to achieve synergistic lethal effects [7], thereby reducing treatment dose and intensity while avoiding the alteration of treated food [8, 9]. Nevertheless, the design of effective combined treatments for microbial control in foods also requires a thorough understanding of their mechanisms of action on bacteria: for this reason, further research is still needed in order to optimize their antibacterial properties.

In recent years, several investigations have focused on the study of microbial genotypic resistance with the purpose of obtaining a better understanding of the mechanisms of cellular response to antimicrobials. Antibiotic resistance studies are an outstanding example thereof [10]. In fact, once the success derived from studying resistant mutant strains was observed, several authors devised laboratory evolution assays designed to obtain resistant strains that would allow for more in-depth studies [11]. The first evolution assays were based on the cyclic exposure of bacterial cell populations to prolonged sub-inhibitory doses [12]. The obtention of mutant strains thus became a useful tool for the study of resistance to antibiotics. Nevertheless, this technique was initially discarded in favor of the study of bacterial behavior against natural antimicrobials, due to the antioxidant properties of the latter [13], which reduced mutagenic frequency and would therefore prevent the occurrence of mutant strains [14]. However, recent studies have reported the emergence of resistant strains to natural antimicrobials: against ICs (carvacrol, citral, and limonene oxide) in *Escherichia coli* [15], *Staphylococcus aureus* [16], *Listeria monocytogenes* [17], and *Salmonella* Typhimurium [18], and against EOs (*Citrus sinensis*) in *S. aureus* [19] by cyclic exposure to prolonged sub-inhibitory doses. In addition, whole-genome sequencing of those strains allowed to identify the mutations responsible for the increased resistance [16–20].

Evolution assays were adapted to lethal treatments in order to investigate resistant strains obtained from survivors to high antibiotic concentrations [21]. This technique was likewise employed in other research areas such as food preservation, and new protocols were designed with the purpose of obtaining resistant mutant strains from the tails of survival curves after the application of lethal treatments. In this way, resistant strains were also obtained under physical food preservation technologies, such as heat [22] and high hydrostatic pressure [23]. Evolution assays have thus also been adapted to the isolation of resistant mutant strains following the application of lethal doses of natural antimicrobial compounds [18].

In this chapter we describe the methodology required to perform evolution assays with natural antimicrobials by two different protocols: (a) by cyclic exposure to prolonged sub-inhibitory doses and (b) by cyclic exposure to short lethal treatments. Our aim is to explain how to obtain and characterize mutant resistant strains against natural antimicrobial compounds. The phenotypic and genotypic study of these strains will allow for a better understanding of the mechanisms of bacterial resistance and, consequently, lead to a more profound knowledge of the mechanisms of antimicrobial action displayed by natural antimicrobial compounds. This information might lead to the design of new and more effective food preservation strategies in the industry.

2 Materials

2.1 Evolution Assay by Cyclic Exposure to Prolonged Sub-inhibitory Doses

1. Cryovial of the bacterial strain to study.
2. Culturing tools: micropipettes, pipette tips, plastic 1.5-mL tubes, petri dishes (90 mm), inoculation loops, and L-shaped spreaders.
3. Growth media: tryptic soya agar and broth supplemented with 0.6% yeast extract (or any other nutritive agar and broth).
4. Natural antimicrobial compound (essential oils, individual constituents, natural extracts, etc.).
5. Phosphate-buffered saline (PBS).
6. Glass test tubes and caps.
7. Vortex.
8. Incubator with orbital shaker.
9. Cryovials.

2.2 Evolution Assay by Cyclic Exposure to Short Lethal Treatments

1. Cryovial of the bacterial strain to study.
2. Culturing tools: micropipettes, pipette tips, plastic 1.5-mL tubes, petri dishes (90 mm), inoculation loops, and L-shaped spreaders.
3. Growth media: tryptic soya agar and broth supplemented with 0.6% yeast extract (or any other nutritive agar and broth).
4. Natural antimicrobial compound (essential oils, individual constituents, natural extracts, etc.).
5. Phosphate-buffered saline (PBS).
6. Glass test tubes, glass flasks (250 mL), and caps.
7. Vortex.
8. Centrifuge.
9. Incubator with orbital shaker.
10. Cryovials.

