SCUOLA DI DOTTORATO
UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Department of

Biotechnology and Biosciences

PhD program: **Converging Technologies for Biomolecular Systems (TeCSBI)** Cycle **XXXIV**

The division of labor in microorganisms: from survival strategies of the species to industrial success stories

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ACADEMIC YEAR 2021/2022

Dedication

I dedicate this thesis to my wife and my children that patiently supported me along my never-ending scientific trail, to my work mates and friends Adriana, Carmine, and Marco, to my loving mother and father, Giuliana and Giuseppe, to my sister and brother Marina, and Paolo. Thank you for your endless love, support, and encouragement throughout my industrial and academic journey.

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Abstract

Actinomycetes and Fungi are frequently organized in small integrated societies (exemplified by colonies) in which a division of labor occurs. The division of labor has the evolutionary outcome of giving a selective advantage to the growing cells and finally to the fertile progeny. The fertile progeny has the task to preserve the species within the limits of natural evolution and colonization of new niches. A considerable part of the population assembled in a fungus or actinomycete colony (up to 80%) is fated to dye since it cannot embed its genome in spores. We have suggested that, in natural microbial isolates, a common characteristic is to segregate phenotypes in which the production of secondary metabolites and enzymes is maximized in the part of the colony which has lost the capacity to reproduce itself (the sterile caste), while reproduction is delegated to another part of the population (the non-sterile caste, generally represented by spores or other durable cells). Based on the above statements, we were digging in the sterile caste having as goal the identification, characterization and long-term preservation of industrially relevant lineages.

In mycelial and polynuclear microorganisms, as fungi and actinomycetes are, simple replication of colonies, or plating by dilution, did not easily allow the separation of genomes. Therefore, to correctly separate the lineages of interest, we have approached the selection of the sterile caste by use of three different techniques: morphological selection of those phenotypes having lost the spore-producing ability (study performed on fungi and actinomycetes), the selection of the sterile caste by use of selective agents (applied to fungi and actinomycetes) and the separation of genomes by massive screening of clones produced with the protoplast technique. More in detail, we used the antibiotic A40926 as selective agent for the identification of high producers of the A40926 glycopeptide (the natural precursor of the clinically relevant Dalbavancin antibiotic). The producer strain, *Nonomuraea gerenzanensis* ATCC 39727, displayed the presence of two distinct populations which were selected by the A40926 antibiotic. The G

population was able to produce twice the A40926 amount in respect of the P population. The G population gave an incomparable industrial advantage.

In the identification of high producers of Teicoplanin (the clinically relevant glycopeptide commercially known as Targocid) in *Actinoplanes teichomyceticus* ATCC 31121, we separated the genomes by use of the protoplast technology, and we performed a massive screening of the resulting clonal populations. In this case, we uncovered both high producers and producers of alternative Teicoplanin antibiotic complexes.

In the producer of the glycopeptide Avoparcin, *Amycolatopsis coloradensis* ATCC 53629**,** we were able to select high producers based on the identification of different non-spore producing colony morphologies and at the same time we were able to uncover biosynthetic dark matter hidden within the genome of the strain. Indeed, the selected morphological variants of this strain, were both able to produce Avoparcin up to a 9 g/L level and to produce an alternative Avoparcin complex with novel and up-to-date unidentified Avoparcin analogues.

Our studies evidenced that the incidence of mutants/variants for the specific traits analyzed (production of enzymes and secondary metabolites) was above any expected random mutation result. We therefore suggested that the sterile caste derived by division of labor in microorganisms should be the first source of improved strains and therefore the initial strategy to be followed in every strain improvement program. Our studies are currently applied to further strategic industrial projects to produce Avoparcin, Avermectin and Vancomycin.

