algorithms

MDPI

Article

Evolutionary Algorithms in a Bacterial Consortium of Synthetic Bacteria

Sara Lledó Villaescusa [1] and Rafael Lahoz-Beltra [2,*]

[1] Department of Biodiversity, Ecology and Evolution (Biomathematics), Faculty of Biological Sciences, Complutense University of Madrid, 28040 Madrid, Spain
[2] Modeling, Data Analysis and Computational Tools for Biology Research Group, Complutense University of Madrid, 28040 Madrid, Spain
* Correspondence: lahozraf@ucm.es

Abstract: At present, synthetic biology applications are based on the programming of synthetic bacteria with custom-designed genetic circuits through the application of a top-down strategy. These genetic circuits are the programs that implement a certain algorithm, the bacterium being the agent or shell responsible for the execution of the program in a given environment. In this work, we study the possibility that instead of programming synthesized bacteria through a custom-designed genetic circuit, it is the circuit itself which emerges as a result of the evolution simulated through an evolutionary algorithm. This study is conducted by performing in silico experiments in a community composed of synthetic bacteria in which one species or strain behaves as pathogenic bacteria against the rest of the non-pathogenic bacteria that are also part of the bacterial consortium. The goal is the eradication of the pathogenic strain through the evolutionary programming of the agents or synthetic bacteria. The results obtained suggest the plausibility of the evolutionary design of the appropriate genetic circuit resulting from the application of a bottom-up strategy and therefore the experimental feasibility of the evolutionary programming of synthetic bacteria.

Keywords: evolution of a bacterial consortium; programming synthetic bacteria; bacterial agents; Gro cell programming language

Citation: Villaescusa, S.L.; Lahoz-Beltra, R. Evolutionary Algorithms in a Bacterial Consortium of Synthetic Bacteria. *Algorithms* **2023**, *16*, 571. https://doi.org/10.3390/a16120571

Academic Editor: Lorenzo Salas-Morera

Received: 8 November 2023
Revised: 6 December 2023
Accepted: 14 December 2023
Published: 17 December 2023

1. Introduction

Today, the convergence of disciplines as diverse as synthetic biology and computer science has led to the acceptance of ideas that were disruptive in the near past. One such insight is the possibility of programming cells to perform specific tasks, creating innovative solutions to problems in areas ranging from medicine to the environment or industry. Although synthetic biology has its origins in the 1970s with the birth and development of genetic engineering and biotechnology, it was not until the year 2000 when this discipline adopted the current approach. At present, the aim of synthetic biology is the design and assembly of artificial biological systems. Consequently, the objective is to obtain new biological components, e.g., proteins, genetic circuits, metabolic networks, etc., taking into account the subsequent possibility of their integration into a recipient organism [1], e.g., a bacterium. This view suggests that synthetic biology was influenced from its origins by the ancient computer hardware and the way in which these early computers were programmed, e.g., the ENIAC [2]. Occasionally, it has been considered that an organism could be programmed as if it were an ENIAC computer (Figure 1) by plugging together these biological components. Therefore, the connection among elements would be the way of programming a synthetic organism, e.g., a bacterium. In this example, the bacteria would be playing the role of a desktop computer case housing the biological hardware, i.e., the program 'written' in these biological components or biobricks. As a result of this approach, it is feasible to program bacteria in

silico as if they were a computer [3,4]. Based on this principle, it is possible to go a step further by programming not isolated cells, such as bacteria, but rather groups of cells, e.g., the so-called xenobots [5]. These synthetic biological entities, created from cells of embryos of the species *Xenopus laevis*, were programmed using a supercomputer to determine their configurations.

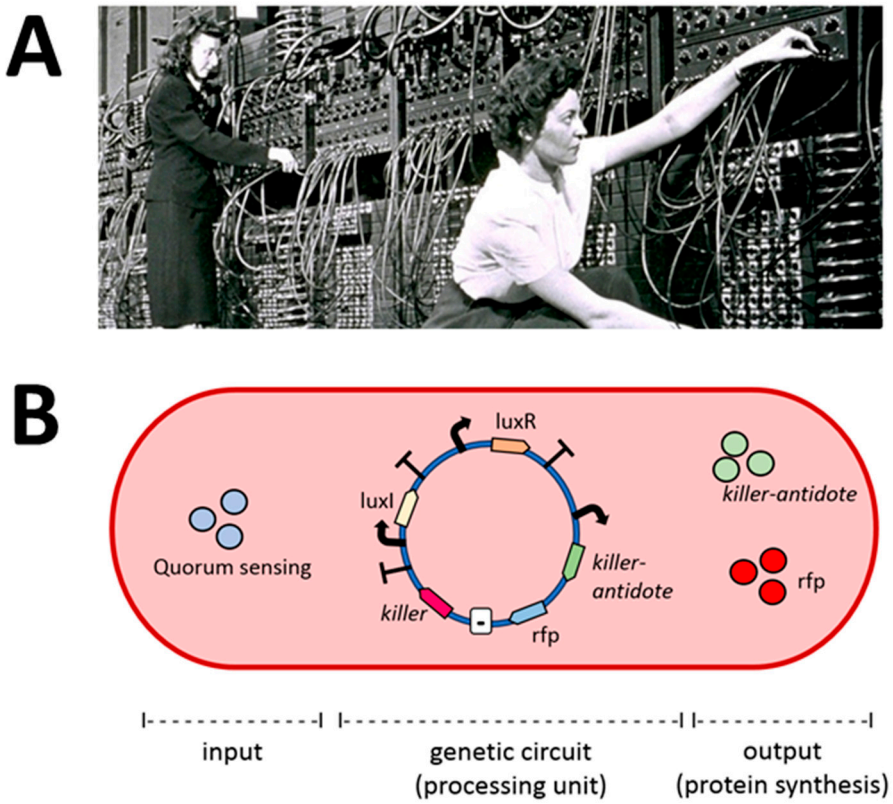

Figure 1. Synthetic bacterium programming. (**A**) The programming of a synthetic organism has been compared on occasions with the programming of the early computers, e.g., ENIAC. (**B**) The input data, e.g., a signal received via quorum sensing, is processed (genetic circuit of a plasmid), producing a signal or output after the expression of one or several genes, e.g., a fluorescent protein, killer antidote, etc.

Bacteria were by their unique features the first unicellular organisms to be programmed. Their programming is made possible by the use of highly characterized functional components, in particular parts of DNA coding for proteins and other regulators that are synthesized and assembled in genetic circuits. These genetic circuits allow the implementation in bacteria of switches, feedback systems, oscillators [6], etc., as well as different AND, XOR, and NOT logic gates [7], etc. The customized design and synthesis of genetic circuits makes it possible to program in synthetic bacteria specific biological functions [8], for example, the biological production of drugs or therapeutic compounds as well as biological devices that can detect and respond to specific signals from the body. This last approach, the application of synthetic biology in therapeutic applications [9], would make it possible to treat infectious diseases, metabolic disorders, and even cancer.

At present, the application of synthetic biology principles and the use of simulators has allowed the design of artificial microbial communities in which bacteria exhibit programmed behaviors [3,4]. In nature, these microbial communities or consortia are a group of bacteria of different species or the same specie but different strains whose individuals interact with each other by sharing resources and exhibiting different behaviors [10]. At present, and in the field of synthetic biology [10], microbial communities

composed of synthetic bacteria have many advantages over cultures composed of only one kind of bacteria, as they allow the division of tasks, spatial organization, and higher robustness to perturbations. Due to these features, microbial consortia are nowadays under study for their possible applications in many different biotechnological processes.

In this paper, westudied several strategies, and therefore algorithms, oriented to the control of a synthetic microbial consortium in which different kinds of bacteria coexist in silico. As stated above, the control of microbial communities is based on an algorithm that is implemented at the 'hardware level' in the related genetic circuit. However, this hardware, i.e., the genetic circuit, is evolutionarily configured through an evolutionary algorithm designed ad hoc, which we named BAGA.

In the experiments we performed, the microbial community is composed of interacting bacteria, including one strain of pathogenic bacteria and the remaining of non-pathogenic bacteria. We studied through simulation experiments the effectiveness of different strategies in order to eliminate the pathogenic bacteria from a community formed by different species or strains of synthetic bacteria. Therefore, the aim of the simulation experiments was to minimize the number of pathogenic bacteria and maximize the number of non-pathogenic bacteria that are present in a microbial consortium or community. In this paper, it is shown that by applying the BAGA evolutionary algorithm, it is possible to find the appropriate genetic circuit that leads to the eradication or progressive elimination of the number of pathogenic bacteria in a microbial consortium. Since the number of possible genetic circuits, i.e., what is known as genotype space, is discrete, the problem to be solved is a combinatorial optimization problem. In consequence, the problem will be solved using a heuristic method such as the BAGA evolutionary algorithm.

In the field of synthetic biology, the creation of new programmable organisms under a bottom-up approach requires heuristic methods coming from the area of combinatorial optimization. Under this approach, the assembly of different parts of an organism in order to optimally or quasi-optimally program the living matter requires appropriate heuristic methods. In this sense, the importance of combinatorial optimization strategies in synthetic biology was recently highlighted [11]. Thus, for example, the engineering of metabolic pathways through microbial strains involves the design and sequential or combinatorial optimization [12] of genetic circuits. In turn, the appropriate regulation of the genetic circuits obtained in silico also requires the use of combinatorial optimization techniques that in the form of software [13] allow the study of the genetic circuit regulation with a computer. In this case, the regulation of a genetic circuit or network is studied either through the analysis of the parameters that affect the genetic network or through the expression of its components, i.e., the genes that define the network.

In the last decade, the application of evolutionary algorithms in synthetic biology optimization, and particularly the so-called genetic algorithms, has received increased attention from researchers. For instance, by applying genetic algorithms, it is possible to design oscillator genetic circuits with specific features [14] and even genetic circuits operating as logic gates [15], e.g., AND, OR, NOT, NAND, etc. However, all these advances are achieved at the level of computer experiments and therefore in silico. The possibility of using evolutionary algorithms in the laboratory, i.e., in the *wet lab*, is so far mainly focused on directed evolution [16]. That is, the selection process proposed by Darwin is emulated in the laboratory through several cycles of DNA mutagenesis and selection of sequences according to a target sequence. The goal is to find a gene sequence yielding to the expression of proteins with optimized functions, for example, enzymes whose catalytic properties are the appropriate ones.

2. Materials and Methods

2.1. Microbial Community or Consortium Meta-Model

Nowadays, most of the synthetic bacteria are programmed according to a *top-down* strategy. That is, the genetic circuitry is computer-designed to suit the task that the bacterium is expected to perform. The programmed expression of the genes through a certain model of regulation of the genetic network will result in a certain cellular behavior that will be displayed by the synthetic bacteria. Once the genetic circuit is designed, it is inserted into a plasmid (Figure 1), i.e., into a circular extrachromosomal DNA molecule that is independent of the chromosomal DNA. To draw an analogy between genetics and a computer, the plasmid plays the role of the code, either a script or a program in a computer. Thus, while the synthetic bacterium is the shell or 'computer' whose dynamic behavior, e.g., the control of cell division, is governed by the chromosomal DNA, the expression of the genes carried by the plasmid plays the role that corresponds to the execution of a program in a computer. The top-down strategy applied to the design of synthetic bacteria is actually the classic protocol used by engineers to design the devices or mechanisms of a car, a microwave oven, or any other machine or device.

In this paper, we propose the design of synthetic bacteria by applying a *bottom-up* strategy. That is to say, bacteria are evolutionarily programmed either as a colony or as a bacterial consortium. A bacterial colony is a bacterial population resulting from the multiplication of a strain or species of bacteria. Likewise, a microbial consortium is a community of microbes formed by two or more groups of bacterial strains or species. Therefore, whether it is a colony or a consortium, we start with some features present in the bacteria that will progressively acquire a particular behavior or cellular response, i.e., output (Figure 1). In our meta-model, the evolution of bacteria is the result of the temporal changes that take place in the colony or microbial community.

In the present work, we studied two possible bottom-up scenarios. On the one hand, we studied the programming of synthetic bacteria through a Darwinian evolutionary model using an evolutionary algorithm that we termed BAGA. In this case, the mutation experienced in bacteria leads to random changes in the genetic circuit present in their plasmid. Depending on the configuration of the resulting genetic circuit, so too will be the adaptability or fitness of the bacterium. If the fitness value increases, the bacterial growth rate of a given species will also increase, and consequently the number of bacteria belonging to that species will grow.

On the other hand, we studied the design of communities of synthetic bacteria considering in this case that the change in the number of bacteria of each strain or species resulted from social interactions between bacteria. That is, assuming bacteria of different species, bacteria of one species interact with bacteria of another species by emitting chemical signals to the environment. Once the signals are emitted, they will have a positive or negative effect on other neighboring bacteria depending on their species. If the molecule released into the environment by an emitting bacterium of a given species has a positive effect on the receiving bacterium of another species, then the bacterial growth rate of the latter will increase, thereby increasing the number of bacteria of that species.

Indeed, whether it is Darwinian evolution or the effect of social interactions among bacterial species, the bottom-up protocol assumes as a guiding principle that evolution, i.e., the change in the number of synthetic bacteria, will be the result of the bacterial growth rate or Malthusian parameter adjustment. In other words, evolution is the result of the change in the value of the parameter of the equation governing the growth or colony size of a bacterial strain.

2.2. Synthetic Bacteria Programming

Synthetic bacteria are agents programmed in Gro 4.0, a cell programming language [17]. Using Gro, we encoded through the appropriate scripts the main features of the synthetic bacteria. Once an initial bacterium is first programmed, the bacterium is then

divided in two, and each daughter bacterium receives a copy of the script. Over the course of the simulation experiment, a bacterial colony is formed, growing with time, running the script in parallel on each of the bacteria. As a result, it is possible to explore the collective behaviors of the colony that emerge from the individual cellular specifications in each bacterium. Specifically, Gro language enables the modeling of different biological events, such as cell division, chemotaxis, and signal diffusion, among other physiological phenomena [17,18]. Although an extended version of Gro was published that allows plasmid design and recombination [19], in the present study, we programmed synthetic bacteria using the original version [17]. The reason for this choice is because plasmid recombination is a feature that was not used in our simulation experiments.