3 Methods

Prior to either of the two evolution assay protocols, it is necessary to obtain a working bacterial culture of the wild-type strain (WT) from which evolution assays will be triggered, as well as to determine the minimum inhibitory concentration (MIC) of the antimicrobial that is being tested. MIC is established as the lowest concentration of the antimicrobial compound capable of inhibiting bacterial growth. The MIC value will be used later on to perform the evolution assays.

The following **steps 1–4** to obtain the **initial working bacterial culture** are common to both evolution protocols:

1. From a cryovial of WT, inoculate and streak over the agar plates surface with an inoculation loop to obtain individual colonies (*see* **Note 1**).
2. Incubate the agar plates for 24 h at 37 °C (*see* **Note 2**).
3. Inoculate a single colony in 5 mL of growth broth in test tube (*see* **Note 3**).
4. Incubate overnight at 37 °C and 130 rpm until a stationary phase culture is obtained (*see* **Note 4**).

It is then necessary to carry out **MIC determination** of the tested natural antimicrobial:

5. Prepare 5 mL test tubes of growth broth with increasing concentrations of the natural antimicrobial, and shake vigorously by vortex (*see* **Note 5**).
6. Inoculate the test tubes with a stationary phase culture at an initial concentration of 1×10^5 CFU/mL (colony-forming units/mL).
7. Prepare positive control tubes with 5 mL of growth broth inoculated at 1×10^5 CFU/mL without antimicrobial, and negative control tubes with 5 mL of growth broth non-inoculated with the natural antimicrobial.
8. Incubate all the test tubes for 24 h at 37 °C and 130 rpm.
9. Observe the turbidity of the growth broth. If the growth medium is cloudy, this means that bacteria have grown, whereas no turbidity indicates that the concentration of the antimicrobial is sufficient to inhibit bacterial growth (*see* **Note 6**).
10. The lowest concentration that has inhibited bacterial growth is established as the MIC.

Use 0.5× of the MIC to carry out the evolution assay by cyclic exposure to prolonged sub-inhibitory doses, and 2× the MIC to conduct the evolution assay by cyclic exposure to short lethal treatments. Figure 1 shows the scheme of the two protocols of evolution assays designed to obtain mutant strains resistant to natural antimicrobial compounds.