Sommario

Gli attinomiceti ed i funghi sono spesso organizzati in piccole società integrate (esemplificate da colonie) caratterizzate da una divisione del lavoro. La divisione del lavoro ha il riscontro evolutivo di dare un vantaggio selettivo alle cellule in accrescimento ed in ultima analisi alla progenie fertile. La progenie fertile ha il compito di preservare la specie nei limiti dell'evoluzione naturale e della colonizzazione di nuove nicchie. Una parte considerevole della popolazione riunita in una colonia di funghi o di actinomiceti (fino all'80%) è destinata a soccombere poiché non può incorporare il proprio genoma nelle spore. Abbiamo suggerito che, partendo da isolati microbici naturali, una caratteristica comune di funghi e batteri filamentosi è quella di segregare fenotipi in cui la produzione di metaboliti secondari ed enzimi è massimizzata in una parte della colonia che ha perso la capacità di riprodursi (la casta sterile), mentre la riproduzione è delegata ad un'altra parte della popolazione (la casta non sterile, generalmente rappresentata da spore o altre cellule durevoli). Sulla base di quanto sopra, abbiamo rivolto i nostri sforzi alla casta sterile avendo come obiettivo l'identificazione, la caratterizzazione e la conservazione a lungo termine di popolazioni clonali industrialmente rilevanti. Tuttavia, nei microrganismi miceliari e polinucleati, come i funghi e gli attinomiceti, la semplice replicazione delle colonie o l'isolamento per diluizione, non consentono la efficace separazione dei genomi. Al fine di separare correttamente le popolazioni clonali d'interesse, ci siamo dedicati alla selezione della casta sterile mediante l'utilizzo di tre diverse tecniche: selezione morfologica di quei fenotipi che hanno perso la capacità di produrre spore (studio eseguito su funghi e attinomiceti e su *Bacillus subtilis*), la selezione della casta sterile mediante l'utilizzo di agenti selettivi (applicato a funghi ed attinomiceti) e la separazione dei genomi mediante screening massivo di cloni prodotti con la tecnica dei protoplasti.

Più in dettaglio, abbiamo utilizzato l'antibiotico A40926 come agente selettivo per l'identificazione di altoproduttori di A40926 (il precursore naturale del

glicopeptide clinicamente rilevante Dalbavancina). Il ceppo produttore, *Nonomuraea gerenzanensis* ATCC 39727, ha mostrato la presenza di due distinte popolazioni che sono state selezionate in presenza di concentrazioni di A40926 sub-inibenti. La popolazione identificata come G è stata in grado di produrre il doppio di A40926 rispetto alla popolazione P. La popolazione G ha quindi mostrato un incomparabile vantaggio produttivo sfruttabile industrialmente.

Nell'identificazione degli altoproduttori di Teicoplanina (il glicopeptide clinicamente rilevante commercialmente noto come Targocid) in *Actinoplanes teichomyceticus* ATCC 31121, sono stati separati differenti fenotipi, grazie alla separazione dei genomi compiuta con l'utilizzo di protoplasti. Uno screening massiccio delle popolazioni clonali risultanti ha permesso di identificare sia altoproduttori che produttori di differenti complex di Teicoplanina.

Nel produttore del glicopeptide Avoparcina, *Amycolatopsis coloradensis* ATCC 53629, siamo stati in grado di selezionare altoproduttori sulla base della identificazione di differenti morfologie incapaci di produrre spore. Contemporaneamente, siamo stati in grado di scoprire "materia oscura biosintetica" nascosta all'interno del genoma del ceppo. Le varianti morfologiche selezionate in questo ceppo sono infatti state in grado di produrre fino a 9 g/L di Avoparcina e di produrre degli analoghi dell'Avoparcina fino ad ora non identificati.

I nostri studi hanno evidenziato che l'incidenza di mutanti/varianti per i tratti specifici analizzati (produzione di enzimi e metaboliti secondari) era al di sopra di qualsiasi incidenza di mutazione casuale attesa. Abbiamo quindi suggerito che la divisione del lavoro nei microrganismi potrebbe essere la prima fonte di origine di ceppi migliorati e quindi la strategia iniziale da seguire in ogni programma di miglioramento dei ceppi. I nostri studi sono attualmente applicati a progetti industriali strategici per la produzione di Avoparcina, Avermectina e Vancomicina.