In this paper, we assume that physiological and biochemical features of synthetic bacteria are encoded in plasmid-resident genes, i.e., in circular DNA molecules that replicate independent of the bacterial chromosome. Therefore, a plasmid is extrachromosomal DNA that we use in synthetic biology to 'program' a bacterium. Unlike the plasmid, the bacterial chromosome is the DNA containing the genes essential for the survival of the bacterium, e.g., to control cell division. Although the parameters affecting cell division can be set with Gro in a simulation experiment, chromosomal DNA is usually not included in the experiments.

2.3. A Preliminary Experiment for Bottom-Up Design of Synthetic Bacteria: GADY

The present work originates from a simulation experiment carried out some time ago and recently published as a pre-print [20]. In order to carry out the study in silico of a microbial community or consortium as simply as possible, we performed a preliminary experiment that we termed GADY (an acronym for get signal; antidote; die when a killer gene is expressed; yellow fluorescence). Since the microbial community was the simplest possible, we assumed a microbial consortium consisting of only two bacterial species: one pathogenic and the other non-pathogenic. The pathogenicity or not of the bacteria was only a label since the aim of this study was not the simulation of a disease caused by bacterial infection. The goal of the experiment was to evaluate an evolutionary algorithm oriented to the eradication of the pathogenic bacterium present in a microbial consortium.

In this model, only pathogenic bacteria undergo Darwinian natural selection, while the non-pathogenic strain remains in the consortium without evolutionary changes.

The algorithm makes the assumption that bacteria communicate with each other by quorum sensing, i.e., a type of communication between bacteria that takes place, for example, in the bacterial strain *E. coli*.

At the beginning of the simulation experiment ($t = 0$), there is a single initial cell or bacterium that divides into two daughter bacteria (Figure 2C). One of the daughter bacteria behaves as non-pathogenic, which sends a chemical signal, while the other daughter bacterium is the pathogenic bacterium that receives the signal (Figure 3). In order to introduce genetic variability in the microbial community, the experiment was conducted by programming synthetic pathogenic bacteria with the genetic mechanism of mutation.

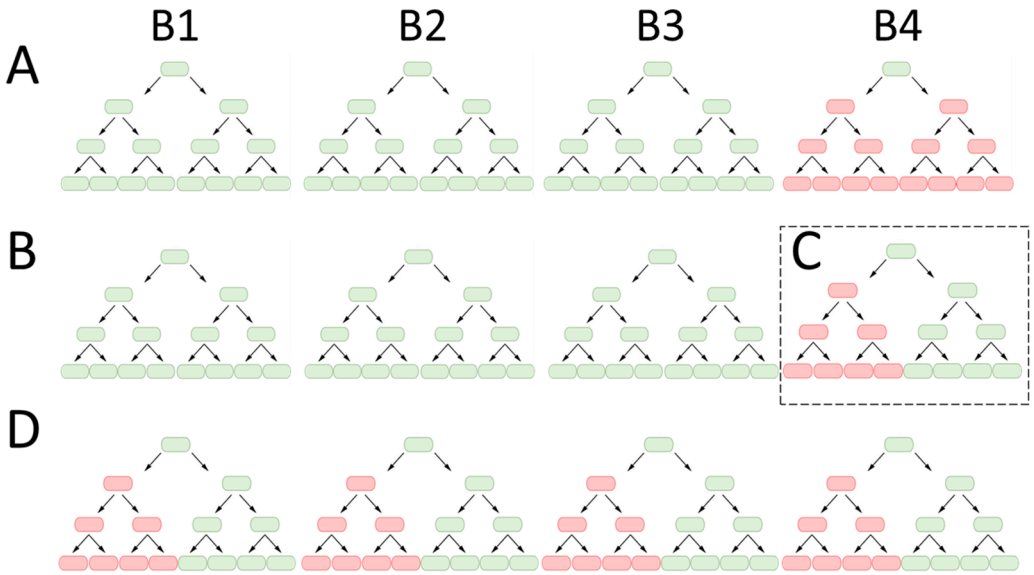

Figure 2. Models of consortia or microbial communities composed of four strains or bacterial species (**B1**–**B4**). Bacterial reproduction occurs in each colony by bipartition; however, some colonies yield pathogenic daughter bacteria, others yield non-pathogenic daughter bacteria, and there are colonies in which there are bacteria of both cell lines. Bacteria displayed in 'green' are non-pathogenic, while 'red' bacteria are pathogenic. (**A**) Experiments 1 and 2. (**B**) Experiment 3. (**C**) GADY experiment. In this case, the simulation experiment was conducted [20] with a single colony of bacteria of the same strain and not with a bacterial community. (**D**) Experiment 4 (for a detailed explanation of the simulation experiments, see text).

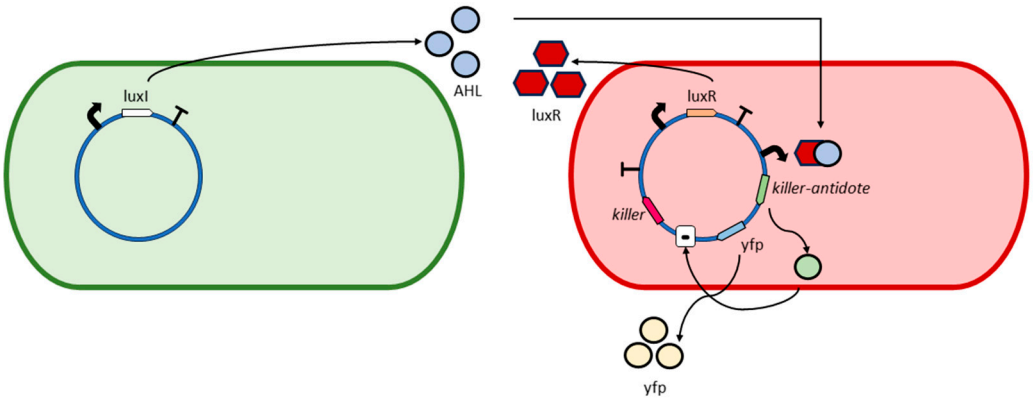

Figure 3. GADY genetic network. (Left) AHL-emitting bacterium ('green' bacterium is the non-pathogenic one) with a plasmid including LuxI gene. (Right) Representation of the gene circuit of the plasmid hosted in the 'red' pathogenic bacterium.

As mentioned above, communication between non-pathogenic and pathogenic bacteria occurs in the microbial community through a signaling mechanism known as quorum sensing. This form of communication, which uses AHL (N-acyl homoserine lactone) as a signaling molecule, is simulated as follows. The genome of the sender bacteria (non-pathogenic) includes a plasmid that is composed of a single luxI gene (Figure 3), whereas the receptor bacteria (pathogenic) host a plasmid consisting of four genes: luxR, killer antidote, killer, and yfp (Figure 3). LuxI in the sender bacterium is responsible for producing AHL, releasing this molecule to the environment. AHL enters into the pathogenic bacterium and binds to luxR, forming a complex. This is followed by the expression of the remaining genes except for the killer gene, whose expression is inhibited by the killer antidote.

In the case of the yfp gene, its expression results in the synthesis of the yellow fluorescent protein, resulting in the emission of yellow fluorescence from the bacteria. The pathogenic bacterium has the luxR gene capable of detecting the AHL inducer signal, i.e., the signal that has been emitted by the non-pathogenic bacterium.

In this model, the future state of the pathogenic bacterium is governed by transition rules in which in addition to the chemical signal (AHL inducer signal) emitted by the non-pathogenic bacterium, i.e., its sister in the neighborhood, the genotype of the pathogenic bacterium is also taken into account. For example, in agreement with [20], the pathogenic bacterium dies if it receives the signal s from its non-pathogenic sister, i.e., get_signal(s) in Gro language, and its genotype is 1 0 1 0 as a result of the expression of the killer gene. This rule is shown below in Gro language:

p.m = PATHOGEN & p.t > delay & (gene [0] = 1 & get_signal(s) > p.r) & gene [1] = 0 & gene [2] = 1 & gene [3] = 0 : {die()}

The GADY experiment is based on the assumption that the pathogenic bacterium is the only one susceptible to undergoing mutations. The ultimate goal of the experiment is to achieve in silico through an evolutionary algorithm the eradication of the pathogenic bacteria by Darwinian natural selection, thus the evolution of the pathogenic bacterium towards a genotype leading to its own eradication by cell suicide. Consequently, the goal of GADY was to evolve a pathogenic bacterium including only the luxR, killer, and yfp genes.

In the following section, we describe the evolutionary algorithm ruling the evolution of pathogenic bacteria.

2.4. BAGA Evolutionary Algorithm

The algorithm termed BAGA, an acronym for bacterial agent genetic algorithm, is a method to perform in silico simulation experiments with synthetic bacterial colonies. This evolutionary algorithm was proposed by us in order to simulate the evolution of pathogenic bacteria in the GADY experiment. BAGA is inspired by the experiment described in [21] and the Gro script described in [22] to simulate evolution. The algorithm was the germ from which an improved version was later published in [3].

The BAGA algorithm assumes the following assumptions: (i) Darwinian selection of bacteria takes place according to their fitness, which influences the growth rate of the bacteria. The higher the fitness, the higher the value of the growth rate. In other words, evolution by natural selection in bacteria is the result of an adjustment of the level of protein expression and its effect on the bacterial growth rate or Malthusian parameter (r); the equation which governs the growth of the bacterial colony is as follows:

$$y(t) = y_0 e^{rt} \quad (1)$$

where $y(t)$ is the number of bacteria and y_0 is the initial size of the bacterial colony (number of bacteria at $t = 0$). (ii) The algorithm includes a reporter circuit (Figure 4) with which the researcher can evaluate the evolution of a bacterial colony. Evaluation is based on a normalized value of the degree of fluorescence emitted by the bacteria. A reporter circuit is a genetic circuit where bacterial fluorescence is the result of the expression of a gene that is expressed after the expression of another gene of interest. In such a case, the bacterium synthesizes a protein that emits fluorescence. For instance, green fluorescent protein (gfp) is a protein that emits green fluorescence when the bacterial colony is exposed to ultraviolet light.

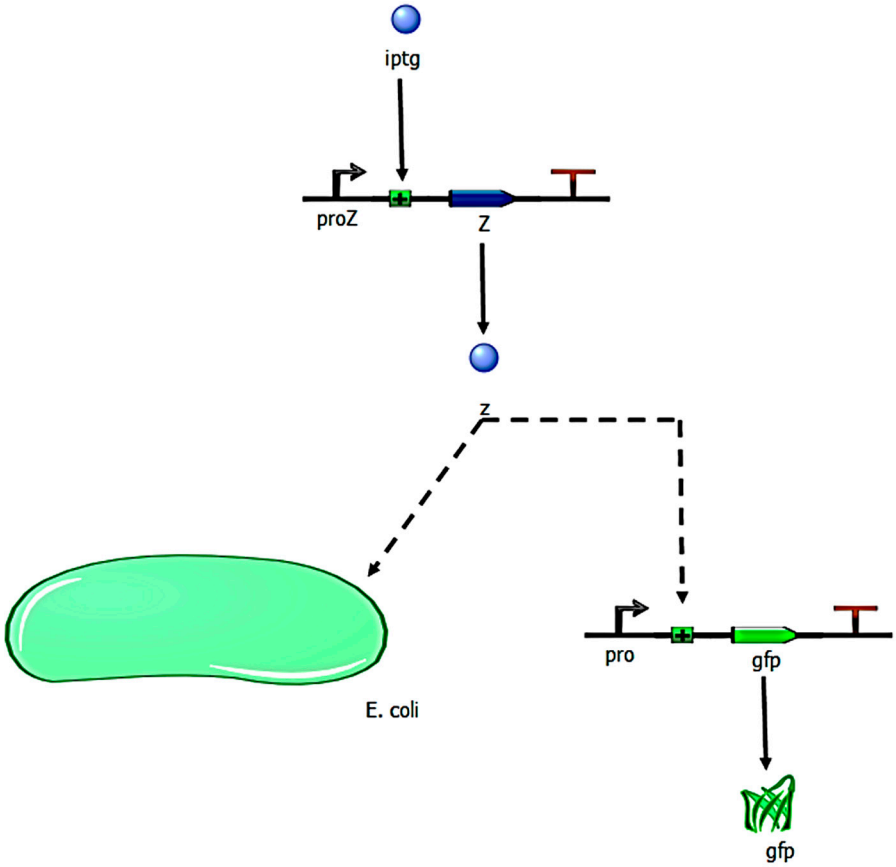

Figure 4. BAGA: an evolutionary protocol inspired by the principles of synthetic biology with *E. coli* bacterium (for explanation, see text).

In summary, the evolutionary algorithm is inspired by Darwinian natural selection at the molecular level whereby the survival of bacteria relies on enzymatic activity. Enzymes are proteins that act as catalysts for chemical reactions by accelerating the reactions' rates, which results from the expression of a gene. In the proposed algorithm, the bacterial selection is followed by a screening step. Thus, bacterial fitness is expressed by a quantitative measure or value of a fluorogenic reporter protein. Hence, the different degree of fluorescence observed in the bacteria of a colony reflects the fitness of each cell and, somehow, the colony evolutionary landscape.

In the following, we describe the main steps of the BAGA algorithm (Figure 5):

(i) Genetic target sequence. In the first step, we set the target sequence. For instance, suppose the problem is of finding the sequence 110. In this instance, the target sequence is declared in Gro language as a list: {1,1,0}.

(ii) Bacterial division. The algorithm assumes a bacterial colony growing according to an exponential or Malthusian growth model. This model does not take into account whether the culture is in a Petri dish, chemostat, or any other container. Each time a bacterium divides into two daughter bacteria, one bacterium retains the parental genes, while the other daughter bacterium undergoes mutations. For example, in the GADY experiment (Figure 2), the non-pathogenic daughter bacterium preserves the parental genes, while the pathogenic daughter bacterium undergoes mutations in its genes. It is also possible for both cells to undergo mutation. In the present version of the algorithm, this is an option to set since it affects the evolution of the bacterial colony.

(iii) Genetic similarity. Once a bacterium divides into two daughter bacteria, the concentration of a hypothetical operon activator (protein with positive control over gene expression) is calculated. An operon is defined in our simulation experiments

as a functional unit of DNA containing one or more genes under the control of a promoter [23]. The algorithm calculates the Hamming distance between the gene sequence inherited by the daughter bacterium and the target sequence. Once the Hamming distance is calculated, its value is translated to a given concentration of iptg (isopropyl β-D-1-thiogalactopyranoside), i.e., an allolactose emulator that activates the lactose operon (operon involved in the metabolism of lactose in *E. coli* and many other bacteria).