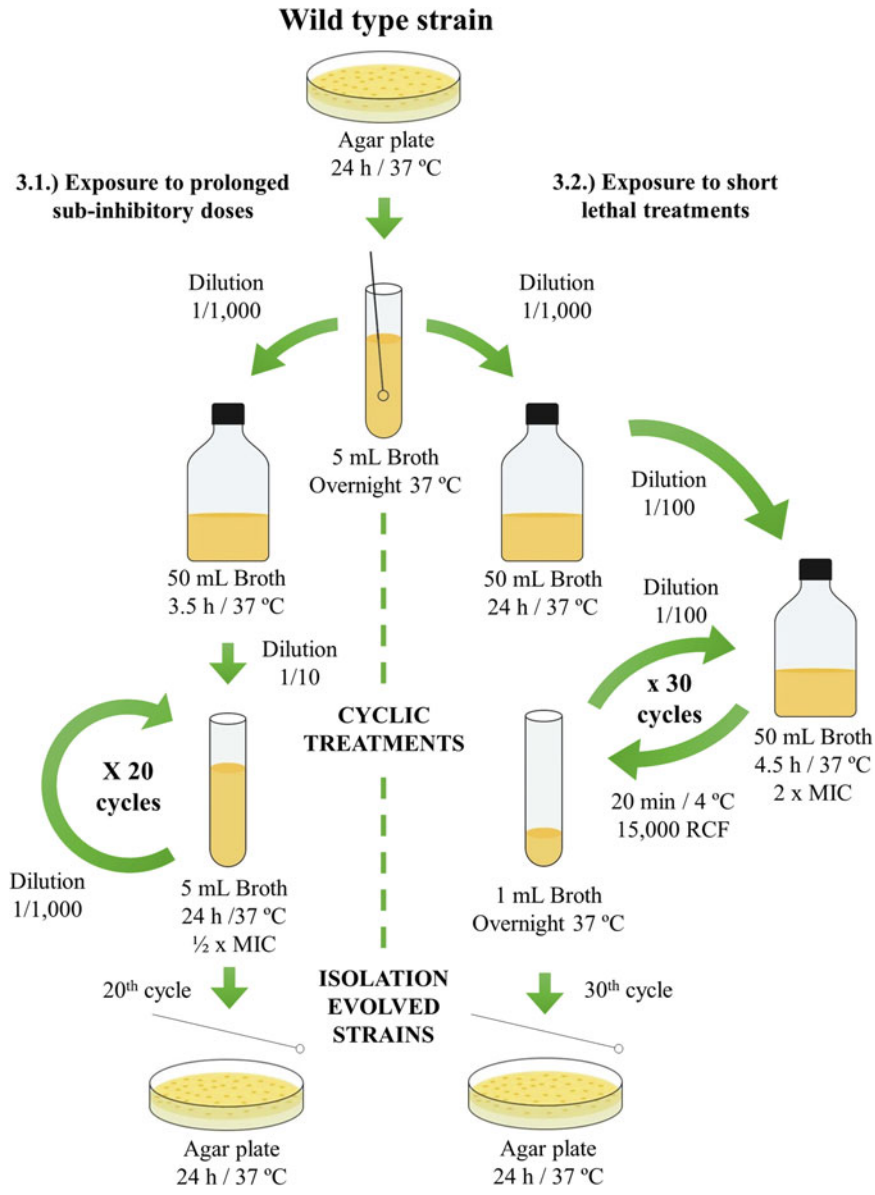

Fig. 1 Diagram of evolution assay (3.1) by cyclic exposure to prolonged sub-inhibitory doses and (3.2) by cyclic exposure to short lethal treatments

3.1 Evolution Assay by Cyclic Exposure to Prolonged Sub-inhibitory Doses

This protocol is based on the application of constant stress to the bacterial population at low concentration. The aim is to allow the occurrence of mutations in the bacterial population that improve its growth fitness in the presence of the antimicrobial agent. Thus, if such mutations occur, the agent's selective presence will facilitate the emergence of such strains in contrast with the WT as well as with other mutants whose mutation is not related to resistance to the agent, thereby allowing the isolation of strains that are resistant to the selected antimicrobial compound (Fig. 2).

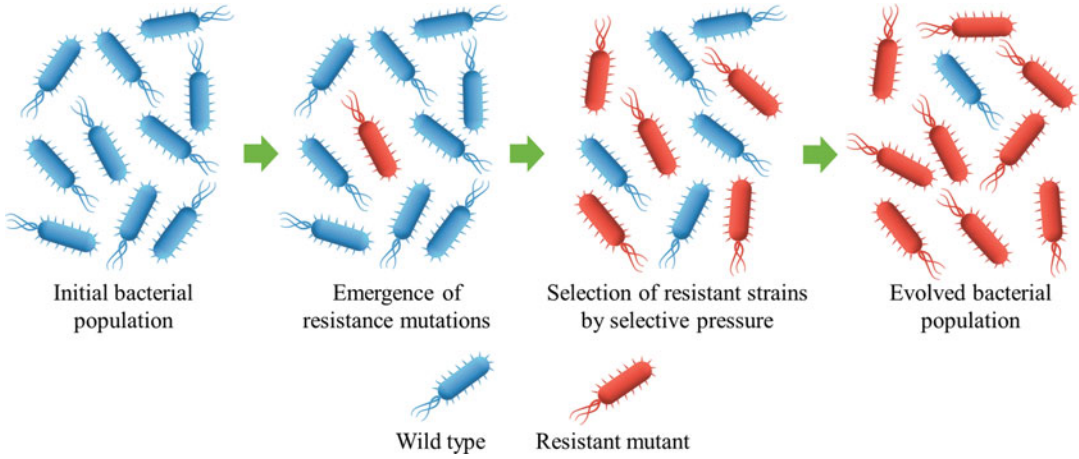

Fig. 2 Selection of resistant mutant strains by cyclic exposure to prolonged sub-inhibitory doses