The algorithm considers iptg molecules as an activator of a hypothetical Z operon (Figure 4), referring to this operon as the 'optimization operon'.

(iv) Fitness. The goodness of the 'solution', i.e., the genetic sequence in the daughter bacterium, is calculated by means of the optimizing operon Z. This operon expresses a Z gene whose transcription product is a Z protein. The concentration z of product Z, i.e., the fitness value, is expressed according to the Hill function:

$$z = \frac{v.\left[iptg\right]^n}{k^n + \left[iptg\right]^n} \tag{2}$$

whose parameters v, k, and n are set empirically depending on the optimization problem. Thus, in our case, the optimization problem is to find the target sequence. Applying this function, we normalize the value of fitness z between 0 and 1.
In the above z function (2), the concentration of iptg was calculated as the difference between L, i.e., the length of the target gene sequence, and the Hamming distance H between the target and the genetic sequence in the daughter bacterium (sequence inserted in the plasmid):

$$[iptg] = L - H \tag{3}$$

(v) Reporter protein. As the evolutionary algorithm governs the evolution of the synthetic bacteria, the bacteria report their fitness. That is, the fitness z of each bacterium is observed by the researcher thanks to the transcription of fluorescent proteins, e.g., gfp, yfp, rfp. The higher the fitness, the better the 'solution' and consequently the more fluorescence the bacterium will emit. That is, Z plays the role of activator for a reporter operon (Figure 4) that expresses a fluorescent protein, e.g., gfp.

(vi) Malthusian parameter update. The Darwinian selection of the bacteria is simulated as follows. Z not only influences the fluorescence emitted by a bacterium but also affects the Malthusian parameter or bacterial growth rate (r), according to the following expression:

$$r = r_0 + \alpha \frac{z}{\beta} \tag{4}$$

where r_0 is the growth rate of bacteria at $t = 0$, and α, β are two parameters for adjusting the r values in simulation experiments. Indeed, the higher the value of r, the larger the e^{rt} term in the exponential Equation (1). As time t goes on, the number of bacteria $y(t)$ with optimum solutions increase, which is detectable through the screening of those bacteria emitting fluorescence. For instance, in the GADY model, the optimum solution is the target sequence representing the genetic circuit of the plasmid that induces cell suicide of the pathogenic bacteria.

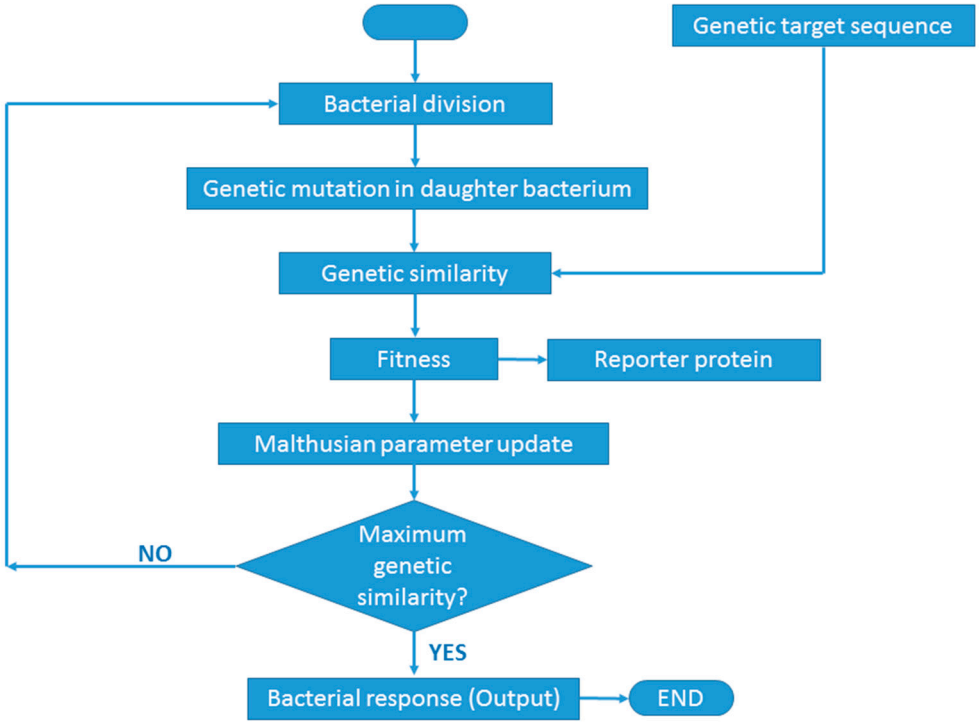

Figure 5. BAGA algorithm (for explanation, see text).

Once the bacteria converge to the optimal solution or the target genotype is expressed, the bacterial response or output will then take place. Note that in the GADY model described in Section 2.3, the bacteria die by cell suicide upon expression of the killer gene.

2.5. Social Interactions Algorithm between Bacterial Agents

The present algorithm was designed with the aim that the number of bacteria of each strain or species increases or decreases as a consequence of the social interactions between species. The interaction between bacteria of different species is the result of the release of chemical signals into the environment. The signal released by an emitting bacterium is received by the receptor bacteria of different species that are in the neighborhood. In Gro and by applying the finite element method, it is possible to simulate the communication between bacteria and therefore their interaction. For this purpose, a signal is defined in a 2D environment specifying the diffusion (k_{diff}) and degradation ($k_{\deg}$) rates of a signal molecule as well as its location on the 2D grid and its concentration at the c_{xy} position. The concentration is updated according to the following expression [17]:

$$\begin{aligned} \Delta c_{x,y} = & - 6\, k_{diff}\, c_{x,y} - k_{\deg}\, c_{x,y} + \\ & k_{diff}\, (0.5\, c_{i+1,y-1} + c_{x+1,y} + 0.5\, c_{x+1,y+1} + c_{x,y-1} + \\ & \quad c_{x,y+1} + 0.5\, c_{x-1,y-1} + c_{x-1,y} + 0.5\, c_{x-1,y+1}) \end{aligned} \quad (5)$$

On the basis of this algorithm, it is possible to define in the bacteria the ability to emit and receive signals. All strains of synthetic bacteria are able to produce a signaling molecule which can have a positive, negative, or neutral effect on the other bacteria in the consortium. Thus, if the interaction between two bacteria is positive (+), the product released by the emitter bacterium will promote the growth of the receiver bacterium:

$$r = r_0 + S_B^+ \quad (6)$$

where S_B^+ is the increase in the growth rate or Malthusian parameter in the signal-receiving bacteria, being (1) the equation governing bacterial colony growth. Conversely, if the interaction is negative (−), then the product released by the emitter bacterium plays the role of a toxic substance. In this case, the bacteria receiving the toxic product, i.e., S_B^-, will die by suicide. Therefore, when a bacterial colony continuously receives negative signals, this species will be gradually eradicated from the bacterial community or consortium.

Finally, an interaction is null (0) if the released product by the emitter bacterium has no effect on other bacteria in the community.

3. Modeling and Simulation of Bacterial Consortia

In this paper, we conducted different experiments simulating a bacterial community or consortium composed of four different types of synthetic bacteria. The notion of bacterial community or consortium [24] we used in our model refers to two or more groups of bacteria interacting symbiotically, i.e., establishing a biological interaction between the bacterial species. The social interactions among bacteria result in a network which confers to the consortium a level of complexity beyond a simple colony of bacteria. In addition to the social interactions between bacteria via quorum sensing, we include in the present model the simulation of the Darwinian evolution of the bacterial community.

In a classical approach, a bacterial consortium is modeled as follows.

In a colony of bacteria, all cells belong to a common bacterial specie or strain *i*; consequently, in a colony, the number of bacteria X_i can be modeled by the following ordinary differential equation (ODE):

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{K}\right) \tag{7}$$

with *k* being the growth rate or Malthusian parameter and *K* the carrying capacity, i.e., the maximum size of the colony that can sustainably live given limited resources. Obviously, the solution of the above ODE is the logistic curve.

Now, when there are several groups of bacteria and they establish social interactions among themselves, then the dynamic behavior of the resulting community or consortium will respond to a system of ODEs:

$$\frac{dX_i}{dt} = k_i X_i\left(1 - \frac{X_i}{K_C}\right) + X_i \sum_{j \neq i} \alpha_{ij} X_j \tag{8}$$

where α_{ij} is the parameter modeling the strength of the interaction between species or strains *i* and *j*, and K_C is the carrying capacity referred to the whole community. According to the model (8), the relationship between two bacteria in the consortium α_{ij} can be positive, negative, or neutral. Indeed, in the Expression (8), the term $X_i \sum_{j \neq i} \alpha_{ij} X_j$ represents the network of interactions among the bacteria of the community.

3.1. Social Interactions Model

In the present paper, one of the aims of the experiments was to simulate a bacterial consortium with different kinds of relationships defined among bacteria, observing in each case the evolution of the bacterial community. The novelty of the present paper is that instead of ODEs, our bacterial consortium model is based on bacterial agents programmed in Gro language. Therefore, instead of simulating the interaction between *i* and *j* bacteria with (8), we applied the procedure described in Section 2.5.

For simplicity, we limited our simulation experiments to the bacterial network shown in Table 1.

Table 1. Bacterial network interactions.

	B1	B2	B3	B4
B1		+	−	−
B2	0		+	−
B3	−	+		−
B4	0	0	0	

According to Table 1, bacteria B1, B2, B3, and B4 in rows receive the signal (+, −, 0) emitted by other bacteria in the community (in columns) as follows:

- The bacterium B1 is positively affected by the B2 bacteria that contribute to its growth, while the products synthesized and released into the medium by the B3 and B4 bacteria are detrimental to the growth of B1.
- The growth of the B2 bacteria is favored by the product eliminated by the B3 bacteria, to the detriment of the product synthesized by the B4 strain.
- Bacteria B3 receive a positive effect on their growth from the product released by bacteria B2, while bacteria of strains B1 and B4 are detrimental to their growth.
- Bacteria B4 are pathogenic, and their growth is not affected by the products released into the medium by the other bacteria.

3.2. Model of Bacterial Genetic Circuit

Bacterial strains were defined assuming that each class of bacteria may or may not behave as pathogenic bacterium. In the consortium, B1, B2, and B3 are non-pathogenic synthetic bacteria, while B4 is the synthetic bacterium that plays the role of pathogenic bacteria. Consequently, and depending on this trait, there will be differences in the plasmid genes of the bacteria programmed with the BAGA algorithm. In the simulation model, non-pathogenic bacteria and pathogenic bacteria have different plasmid types (Figure 6).

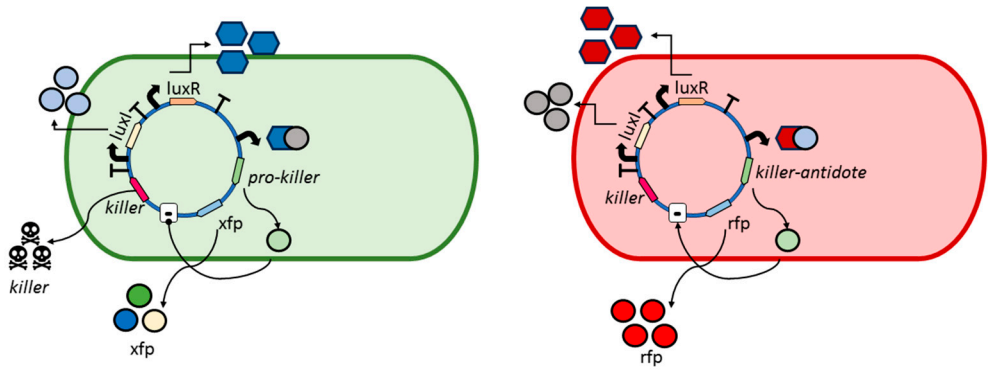

Figure 6. Experimental genetic circuit of the plasmid. (Left) Non-pathogenic 'green' bacterium synthesizes different chemical signals or products as a result of the luxI gene and receives the signal of other products due to the luxR gene. If the bacteria come into contact with toxic chemical products, they die because of the expression of the pro-killer gene. The bacteria emit fluorescence as a result of the presence of fluorescent protein, which is sensitized by the *x*fp gene (e.g., gfp, yfp, cfp). (Right) Pathogenic 'red' bacterium carrying a plasmid genetic circuit with five genes: luxI to yield different products, luxR to synthesize luxR, killer antidote to inhibit the expression of the killer gene (preventing its death), and the rfp gene, whose expression gives rise to the emission of red fluorescence.

Non-pathogenic bacteria have five genes. These include the luxI gene for synthesis of chemical signals or products and luxR for luxR synthesis, which will form a molecular

complex with the products of other bacteria, expressing the rest of the genes. They also include a pro-killer gene so that when the bacterium receives a toxic product or chemical signal S_B^- present in the medium, then the bacterium expresses the killer gene causing bacterial death (cell suicide). In addition, when the *x*fp gene is expressed, an *x* protein (e.g., gfp, yfp, rfp) that emits fluorescence is synthesized by the bacterium. Fluorescence can be observed in the laboratory (in our case via simulation) through its color, e.g., green, yellow, red, etc.

Pathogenic bacteria have most of the genes of non-pathogenic bacteria (Figure 6), except that instead of having a pro-killer gene, they have a killer-antidote gene whose expression inhibits the expression of the killer gene, thereby preventing the suicide of the bacterium.

In order to facilitate the observation of the pathogenic and non-pathogenic bacteria in the community, each kind of bacterium exhibits a different type of fluorescence: bacterium B1 synthesizes gfp (green fluorescent protein), bacterium B2 synthesizes yfp (yellow fluorescent protein), bacterium B3 synthesizes cfp (cyan fluorescent protein), and bacterium B4 synthesizes rfp (red fluorescent protein).

3.3. Simulation Experiments

In this paper, we designed four classes of bacterial consortia or communities based on Figure 2 and the community or microbial consortium (CMC) graph (see Appendix A). The CMC graph displays interactions between bacterial colonies, which were simulated in four experiments, respectively.

The aim of the simulation experiments was to study how the number of bacteria of each bacterial strain, i.e., B1, B2, B3, and B4, varies either due to social interactions (Section 2.5) or as a result of Darwinian evolution (Section 2.4). It is important to emphasize that whether a bacterium is pathogenic or not is only a tag, and therefore, neither the infection nor the possible resulting disease were simulated. Simulation experiments were conducted by setting initially ($t = 0$) a consortium with a colony size of 50 bacteria for B1, B2, B3, and B4 strains. Consequently, the initial community size was 200 bacteria.

In agreement with the CMC graph, the consortium in the first two experiments (Experiments 1 and 2, Figure 2A) is governed exclusively by the social interactions defined in Table 1, with B1, B2, and B3 being non-pathogenic bacteria and B4 being the pathogenic bacteria. Therefore in both experiments, the genetic circuits of the plasmids depicted in Figure 6 do not play a role during the experiments in silico.

3.3.1. Experiment 1

In the first simulation experiment—Experiment 1—the bacterial community has been defined assuming that none of the bacteria undergo mutations, and therefore no bacteria evolve according to the BAGA evolutionary algorithm (Section 2.4). In consequence, the changes experienced by the Malthusian parameter *r* in B*i* bacteria are the result of the effects of the positive interactions (6) with other B*j* agents. Consequently, the increase (colony size) experienced by bacteria is only due to the social interactions between *i* and *j* bacteria (Figure 7).

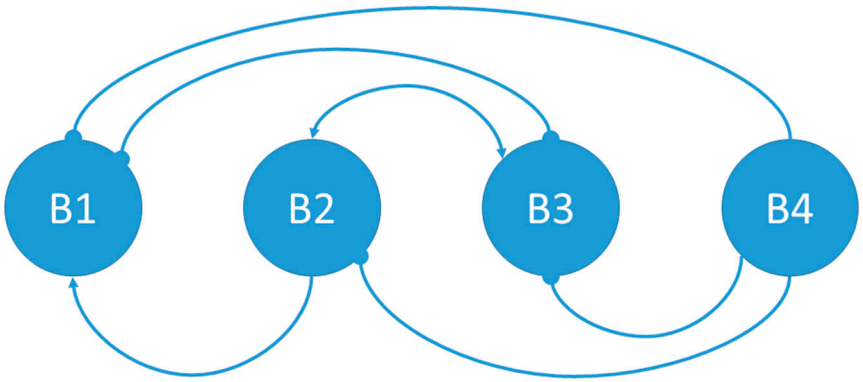

Figure 7. Graph depicting the social interactions in the bacterial community in Experiment 1. The nodes stand for the bacterial strains, and the arcs represent social interactions through the release and reception of chemical signals (+ signal: arcs with arrow termination, − signal: arcs with circular termination).

The bacterial growth rate r_0, i.e., the Malthusian parameter at $t = 0$, was set to 0.045 and 0.5 for non-pathogenic (B1, B2, B3) and pathogenic (B4) bacteria, respectively. In addition, k_4 was equal to 0.2, regulating in the bacterial colony the effect of a negative interaction S_B^-. The chemical signals emitted by each bacterium were set with the same parameter values, and the concentration of the chemical signals $\left[S_B^+\right]$ and $\left[S_B^-\right]$ was updated with time according to the algorithm (5) described in [17].

3.3.2. Experiment 2

A second experiment, Experiment 2, was conducted with a similar bacterial community (Figure 2A) to the preceding one but including in the simulation the presence of an antibiotic in the medium (Figure 8). The initial ($t = 0$) bacterial growth rate (r_0) or Malthusian parameter was 0.045 and 0.2 for non-pathogenic and pathogenic bacteria, respectively. Likewise, in the case of negative social interactions, k_4 was equal to 0.2.

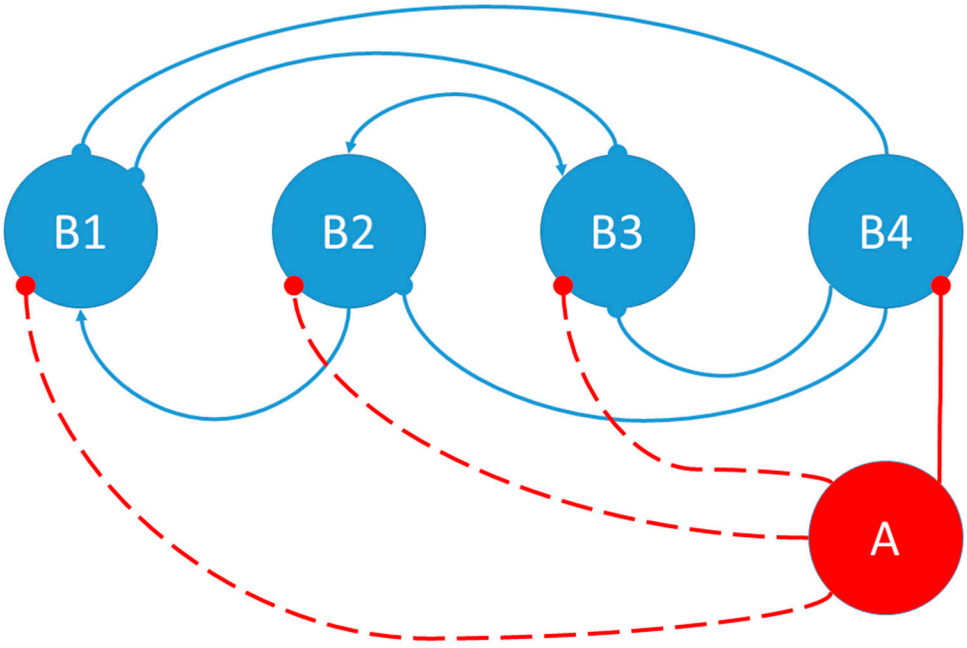

Figure 8. Graph depicting social interactions in the bacterial community of Experiment 2. The nodes stand for the bacterial strains, and the arcs represent social interactions through the release and

reception of chemical signals (+ signal: arcs with arrow termination, − signal: arcs with circular termination). Node A represents the antibiotic with a lower effect on non-pathogenic bacteria B1, B2, and B3 than on pathogenic bacteria B4.

The antibiotic was taken up by all types of synthetic bacteria, especially by the pathogenic bacterium B4. Therefore, in the present simulation experiment, it is expected that the number of pathogenic bacteria will progressively decrease with respect to the number of non-pathogenic bacteria.

In contrast with the above experiments, in Experiments 3 and 4, the number of bacteria of each species is influenced by selection pressure, evolving the community by Darwinian natural selection. In this case, the genotypes of the pathogenic and non-pathogenic bacteria represented in Figures 9 and 10 play a fundamental role according to their fitness during the evolution of the bacterial community. The mutations experienced in the plasmid genes depicted in Figure 6 are the source of variability on which the natural selection mechanism selects or does not select a synthetic bacterium.

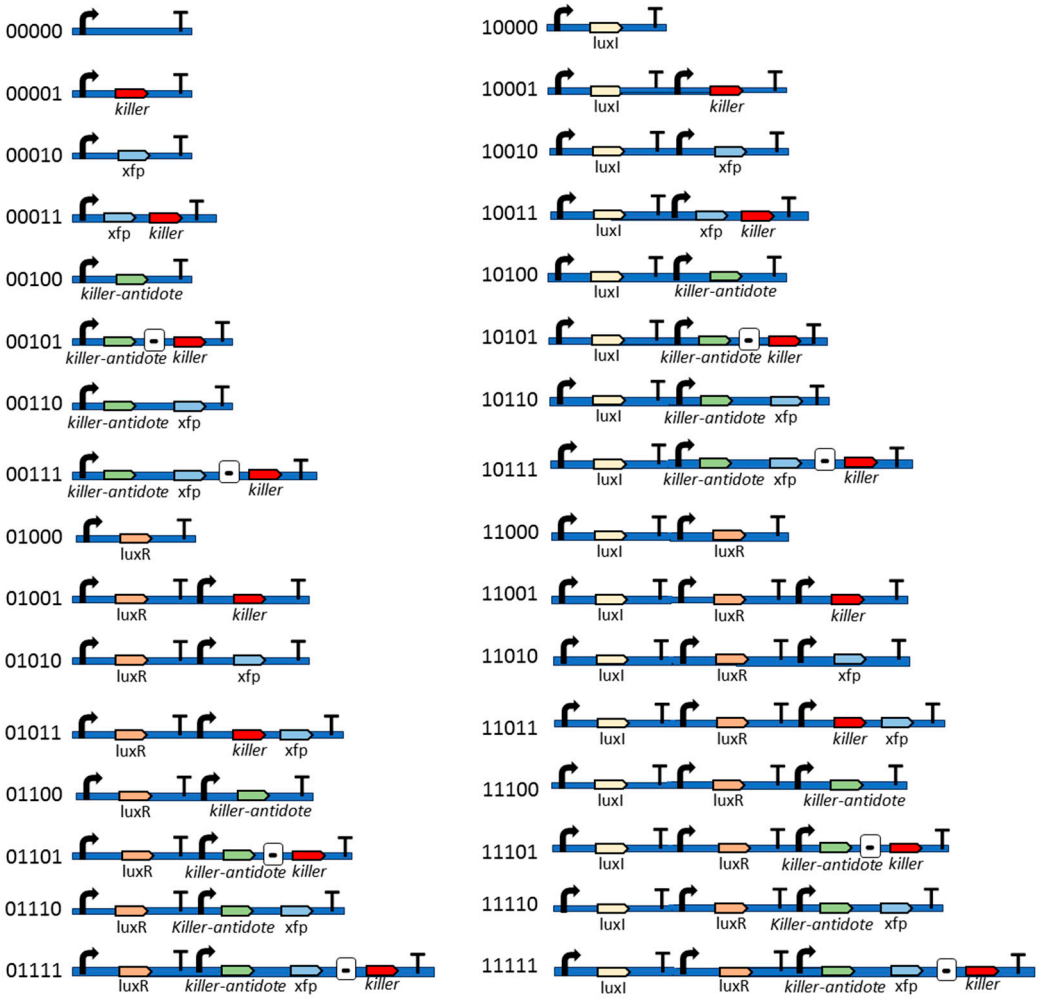

Figure 9. Space of genotypes in the pathogenic bacteria and their representation in binary code.

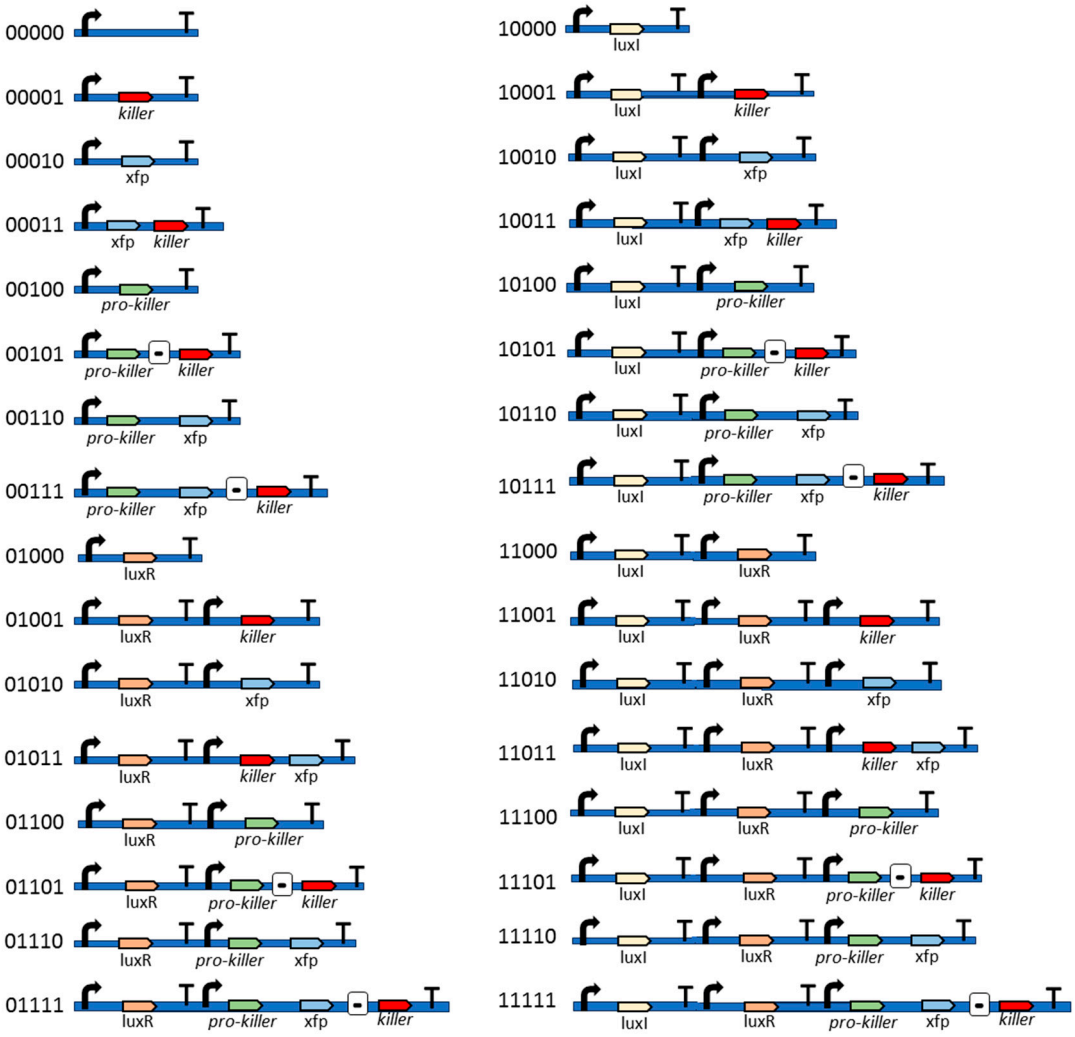

Figure 10. Space of genotypes in the non-pathogenic bacteria and their representation in binary code.

3.3.3. Experiment 3

The following experiment–Experiment 3—is again based on the bacterial interactions (Table 1) among the four strains or bacterial species, but one of the strains of the consortium is also subject to Darwinian natural selection (Figure 2B). Thus, pathogenic bacteria B4 undergo mutations (Figure 11), evolving B4 according to the experiment GADY (Figure 2C) described in Section 2.3. Consequently, in this experiment, the bacterial community evolves as a result of social interactions (B1, B2, B3, B4) as well as Darwinian evolution (B4) through the BAGA evolutionary algorithm.