To carry out the evolution assay by cyclic exposure to prolonged sub-inhibitory doses, the following steps should be taken (continue from **step 4**):

5. Dilute working bacterial culture of WT 1:1,000 into 50 mL growth broth and incubate for 3.5–4 h at 37 °C and 130 rpm until an exponential phase culture is obtained (*see* **Note 7**).
6. Once grown, inoculate 5 mL growth broth in test tubes at an initial concentration of 10^6 CFU/mL in the presence of 0.5× of the MIC of the antimicrobial compound.
7. Incubate for 24 h at 37 °C and 130 rpm until stationary phase is reached (*see* **Note 4**).
8. Return to **step 6**. After 20 cycles, continue with **step 9** (*see* **Note 8**).
9. After the 20th cycle, dilute the bacterial culture in PBS, and inoculate and spread on agar plates to obtain individual colonies.
10. Incubate the agar plates for 24 h at 37 °C (*see* **Note 2**).
11. After the incubation on agar plates, select several colonies and store them in cryovials (*see* **Note 9**).

3.2 Evolution Assay by Cyclic Exposure to Short Lethal Treatments

This protocol is based on the application of short cyclic treatments to the bacterial population at high concentration. Its goal is to provoke the emergence of mutations associated with resistance to lethal treatments of the antimicrobial agent. Evolved bacteria that have suffered mutations implying increased resistance will survive lethal treatments based on the resistance of the WT. In this way, the lethal treatments applied will inactivate the most sensitive cells, while sparing the mutant strains that are resistant to antimicrobial compounds (Fig. 3).

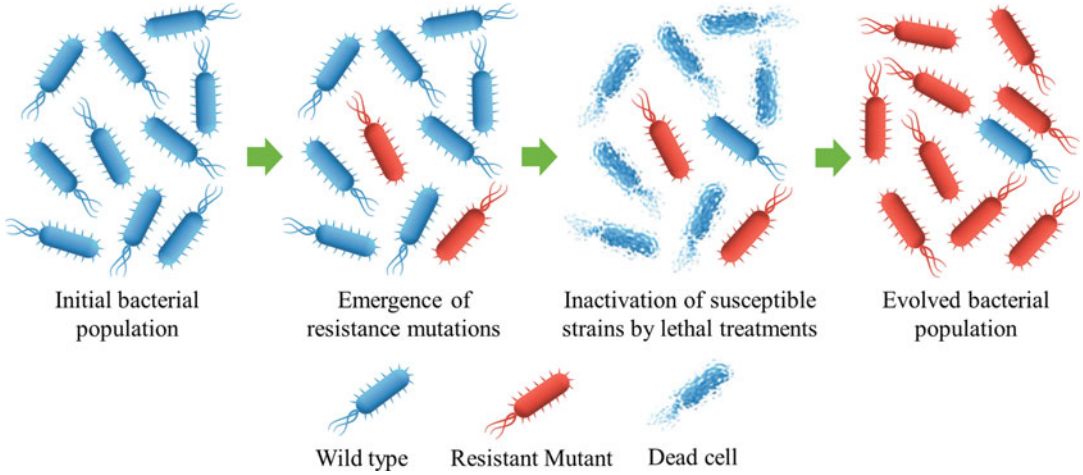

Fig. 3 Selection of resistant mutant strains by cyclic exposure to short lethal treatments

To carry out the evolution assay by cyclic exposure to short lethal treatments, the following steps should be taken (continue from **step 4**):

5. Dilute working bacterial culture of WT 1:1,000 into 50 mL growth broth and incubate for 24 h at 37 °C and 130 rpm until a stationary phase culture is obtained (*see* **Note 4**).
6. Once incubated, dilute stationary phase culture 1:100 (initial concentration of 10^7 CFU/mL) in glass flasks containing 50 mL growth broth in presence of 2× the MIC of the antimicrobial compound.
7. Apply the treatment while maintaining the flask at 37 °C for 4.5 h (*see* **Note 10**).
8. Centrifuge the treated cells for 20 min at 15,000 RCF and 4 °C, wash twice with fresh growth broth, and resuspend in 1 mL of growth broth in test tube.
12. Incubate test tubes overnight at 37 °C and 130 rpm until stationary phase is reached (*see* **Note 4**).
13. Return to **step 6**. After 30 cycles, continue with **step 14** (*see* **Note 8**).
14. After the 30th cycle, dilute the bacterial culture in PBS, and inoculate and spread on agar plates to obtain individual colonies.
15. Incubate the agar plates for 24 h at 37 °C (*see* **Note 2**).
16. After the incubation on agar plates, select several evolved colonies and store them in cryovials (*see* **Note 9**).