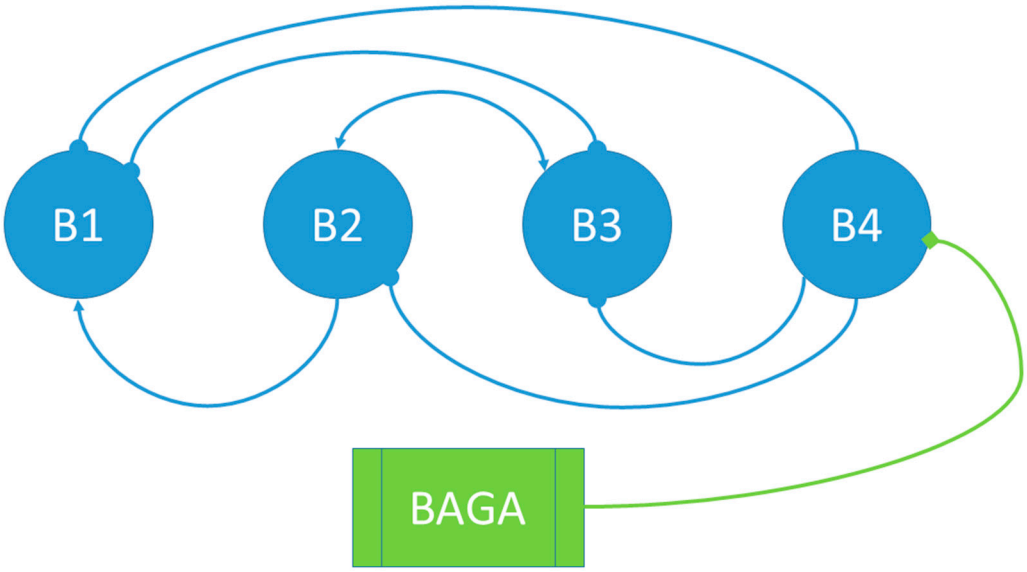

Figure 11. Graph depicting the bacterial community in Experiment 3. The nodes stand for the bacterial strains, and the arcs represent social interactions through the release and reception of chemical signals (+ signal: arcs with arrow termination, − signal: arcs with circular termination). Note how the size of the colonies of non-pathogenic bacteria B1, B2, and B3 depends on their social interactions, while the size of the colony of pathogenic bacteria B4 depends on its adaptability or fitness: B4 is the only colony that exhibits mutations and therefore evolves through the BAGA evolutionary algorithm (green line).

The initial (t = 0) bacterial growth rate was r_0 = 0.045 for pathogenic bacteria B4 and 0.03 for non-pathogenic bacteria B1, B2, B3.

Mutation is the source of variability in the pathogenic B4 bacteria, presenting the synthetic bacteria the genotypes shown in Figure 9. Therefore, the Malthusian parameter (r) of bacteria B4 was updated according to the Expression (4), selecting α = 2 and β = 10 as parameter values (see Appendix A). In constrast, for non-pathogenic bacteria, the value of the growth rate is updated according to their social interactions (6) because B1, B2, and B3 bacteria do not undergo mutations and therefore do not evolve by natural selection (see Appendix A). The target genotype is 11011 for the pathogenic B4 bacterium (Figure 9), with 1 being the presence and 0 the absence of a given gene. In B4, the fitness value is higher as the genome of the mutant bacterium is closer to the desired genotype, i.e., the genotype that leads to the elimination or suicide of the bacterial pathogenic agent. Now, since the colony of species B4 divides according to the GADY model, this means that the chemical substance produced and released into the environment by the non-pathogenic cell line will affect the bacteria of the pathogenic line through state transition rules. This means that a pathogenic bacterium B4 dies if two conditions are satisfied: the pathogenic bacterium (1) receives a chemical signal from a non-pathogenic sister bacterium in its vicinity and (2) presents a genotype, i.e., a genetic circuit, which is appropriate (11011), thus, the genotype in which the killer gene is expressed and will induce the suicide of the pathogenic bacterium. For instance:

p.m = PATHOGEN & p.t > delay & (gene [0] = 1 & gene [1] = 1 & get_signal(product_4) > p.r) & gene [2] = 0 & gene [3] = 1 & gene [4] = 1 : {yfp: = 100*volume, die()}

In this experiment, in order to make it easier to visualize during simulation, non-pathogenic bacteria B1, B2, and B3 produced gfp, emitting green fluorescence. In contrast, pathogenic bacteria B4 expressed the red fluorescent proteins (rfp). Therefore, in the bacterial community or consortium, pathogenic and non-pathogenic bacteria were easily detected by emitting red or green fluorescence, respectively.

3.3.4. Experiment 4

We conclude this section by describing the last of the experiments, Experiment 4. The aim of this experiment was to simulate a bacterial community in which the interactions among bacteria as well as the evolutionary potential of the bacteria give rise to a bacterial community with a complexity as close as possible to a real bacterial consortium. This is a completely different experiment from the previous ones, since the social interactions are quite different from the previous ones defined in Table 1.

In this bacterial community (Figure 2D), we apply the GADY model to the four bacterial strains but with the particularity that the objective is still the eradication of the B4 bacterium. That is, although in each colony one bacterial cell line is pathogenic after each cell division or cell bipartition, the goal remains the elimination of the B4 strain in the course of the evolution of the bacterial consortium. Accordingly, natural selection acts on a consortium of bacteria that are all pathogenic. Now, while B4 may exhibit one of the genotypes in Figure 9, pathogenic bacteria B1, B2, and B3 behave as if they are non-pathogenic, with their genotype space as shown in Figure 10. The positive social interactions between the pathogenic bacteria appearing in colonies B1, B2, and B3 take effect through the Malthusian parameter (6) as in previous experiments. However, and according to Figure 12, the negative social interactions take effect through transition rules. In other words, for negative social interactions, synthetic bacteria behave as in Experiment 3, as a finit—state machine as it occurs in the GADY protocol. The following is an example (Figure 10) of the transition rule for the present experiment, where the bacterium emits green fluorescence and dies:

p.m = PATHOGEN & p.t > delay & get_signal(product_3) > p.r | get_signal(product_1) > p.r & (gene [0] = 1 & gene [1] = 1 & get_signal(product_2) > p.r) & gene [2] = 1 & gene [3] = 0 & gene [4] = 1 : {yfp: = 100*volume, die()}

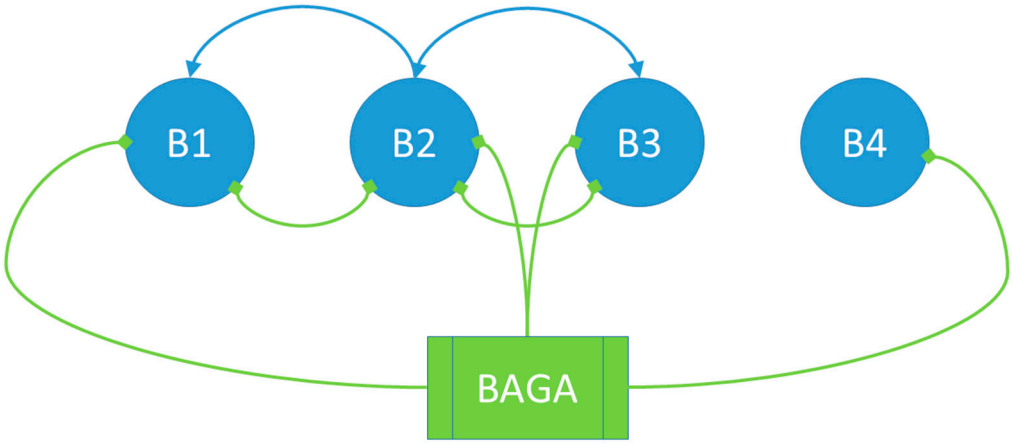

Figure 12. Graph depicting the bacterial community in Experiment 4. The nodes stand for the bacterial strains. In this bacterial consortium, B1, B2, B3, and B4 undergo mutations and evolve according to the BAGA algorithm. Therefore, the four bacterial colonies of the consortium are regulated by the GADY model: non-pathogenic bacteria emit a chemical signal that affects their pathogenic sister bacteria depending on the genotype of the sister bacterium (green lines), which is modeled with state transition rules. In the community, B1, B2, and B3 exhibit positive social interactions (blue arcs with arrow termination).

In this experiment, and in order to be able to distinguish one bacterial species from another, the synthetic bacteria B1, B2, B3, and B4 express in their genome the fluorescent proteins gfp, yfp, cfp, and rfp, respectively.

Note how in the course of evolution (Figure 13), the pathogenic bacteria B1, B2, and B3 acquire resistance to the products or toxic chemical signals released by other bacteria, with the pathogenic strain B4 gradually becoming eradicated.

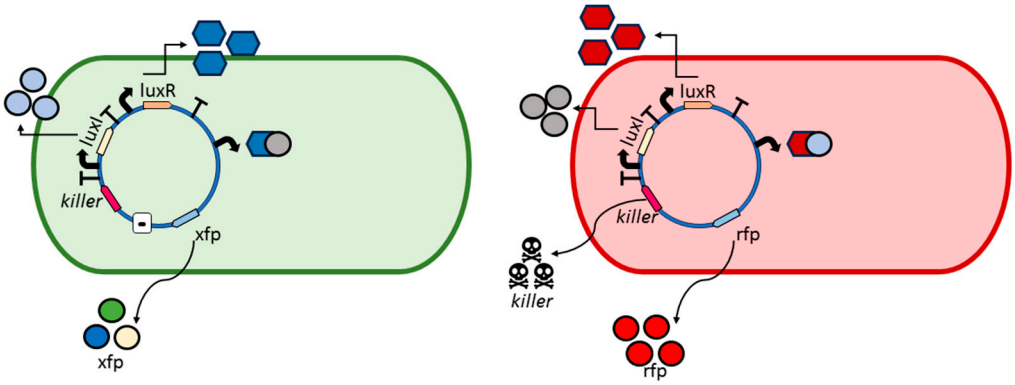

Figure 13. (Left) Pathogenic bacterium B1, B2, and B3 ('green' bacterium), with the mutated genotype which allows the acquisiton of resistance to the products or toxic chemical signals released by other bacteria. Note that since the pro-killer gene is not present, the killer gene will not be expressed, surviving the bacterium. (Right) Pathogenic bacterium B4 ('red' bacterium), with the mutated genotype without the killer-antidote gene; therefore, the killer gene will be expressed, resulting in the killing of the bacterium.

4. Results

The results obtained in the simulation experiments sufficiently capture the biological elements and principles that explain the evolution of a microbial community or consortium composed of bacterial colonies of different species. One of these elements is the model we adopted of the bacterial genetic circuit which includes the genes that allow communication between bacteria by the known mechanism of quorum sensing. When in the bacterial consortium environment there is no selection pressure, then the bacteria evolve through social interactions. When social interactions are positive, then an increase in the growth rate or Malthusian parameter occurs, increasing the number of bacteria and in consequence the size of the colony. Otherwise, if the social interactions are negative, then the death of the bacteria is promoted, decreasing the size of the colony. Now, if the environment imposes a Darwinian selection pressure on any of the colonies of the consortium, then these bacterial colonies will evolve according to the BAGA evolutionary agorithm. In this scenario, the model of bipartition or cell division is the GADY model, in which the future of the lineage of pathogenic bacterial cells is governed by state transition rules. In a transition rule, it is not only the reception of a particular chemical signal taken into consideration but also the genotype of the bacterium that receives the signal. The fact that some bacteria play the role of pathogens, while others do not, is a prerequisite that facilitated the present study by narrowing it down to the elimination of pathogenic microorganisms, i.e., to a methodology which is commonly known as bioremediation.

In summary, the bacterial community or consortium meta-model presented in this paper brings together the elements and principles with which it is possible to design and simulate bacterial communities with the desired complexity.

In the first experiment (Experiment 1), we simulated a bacterial network formed by four types of bacteria, B1, B2, B3, and B4, where B4 was pathogenic and the remaining B1, B2, and B3 were non-pathogenic, and we observed how B4 had a detrimental influence on the rest of the synthetic bacteria (Figure 14). For this reason, and despite starting the simulation ($t = 0$) with the same number of bacteria of each species, towards the end of the experiment, a significant decrease in the number of non-pathogenic bacteria (B1, B2, and B3) was observed with respect to the pathogenic bacteria B4. This is due to the fact that pathogenic bacteria synthesize a product or chemical signal that is released into the medium which kills the rest of the non-pathogenic bacteria when they come into contact with this product. In addition, B1 and B3 bacteria release chemical signals to the medium that lead to a negative interaction (Table 1) between these agents.

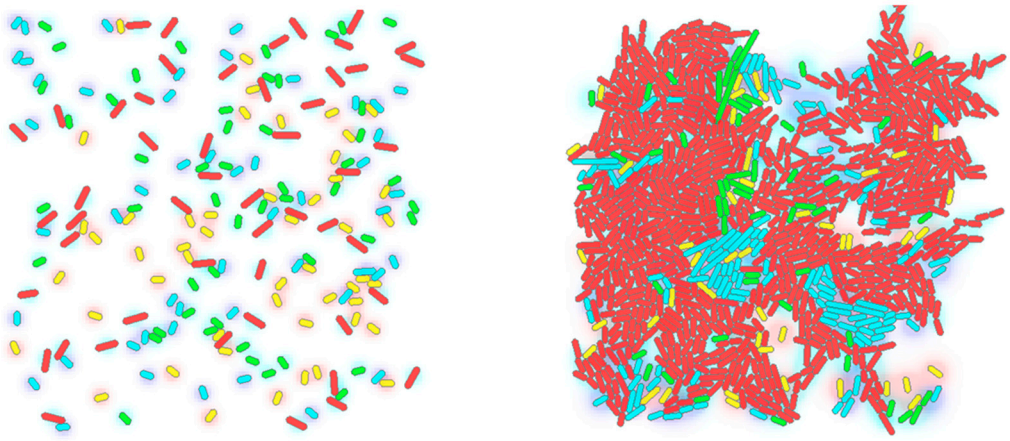

Figure 14. Bacterial community of Experiment 1 composed of pathogenic bacteria B4 expressing rfp (red) and non-pathogenic B1 (green), B2 (yellow), and B3 (cyan) bacteria emitting fluorescence as a consequence of the synthesis of gfp, yfp, and cfp proteins, respectively. Bacterial consortium after (left) 1.02 time units and (right) 5.68 time units. Note the AHL inhibition halo as well as the chemical signals or products released into the environment (light-pink color) around the bacteria comprising the consortium.

The results obtained in this experiment also depend on the mortality rate, the effect of this rate being evident when the bacterial agents come into contact with some of the products eliminated by other bacteria with which the interaction is negative. In the experiment, we considered that the microbial death rate was the same in both non-pathogenic and pathogenic bacteria. However, the growth rate we set in the bacterial agents was higher in the pathogenic bacteria than in the non-pathogenic bacteria.

The second experiment in silico (Experiment 2) includes the effect of an antibiotic in the medium in order to mitigate the negative effect of the pathogenic bacteria on the non-pathogenic bacteria. Figure 15 shows a decrease in the number of pathogenic bacteria and a subsequent increase in the number of the other bacterial types in the consortium, i.e., the non-pathogenic bacteria. Despite the effect of the antibiotic on the pathogenic bacteria B4, the antibiotic also affects the other cell types (B1, B2, and B3) because the antibiotic is not specific to pathogenic bacteria. However, the results obtained show that the decrease in the number of pathogenic B4 bacteria favors the growth of the number of non-pathogenic B1, B2, and B3 bacteria.

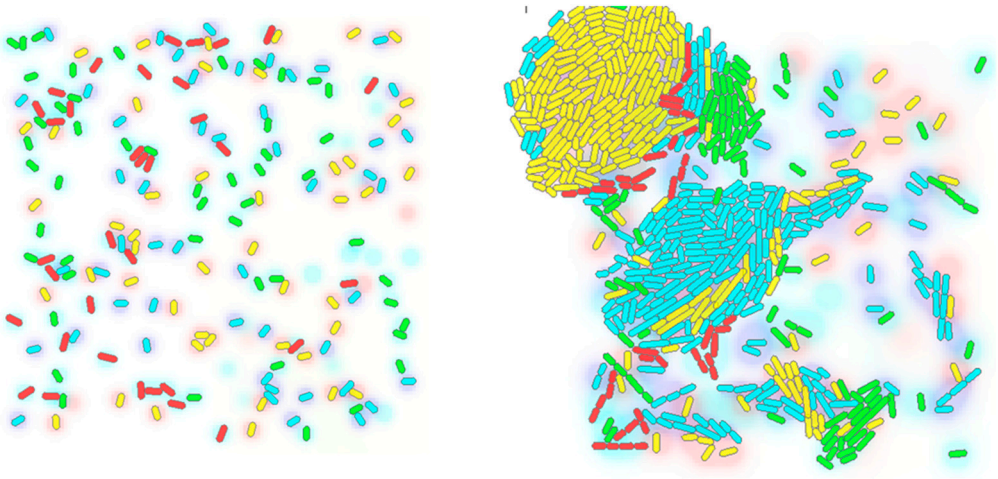

Figure 15. Bacterial community of Experiment 2 composed of pathogenic bacteria B4 expressing rfp (red) and non-pathogenic B1 (green), B2 (yellow), and B3 (cyan) bacteria emitting fluorescence as a consequence of the synthesis of gfp, yfp, and cfp proteins, respectively. Effect of the antibiotic on the bacterial consortium after (left) 1.36 time units and (right) 6.54 time units. Note the AHL

inhibition halo and the products or chemical signals released into the environment (light-pink color) around the bacterial consortium.

In Experiment 3, the results obtained show that the eradication of the pathogenic strain B4 was successfully achieved (Figure 16). However, instead of killing the B4 agent through the effect of an antibiotic present in the medium, which also killed the non-pathogenic synthetic bacteria, this goal was achieved through mutations of the plasmid genes in the pathogenic bacterium, thus due to the evolution of the B4 pathogen driven by the BAGA evolutionary algorithm. The pathogen evolved to a genotype which led to its elimination through cell suicide. The target genotype for the pathogenic bacterium to commit suicide was genotype 11011, i.e., the plasmid, which includes the luxR gene, lacks the killer-antidote gene and includes the killer and fluorescent protein genes.

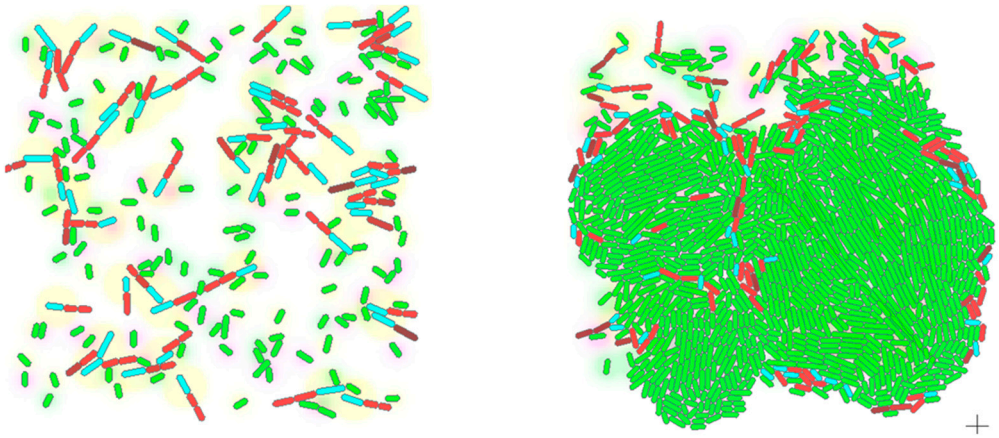

Figure 16. Bacterial community of Experiment 3: Green bacteria (gfp) are non-pathogenic bacterial types (B1, B2, B3), while pathogenic bacteria emit red fluorescence (rfp). Effect of the pathogenic bacteria evolution to its own detriment after (left) 2.75 time units and (right) 13.15 time units. Note the AHL inhibition halo and the products or chemical signals released into the environment (light-pink/yellow color) around the bacterial consortium.

In this experiment, in order to make easier the observation of the three types of non-pathogenic bacteria B1, B2, and B3, these only produce gfp, emitting green fluorescence.

However, and throughout the evolution of pathogenic B4 bacteria, there are periods of time when, after bacterial division, a pathogenic bacterium divides into two daughters, and different kinds of fluorescence are observed (Figure 16). It is interesting to note that those bacteria that emit cyan are the pathogenic bacteria defined in the initial population ($t = 0$). Furthermore, the bacteria emitting yellow fluorescence are the pathogens that are mutating, and the bacteria emitting red are the pathogenic descendants of the initial bacteria emitting cyan. Thus, these bacteria emit their fluorescence transiently after the cellular division of a pathogenic bacterium.

In Experiment 4, the last experiment, we simulated a bacterial community in which all bacterial species or strains evolve (Figure 17): non-pathogenic bacteria evolve by acquiring resistance to the products or chemical signals released into the environment by the other bacteria, and pathogenic bacteria evolve as described in the GADY experiment. In non-pathogenic bacteria, the desired or target genotype, i.e., that which endows the bacteria with resistance to these products, is the genotype that does not express the pro-killer gene, and consequently the killer gene is inhibited when the bacterial agents come into contact with the chemical signals or 'toxic' products. Otherwise, the presence of the pro-killer gene would lead to their death by cell suicide.

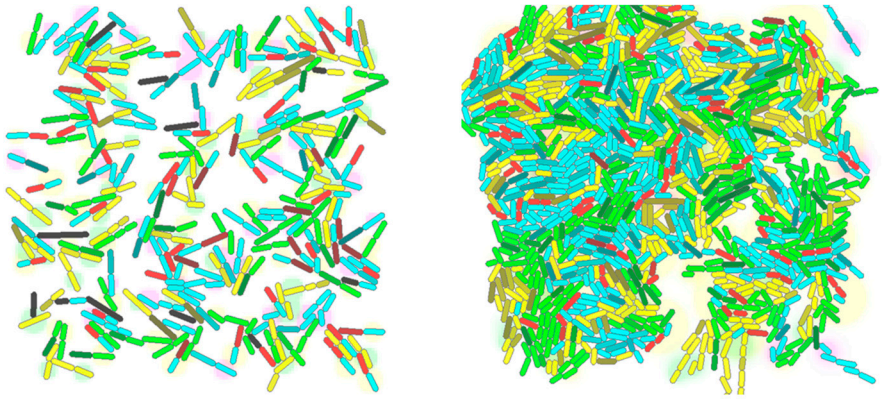

Figure 17. Bacterial community of Experiment 4 composed of pathogenic bacteria: B4 expressing rfp (red) and B1 (green), B2 (yellow), and B3 (cyan) bacteria emitting fluorescence as a consequence of the synthesis of gfp, yfp, and cfp proteins, respectively. Effect of the bacterial community evolution to the detriment of pathogenic bacteria after (left) 1.5 time units and (right) 4.06 time units. Note the AHL inhibition halo and the product released into the environment (light-pink color) around the bacterial consortium.

As shown in Figure 17, in this experiment, each bacterium synthesizes a different fluorescent protein resulting from the expression of its corresponding *x*fp gene, with rfp synthesis and red fluorescence emission in the pathogenic bacterium B4. We observed oscillations of the fluorescent protein, and therefore changes in the intensity of the emitted fluorescence, resulting from the mutations of the synthetic bacteria. At the end of the simulation, it is observed that the pathogenic bacteria B4 decreases in number, increasing the number of non-pathogenic bacteria as they are resistant to those neighboring bacteria with which they have negative interactions. It is interesting to note that in the present experiment, and because of the evolutionary dynamics of the bacterial community, non-pathogenic bacteria may acquire a genotype with which to obtain resistance to the toxic products released by neighboring bacteria.

Figure 18A shows how in the absence of an evolutionary algorithm governing the bacterial community or consortium, the number of pathogenic bacteria increases progressively. In this scenario, the size of the consortium colonies changes, i.e., bacterial colonies evolve, due only to social interactions between bacteria. In consequence, the pathogenic strain becomes numerically dominant over non-pathogenic bacteria in the consortium. However, the presence of antibiotics in the medium (Figure 18B) is able to slow down their growth, and the size of the colony of pathogenic bacteria reaches a similar size to the non-pathogenic bacterial colonies. It is evident that this effect is due to the fact that the presence of antibiotics in the environment implies, from a Darwinian point of view, a selection pressure on bacterial colonies.

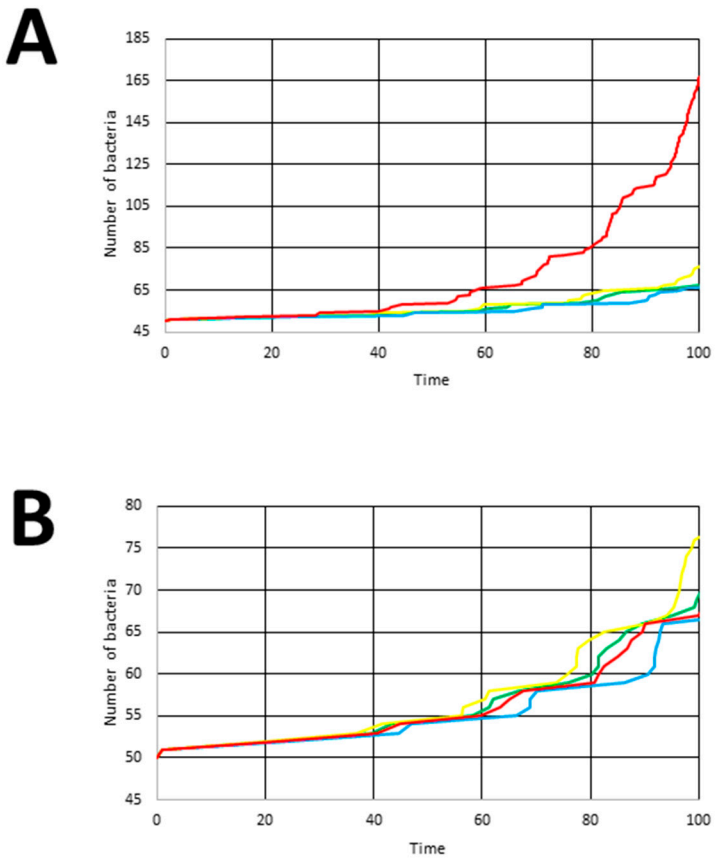

Figure 18. Number of bacteria over time in the absence of an evolutionary algorithm governing the bacterial community or consortium: (**A**) without antibiotics (Experiment 1) and (**B**) with antibiotics in the environment (Experiment 2). Pathogenic B4 bacteria emitting red fluorescence and non-pathogenic bacteria emitting green (B1 bacteria), yellow (B2 bacteria), and cyan (B3 bacteria) fluorescence.

Nevertheless, it is remarkable to note (Figure 19) how random changes in the genome, i.e., mutations, and the consequent effect of mutations on the fitness value of synthetic bacteria leads to the elimination of the pathogenic bacteria. In other words, in this scenario, Darwin's selection mechanism comes into play, completely changing the microbial consortium environment.

As shown in Figure 19A, when only pathogenic bacteria are suitable for evolution, i.e., when pathogenic bacteria evolve according to the GADY protocol, then the results are very similar to those obtained when the pathogenic bacteria are killed by the use of antibiotics. However, when all the bacteria in the community or consortium, i.e., pathogenic and non-pathogenic bacteria, undergo evolutionary changes based on the GADY model, then pathogenic bacteria are successfully eliminated or eradicated by cell suicide (Figure 19B).