Finally, the isolated strains obtained either by cyclic exposure to prolonged sub-inhibitory doses or by short lethal treatments must

be phenotypically and genotypically characterized. The WT must be used as control to evaluate the resistance of the evolved strains, and, likewise, in order to carry out the comparisons among the genomes with the purpose of finding the mutations that have occurred during evolution assays responsible for the increased resistance.

On the one hand, it is recommended to first evaluate the resistance of the evolved strains against the natural antimicrobial used in the evolution assay at both bacteriostatic and bactericidal concentrations. For this purpose, MIC and minimum bactericidal concentration (MBC) can be determined, survival curves to lethal treatments can be obtained, and results can be compared with the WT. These methodologies are explained by Berdejo et al. [16]. Increased resistance may also occur against other natural antimicrobials not used in the evolution assay [15]; thus the same methodology can be used to test other antimicrobials. According to recent results in evolved strains, an antibiotic susceptibility test should be performed due to the fact that increased cross-resistance to antibiotics has been observed [18], and these compounds probably have similar mechanisms of action. In addition, resistant strains isolated by natural antimicrobials have also demonstrated increased resistance to other food preservation technologies such as heat or pulsed electric fields [15]. Such results will provide more information on the behavior of the evolved strains, as well as on the direct resistance and cross-resistance which will have emerged in the evolution assays between natural antimicrobials, antibiotics, and food preservation technologies.

On the other hand, whole-genome sequencing (WGS) of WT and of the evolved resistant strains, followed by comparison between them, will allow a determination of the genetic modifications that cause the increase in resistance [16, 18]. These results will thus provide knowledge regarding cellular response and resistance mechanisms (cellular targets, repair systems, etc.) against the antimicrobial compound, which might help to optimize their use as preservatives in the food industry or as cleaning and disinfection treatments.

4 Notes

1. For evolution studies it is recommended to always use the same cryovial, or original strain, to avoid the occurrence and accumulation of random mutations in the WT, which make it more difficult to study genotypic resistance.
2. Incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.

3. Check the size and shape of the colonies, and verify the homogeneity in the agar plate to avoid microbial contamination.
4. To obtain a stationary phase culture, incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.
5. The concentration range should be adjusted based on the natural antimicrobial's bacteriostatic activity and on the resistance of the bacteria under study. Based on our experience, the range for ICs with high antimicrobial activity, such as carvacrol or thymol, or EOs, such as oregano or thyme EO, lies between 50 and 300 μL/L (with intervals of 50 μL/L). The range used for other less active compounds such as limonene oxide or citrus EOs, such as orange or lemon EO, is from 500 to 2,000 μL/L (with intervals of 100 μL/L).
6. To obtain an objective measurement, it is recommended to read the optical density at 595 nm (OD_{595}). 10% of the OD_{595} value of the positive control has been established as the lower limit to consider that a bacterial strain was grown [11].
7. To obtain an exponential phase culture, incubation temperature and time should be modified according to the optimal growth conditions of the bacteria.
8. Depending on the mutation frequency of the bacteria, the natural antimicrobial used, and treatment conditions, the evolution assay should be prolonged until increased resistance is observed. For this reason, it is recommended to perform an antimicrobial resistance test every three cycles to detect the emergence of resistant strains.
9. The population may be genotypically heterogeneous depending on the number of evolutionary cycles: for this reason, it is recommended to evaluate several colonies in order to select the one that is most resistant.
10. Time and temperature of the lethal treatment can be modified according to the susceptibility of the WT to the antimicrobial used. This treatment's design seeks to inactivate a large part of the bacterial population (>5 $\log_{10}$ cycles of reduction), while nevertheless allowing for the recovery of surviving cells before proceeding to the evolution assay.

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