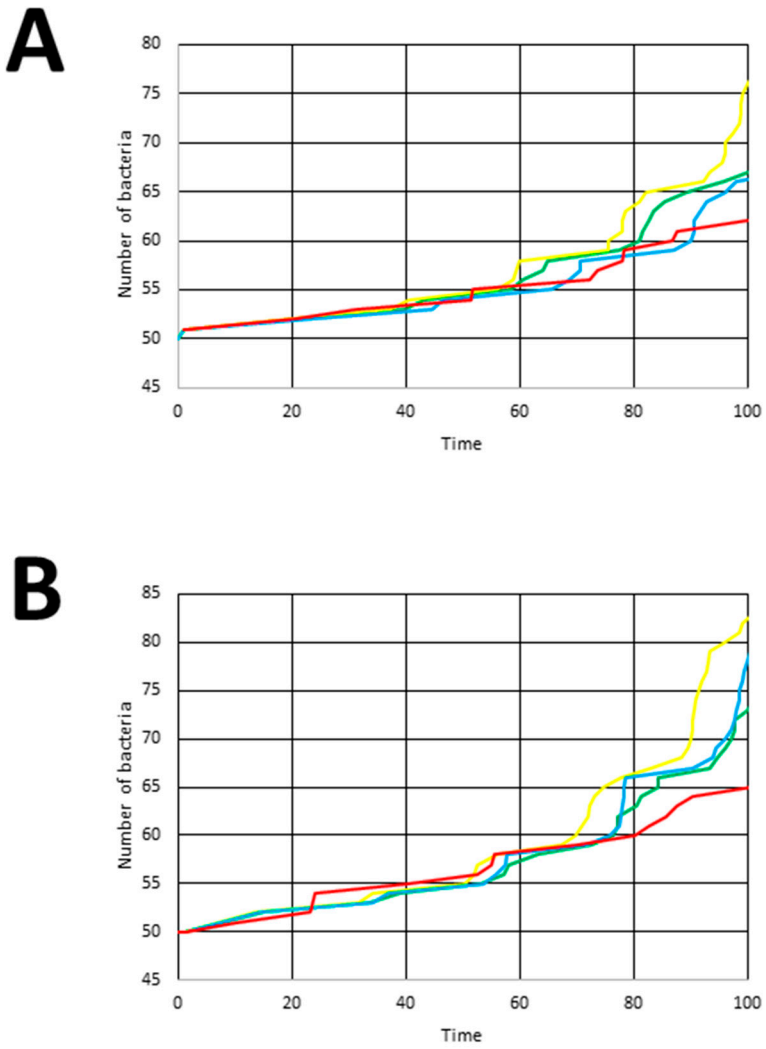

Figure 19. Number of bacteria over time (**A**) when only pathogenic bacteria B4 undergo evolutionary changes driven by the BAGA algorithm (Experiment 3) and (**B**) in an environment where both pathogenic (B4) and non-pathogenic bacteria (B1, B2, B3) undergo Darwinian evolution (Experiment 4) ruled by the BAGA algorithm. Pathogenic B4 bacteria emitting red fluorescence and non-pathogenic bacteria emitting green (B1 bacteria), yellow (B2 bacteria), and cyan (B3 bacteria) fluorescence.

5. Discussion

In this paper, we proposed the design of different experimental protocols to simulate the evolution in silico of a community or consortium composed of synthetic bacteria. In this study, we modeled a synthetic bacterium as a 'shell' which includes by default the dynamic behavior of an *E.coli*-like bacterium, i.e., its growth, cell division, etc., and which can be programmed by hosting a script representing a plasmid's genetic circuit. The species or strains of bacteria were programmed with different bioremediation algorithms based on synthetic biology and computational principles and rules. Since bacteria inherit from the cells from which they originate the program hosted in the parental bacteria, a bacterial colony, or as in our case a bacterial community, represents a form of parallel computation where each synthetic bacterium emulates the core of a computer processor. The parallelism requirement is satisfied with the Gro cellular programming language and its simulator. In this paper, this simulator was the most appropriate way to solve a combinatorial optimization problem: given a bacterial community or consortium, the objective of the experiments was to evaluate different algorithms with which to achieve the minimization of a pathogenic bacterial strain and the maximization of its non-

pathogenic neighbors. However, in nature, algorithms emerge from the genetic circuits that govern the behavior or state of each individual and from the social interactions between these individuals.

In summary, the main goal of our work was to simulate a community or consortium of bacteria in which the number of bacteria of each species or strain is the result of (i) the social interactions among bacterial agents or (ii) the Darwinian evolution of bacteria. According to this objective, we presented four scenarios, and therefore algorithms, with the aim of eliminating the bacterial agent which played the role of a pathogenic bacterium in the simulations.

However, the motivation of this work was to introduce a methodology with which it is possible to study the evolution of a community of agents governed either by the interactions between agents or by the Darwinian evolution of these agents, or even by both factors. It is evident that this situation may be of interest in many areas other than biology, such as sociology, economics, etc.

From a biological perspective, the modeling and simulation of a bacterial colony is a classical issue that was addressed by applying different simulation techniques, such as cellular automata [25], multi-agent systems [26], or artificial life techniques [27]. However, the current challenge is the simulation of the formation and growth of the so-called biofilm, i.e., a microbial ecosystem organized on a surface. This interest arises because biofilms are responsible for the development of some infectious diseases, antibiotic resistance, etc. In our model, a biofilm would be composed of one or several species or strains of bacteria, resembling the consortium or bacterial community simulated in this paper. In a similar way to the formation and growth of a bacterial colony, the formation of a biofilm can also be addressed by different modeling techniques, e.g., cellular automata [28]. However, the differential equations approach [29,30] is the most common method used to simulate the growth of biofilms.

Therefore, we believe that by including in our meta-model the phenomena involved in the formation of a biofilm, we could successfully simulate its formation as well as the changes it undergoes over time.

Whether in a consortium or in a biofilm, cell-to-cell communication between bacteria represents today a subject of study of great interest. Nowadays, it is thought that cell-to-cell communication based on molecular-level mechanisms is the origin of the social intelligence found in bacteria. In addition to quorum sensing, other forms of communication such as chemotactic signaling or plasmid exchange [31] explain how bacteria self-organize in highly structured communities. These bacterial communities are a form of organization that allows microorganisms to acquire an identity, the recognition of other colonies, etc.

Our work includes an evolutionary algorithm that we named BAGA and that is based on the communication mechanism between bacteria known as quorum sensing. In the future, the design of evolutionary algorithms with other forms of communication between bacteria different from quorum sensing will result in simulations with which it will be possible to study in silico relevant issues, for instance, the development of biofilms, the design of cellular patterns with practical purposes [32], etc.

Many of the ideas described in this paper will be in the future the basis for the use of microbial communities in biotechnology.

Today, synthetic biology under the influence of ideas coming from the fields of engineering and computer science [33–36] has developed the tools to enable the implementation in a real laboratory—the wet lab—of many proposals modeled and simulated in silico, as is the case of this work. These bacteria will be responsible for executing in vivo sophisticated algorithms designed to solve crucial problems such as the biodegradation of environmental pollutants [37], the design of probiotics [38], or even the design of microbial agents capable of inducing the regression of a cancerous tumor [39].

The present work was limited to the design of a meta-model and its in silico simulation. However, the possibility that in the future, other research groups will adopt

our meta-model for its implementation in real bacteria in the wet lab is not remote. Living microorganisms such as bacteria used in synthetic biology are fully functional with biological sensing, production, and containment devices. Bacteria include sensors, regulators, memory circuits, and switch-off triggers [36]. That is, bacteria have many advantages by interacting directly with the human body: they are programmable to perform specific tasks, respond to environmental stimuli, and are more effective in the treatment of complex and multifactorial diseases [40]. For example, there are already real synthetic bacteria capable of breaking down the excess phenylalanine responsible for phenylketonuria [1], an inherited disease that over time can lead to serious health problems. In [1], other examples are also described of treatments known as bactotherapy, i.e., treatments based on the administration to the patient of synthetic bacteria like those we studied in silico in this work.

At present, and in a similar way to is happening with generative artificial intelligence, synthetic biology has opened an ethical debate about genetic modifications and the use of synthetic organisms for therapeutic purposes. It is now urgent to study at a social and philosophical level the potential risks of the design and creation of new life forms, evaluating their possible risks and benefits [41]. One of these potential risks could be the possibility of synthetic bacteria escaping human control and becoming integrated in nature, competing with and even eliminating their wild counterparts. Indeed, as synthetic bacteria share physiological principles of non-synthetic bacteria, a colony of synthetic bacteria could increase in size according to the exponential or Malthusian model. That is, in certain environments and due to their greater adaptability or fitness [42], the Malthusian parameter or growth rate would increase, growing exponentially the number of synthetic bacteria. However, the possibility of designing synthetic bacteria carrying a killer gene [43,44] that induces cell suicide, as we designed in the present paper, opens the possibility of reducing or avoiding these potential risks inherent to the design and application of synthetic bacteria.

In this regard, in the post-COVID-19 era, there is a growing concern about this issue, as reported in a recent article [45]. Let us hope that the advances in synthetic biology will be for the good of all, helping to solve many of the problems facing humankind in the 21st century.

Author Contributions: S.L.V. collaborated on the revision and carried out the programming in Gro language of the synthetic bacteria. She also designed the experimental methodology and conducted the simulation experiments that were used in her undergraduate final project in the biology bachelor's degree from the Faculty of Biology, Complutense University of Madrid. R.L.-B. devised the general problem, supervised the work of the first author, and wrote this paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: This work does not include data available to the public.

Acknowledgments: S.L.V. was granted a scholarship under the program 'Becas para Estudiantes de Enseñanzas Postobligatorias 2022/2023, Ministerio de Educación y Formación Profesional (España)', allowing her to carry out the present work.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

In this Appendix, we provide some details on how we drew up each microbial community the corresponding community or microbial consortium (CMC) graph, explaining how this graph is translated into the calculations allowing the updating of the number of bacteria in each colony according to their relationships with other colonies in the consortium. Figures 7, 8, 11 and 12 show in a CMC graph the relationships between the colonies that constitute the bacterial community or consortium. The graphs show the colonies with nodes and the interactions between nodes with arcs. Figure A1 represents

four different classes of node–arc junctions, which are the ones we used in the four models of bacterial communities simulated in silico in this paper.

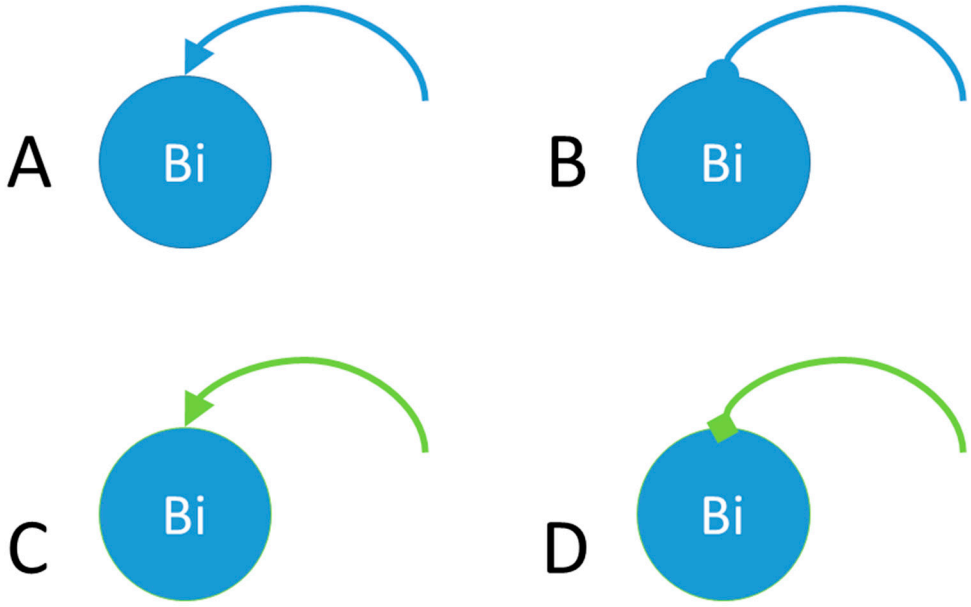

Figure A1. Node–arc junctions used to represent the relationships between bacterial colonies in a community or consortium (for an explanation, see text).

The evolution of the microbial consortium, i.e., the update of the number of bacteria in each colony, is simulated based on the graph representing the inter-colony relationships in the consortium.

In the experiments in which the microbial community is such that only social interactions between colonies are established (Experiments 1 and 2, Figures 7 and 8), the state or number of bacteria in a colony B*i* is the result of its interaction with another colony B*j*, using in such a case the nodes–arcs A and B in Figure A1.

If a colony B*i* receives a chemical signal or product S_B^+ released into the environment by a colony B*j* and this signal has positive effects on the bacteria B*i* (Figure A1A), then the number of bacteria in the colony B*i* is updated according to

$$r = r_0 + \left[S_B^+\right]$$

where r_0 is the growth rate of bacteria at $t = 0$, r is the Malthusian parameter, and $\left[S_B^+\right]$ is the concentration of the chemical signal. However, when colony B*i* receives a chemical signal from colony B*j* with 'toxic' or detrimental effects, i.e., receives S_B^-, then the number of bacteria B*i* decreases according to the equation

$$D_R = k_4\left[S_B^-\right]$$

where D_R is the rate of microbial death (related to the bacterial colony) and k_4 is a cell death rate, the value of which was 0.2 in the simulation experiments.

An example in Gro language is shown below. In this example, bacterial colony B1 emits a positive signal sB1 to the environment of the bacterial consortium and receives from colony B2 a positive chemical signal sB2 and from B3 a toxic signal or product sB3 (Figure A2).

```
program bacteria_1() := {
        gfp := 0;
        true : {
                set("ecoli_growth_rate",((g_rate_ECOLI) + (get_signal(sB2)))),
                gfp := 100*volume,
                emit_signal(sB1,1), // emits the signal product
                // the signals it absorbs:
                absorb_signal(sB2,0.1),
                absorb_signal(sB3,0.1),
        }
        rate( k4 * get_signal(sB3) ) : {
        die(); //if it captures the product of the bacteria 3 it dies
        };
        };
```

Figure A2. Gro language script illustrating a microbial consortium in which the colony of bacteria B1 is associated with colonies B2 and B3.

However, when a colony B*i* evolves under the Darwinian selection mechanism through the BAGA algorithm and the rest of the colonies B*j* evolve by social interactions as explained above, then B*i* is updated as follows. In agreement with Figure 2C, the lineage of pathogenic 'red' bacteria is updated according to the expression (4):

$$r = r_0 + \alpha \frac{z}{\beta}$$

the meaning of which is explained above.

Let us now assume the scenario of a microbial community in which the bacterial colony B4 evolves according to the GADY model (Figure 2C), while the rest of the non-pathogenic bacteria B1, B2, and B3 evolve according to the previous model, i.e., through social interactions between colonies. An example of a script in Gro language is shown below (Figure A3), in which after the cell division of a bacterium, the non-pathogenic daughter bacteria release a chemical signal sB4 that is received by the pathogenic sister bacteria of the pathogenic lineage. Once this signal or product is received, the future state of the pathogenic daughter bacteria lineage is set according to their genotype, as shown in the two transition rules at the end of the script (Figure A3).

```
program bacteria_4() := {

// PATHOGEN DETECTS SIGNAL

  // mutation

  p.m = PATHOGEN : {
     gene[0]:=rand(2),
     gene[1]:=rand(2),
     gene[2]:=rand(2),
     gene[3]:=rand(2),
     gene[4]:=rand(2),

   // Fitness
   H := ( (gene[0]-genet[0])^2 +(gene[1]-genet[1])^2 +(gene[2]-genet[2])^2 +(gene[3]-genet[3])^2 +(gene[4]-genet[4])^2 ),
   iptg:=L-H,
   z := (v * (iptg^n)) / ( (k^n) + (iptg^n)),
   g_rate_PATHOGEN := 0.03 + 2*(z/10),

  }

  // detection radius

  delay:=50;

  p.m = PATHOGEN & p.t>delay & (gene[0]=1 & gene[1]=1 & get_signal(sB4)>p.r) & gene[2]=0 & gene[3]=0 & gene[4]=0  : { skip() }
  p.m = PATHOGEN & p.t>delay & (gene[0]=1 & gene[1]=1 & get_signal(sB4)>p.r) & gene[2]=0 & gene[3]=0 & gene[4]=1  : { yfp:=100*volume }

};
```

Figure A3. Gro language script illustrating a microbial consortium in which the colony of bacteria B4 evolves according to the GADY model, releasing sB4. The signal will have one effect or another on the pathogenic bacteria of the same colony depending on the genotype of the latter.

In this case, and depending on the symbol B*i*, the state transition rule that is applied to the pathogenic bacteria lineage of the colony B*i* will be different. That is, if the colony B*i* is the symbol of Figure A1C, then the chemical signal received by the pathogenic bacterial lineage, e.g., the product sB4, will produce a positive effect on the pathogenic bacteria:

p.m = PATHOGEN & p.t > delay & (gene [0] = 1 & gene [1] = 1 & get_signal(sB4) > p.r) & gene [2] = 0 & gene [3] = 0 & gene [4] = 1 : {yfp: = 100*volume}

Now, if colony B*i* is depicted by the symbol in Figure A1D, then the chemical signal that is received in the pathogenic bacteria lineage, e.g., product sB4, will yield a negative effect on such bacteria:

p.m = PATHOGEN & p.t > delay & (gene [0] = 1 & gene [1] = 1 & get_signal(sB4) > p.r) & gene [2] = 0 & gene [3] = 1 & gene [4] = 1 : {die()}

References

1. Liu, Y.; Feng, J.; Pan, H.; Zhang, X.; Zhang, Y. Genetically engineered bacterium: Principles, practices, and prospects. *Front. Microbiol.* **2022**, *13*, 997587. https://doi.org/10.3389/fmicb.2022.997587.
2. Lahoz-Beltra, R. *Turing: Del Primer Ordenador a la Inteligencia Artificial*; Nivola: Madrid, Spain, 2005.
3. Gargantilla Becerra, A.; Gutiérrez, M.; Lahoz-Beltra, R. A synthetic biology approach for the design of genetic algorithms with bacterial agents. *Int. J. Parallel Emergent Distrib. Syst.* **2021**, *36*, 275–292.
4. Gargantilla Becerra, A.; Gutiérrez, M.; Lahoz-Beltra, R. Computing within bacteria: Programming of bacterial behavior by means of a plasmid encoding a perceptron neural network. *BioSystems* **2022**, *213*, 104608. https://doi.org/10.1016/j.biosystems.2022.104608.
5. Kriegman, S.; Blackiston, D.; Levin, M.; Bongard, J. A scalable pipeline for designing reconfigurable organisms. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 1853–1859. https://doi.org/10.1073/pnas.1910837117.
6. English, M.A.; Gayet, R.V.; Collins, J.J. Designing biological circuits: Synthetic biology within the operon model and beyond. *Annu. Rev. Biochem.* **2021**, *90*, 221–244.
7. Singh, V. Recent advances and opportunities in synthetic logic gates engineering in living cells. *Syst. Synth. Biol.* **2014**, *8*, 271–282. https://doi.org/10.1007/s11693-014-9154-6.
8. Xia, P.F.; Ling, H.; Foo, J.L.; Chang, M.W. Synthetic genetic circuits for programmable biological functionalities. *Biotechnol. Adv.* **2019**, *37*, 107393. https://doi.org/10.1016/j.biotechadv.2019.04.015.

9. Abil, Z.; Xiong, X.; Zhao, H. Synthetic biology for therapeutic applications. *Mol. Pharm.* **2015**, *12*, 322–331. https://doi.org/10.1021/mp500392q.
10. McCarty, N.S.; Ledesma-Amaro, R. Synthetic biology tools to engineer microbial communities for biotechnology. *Trends Biotechnol.* **2019**, *37*, 181–197. https://doi.org/10.1016/j.tibtech.2018.11.002.
11. Naseri, G.; Koffas, M.A.G. Application of combinatorial optimization strategies in synthetic biology. *Nat. Commun.* **2020**, *11*, 2446. https://doi.org/10.1038/s41467-020-16175-y.
12. Jeschek, M.; Gerngross, D.; Panke, S. Combinatorial pathway optimization for streamlined metabolic engineering. *Curr. Opin. Biotechnol.* **2017**, *47*, 142–151.
13. Schreiber, J.; Arter, M.; Lapique, N.; Haefliger, B.; Benenson, Y. Model-guided combinatorial optimization of complex synthetic gene networks. *Mol. Syst. Biol.* **2016**, *12*, 899. https://doi.org/10.15252/msb.20167265.
14. Chen, B.S.; Chen, P.W. GA-based design algorithms for the robust synthetic genetic oscillators with prescribed amplitude, period and phase. *Gene Regul. Syst. Biol.* **2010**, *4*, 35–52. https://doi.org/10.4137/grsb.s4818.
15. Chuang, C.H.; Lin, C.L.; Chang, Y.C.; Jennawasin, T.; Chen, P.K. Design of synthetic biological logic circuits based on evolutionary algorithm. *IET Syst. Biol.* **2013**, *7*, 89–105. https://doi.org/10.1049/iet-syb.2012.0048.
16. Currin, A.; Parker, S.; Robinson, C.J.; Takano, E.; Scrutton, N.S.; Breitling, R. The evolving art of creating genetic diversity: From directed evolution to synthetic biology. *Biotechnol. Adv.* **2021**, *50*, 107762. https://doi.org/10.1016/j.biotechadv.2021.107762.
17. Jang, S.S.; Oishi, K.T.; Egbert, R.G.; Klavins, E. Specification and simulation of multicelled behaviors. *ACS Synth. Biol.* **2012**, *1*, 365–374. https://doi.org/10.1021/sb300034m.
18. Oishi, K.; Klavins, E. A framework for implementing finite state machines in gene regulatory networks. *ACS Synth. Biol.* **2014**, *3*, 652–665.
19. Gutiérrez, M.; Gregorio-Godoy, P.; Pérez Del Pulgar, G.; Muñoz, L.; Sáez, S.; Rodríguez-Patón, A. A new improved and extended version of the multicell bacterial simulator gro. *ACS Synth. Biol.* **2017**, *6*, 1496–1508.
20. Sanchez Iñiguez, U.; Lledo Villaescusa, S.; Lahoz-Beltra, R. GADY algorithm: Towards an evolutionary protocol for bottom-up design of synthetic bacteria. *bioRxiv* **2023**. https://doi.org/10.1101/2023.11.07.566064.
21. Dekel, E.; Alon, U. Optimality and evolutionary tuning of the expression level of a protein. *Nature* **2005**, *436*, 588–592.
22. Klavins, E. gro. The Cell Programming Language. Available online: http://depts.washington.edu/soslab/gro/index.html (accessed on 6 November 2023).
23. Sadava, D.E.; Hillis, D.M.; Heller, H.C.; Berenbaum, M. *Life: The Science of Biology*, 9th ed.; W. H. Freeman: New York City, NY, USA, 2009; p. 349.
24. Marino, S.; Baxter, N.T.; Huffnagle, G.B.; Petrosino, J.F.; Schloss, P.D. Mathematical modeling of primary succession of murine intestinal microbiota. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 439–444. https://doi.org/10.1073/pnas.1311322111.
25. Li, B.; Wang, J.; Wang, B.; Wenhan, L.; Wu, Z. Computer simulations of bacterial-colony formation. *Europhys. Lett.* **1995**, *30*, 239–243. https://doi.org/10.1209/0295-5075/30/4/009.
26. Kreft, J.U.; Booth, G.; Wimpenny, J.W.T. BacSim, a simulator for individual-based modelling of bacterial colony growth. *Microbiology* **1998**, *144*, 3275–3287. https://doi.org/10.1099/00221287-144-12-3275.
27. Thai Dam, D.; Lahoz-Beltra, R. MICRORAM: A simulation model of a colony of bacteria evolving inside an artificial world. *Int. J. Inf. Theor. Appl.* **2014**, *21*, 328–338. Available online: http://www.foibg.com/ijita/vol21/ijita21-04-p04.pdf (accessed on 5 December 2023).
28. Wimpenny, J.W.T.; Colasanti, R. A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. *FEMS Microbiol. Ecol.* **1997**, *22*, 1–16. https://doi.org/10.1111/j.1574-6941.1997.tb00351.x.
29. Ghosh, P.; Mondal, J.; Ben-Jacob, E.; Levine, H. Mechanically-driven phase separation in a growing bacterial colony. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E2166–E2173. https://doi.org/10.1073/pnas.1504948112.
30. Rudge, T.J.; Steiner, P.J.; Phillips, A.; Haseloff, J. Computational modeling of synthetic microbial biofilms. *ACS Synth. Biol.* **2012**, *1*, 345–352. https://doi.org/10.1021/sb300031n.
31. Ben Jacob, E.; Becker, I.; Shapira, Y.; Levine, H. Bacterial linguistic communication and social intelligence. *Trends Microbiol.* **2004**, *12*, 366–372. https://doi.org/10.1016/j.tim.2004.06.006.
32. Mitchell, R.J.; Lee, S.K.; Kim, T.; Ghim, C.M. Microbial linguistics: Perspectives and applications of microbial cell-to-cell communication. *BMB Rep.* **2011**, *44*, 1–10. https://doi.org/10.5483/BMBRep.2011.44.1.1.
33. Fellermann, H.; Markovitch, O.; Gilfellon, O.; Madsen, C.; Phillips, A. Toward programmable biology. ACS synthetic biology 2016, 5(8), 793–794. https://doi.org/10.1021/acssynbio.6b00213.
34. Gao, C.; Xu, P.; Ye, C.; Chen, X.; Liu, L. Genetic Circuit-Assisted Smart Microbial Engineering. *Trends Microbiol.* **2019**, *27*, 1011–1024. https://doi.org/10.1016/j.tim.2019.07.005.
35. Han, Y.H.; Kim, G.; Seo, S.W. Programmable synthetic biology tools for developing microbial cell factories. *Curr. Opin. Biotechnol.* **2023**, *79*, 102874. https://doi.org/10.1016/j.copbio.2022.102874.
36. Pedrolli, D.B.; Ribeiro, N.V.; Squizato, P.N.; de Jesus, V.N.; Cozetto, D.A.; Team AQA Unesp at iGEM 2017. Engineering microbial living therapeutics: The synthetic biology toolbox. *Trends Biotechnol.* **2019**, *37*, 100–115. https://doi.org/10.1016/j.tibtech.2018.09.005.
37. Xiang, L.; Li, G.; Wen, L.; Su, C.; Liu, Y.; Tang, H.; Dai, J. Biodegradation of aromatic pollutants meets synthetic biology. *Synth. Syst. Biotechnol.* **2021**, *6*, 153–162. https://doi.org/10.1016/j.synbio.2021.06.001.

38. Lebovich, M.; Zeng, M.; Andrews, L.B. Algorithmic programming of sequential logic and genetic circuits for recording biochemical concentration in a probiotic bacterium. *ACS Synth. Biol.* **2023**, *12*, 2632–2649. https://doi.org/10.1021/acssynbio.3c00232.
39. Chowdhury, S.; Castro, S.; Coker, C.; Hinchliffe, T.E.; Arpaia, N.; Danino, T. Programmable bacteria induce durable tumor regression and systemic antitumor immunity. *Nat. Med.* **2019**, *25*, 1057–1063. https://doi.org/10.1038/s41591-019-0498-z.
40. Brennan, A.M. Development of synthetic biotics as treatment for human diseases. *Synth. Biol.* **2022**, *7*, ysac001. https://doi.org/10.1093/synbio/ysac001.
41. Schmidt, M.; Ganguli-Mitra, A.; Torgersen, H.; Kelle, A.; Deplazes, A.; Biller-Andorno, N. A priority paper for the societal and ethical aspects of synthetic biology. *Syst. Synth. Biol.* **2009**, *3*, 3–7. https://doi.org/10.1007/s11693-009-9034-7 .
42. Concepción-Acevedo, J.; Weiss, H.N.; Chaudhry, W.N.; Levin, B.R. Malthusian parameters as estimators of the fitness of microbes: A cautionary tale about the low side of high throughput. *PLoS ONE* **2015**, *10*, e0126915. https://doi.org/10.1371/journal.pone.0126915.
43. Granato, E.T.; Foster, K.R. The evolution of mass cell suicide in bacterial warfare. *Curr. Biol.* **2020**, *30*, 2836–2843.e3. https://doi.org/10.1016/j.cub.2020.05.007.
44. Rottinghaus, A.G.; Ferreiro, A.; Fishbein, S.R.S.; Dantas, G.; Moon, T.S. Genetically stable CRISPR-based kill switches for engineered microbes. *Nat. Commun.* **2022**, *13*, 672. https://doi.org/10.1038/s41467-022-28163-5.
45. Wang, G.; Kong, Q.; Wang, D.; Asmi, F. Ethical and social insights into synthetic biology: Predicting research fronts in the post-COVID-19 era. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1085797. https://doi.org/10.3389/fbioe.2023.1085797.