Introduction

Division of labor is a common evolutionary strategy that can be found at nearly every level of the biological organization, from the individuals of a society to the cells of a single multicellular organism. Despite the apparent biological simplicity and the ability to be self-standing organisms, even microbes have evolved a division of labor among colony members which turns out in the possibility to influence and modify their local environment at their own benefit. Beneficial collective actions performed by microorganism communities include the secretion of nutrient-degrading enzymes [1], iron-scavenging siderophores [2], biosurfactants for group motility [3], and structural components for biofilm formation [4,5], and the formation of spores [6]. In certain cases, subpopulations of cells specialize to perform different tasks such as producing antibiotics [7] or fixing nitrogen [8]. The prerequisites for the division of labor in microorganisms are the exhibition of different phenotypes (task allocation), the cooperation between phenotypes, and a fitness benefit for the species deriving from the interaction [9]. Indeed, differentiated colonies have higher fitness than those lacking a differentiation. This benefit results from the efficiency of dividing tasks between cells rather than having a single cell either switching between these tasks or carrying them out simultaneously [9–13]. The division of labor occurs at the phenotypic level, at the genotypic level or at both, being determined respectively by differences in gene expression resulting in tasks allocation, differences in the genotype determining the specialist task, or a combination of the two [14] (Figure 1).

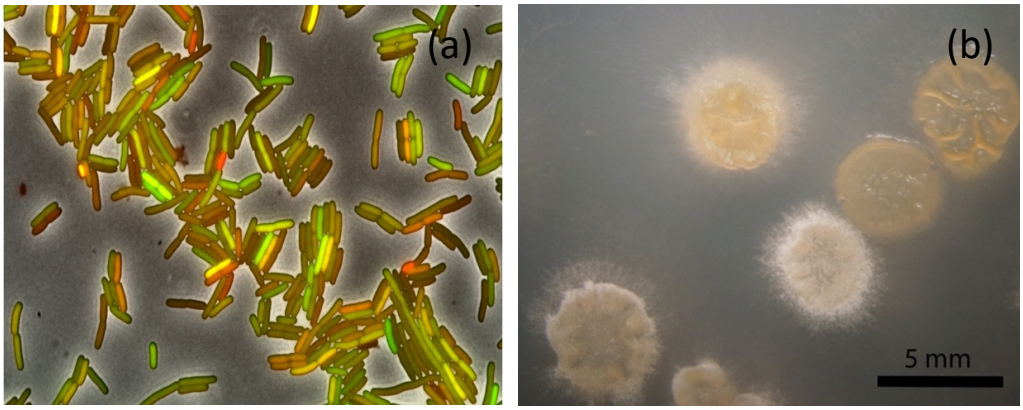

Figure 1. Division of labor can be deermined by transient differential gene expression (adapted from [15]) (a) or arise from the rearrangement of genomes which determines stable phenotypes (b) [16].

From the evolutionary point of view when phenotypic cell-to-cell differences are heritable, bacterial lineages are formed. The new bacterial lineages became the key to anticipate future challenges such as the colonization of new ecological niches, being those niches a new substrate to degrade, a new harsh environmental condition or an industrial bioreactor. In this work we have analyzed the benefits of the division of labor in view of its application to the industrial production of metabolites and enzymes by filamentous fungi and bacteria.

Filamentous microorganisms as a model of division of labor

Microbes that display obligate patterned multicellularity, offer the most dramatic examples of divisions of labor. This is because these groups are characterized by terminal differentiation into reproductive and non-reproductive cells that mimic the divisions between germ and soma in plants or animals [17–20]. Members of the order actinomycetales and of the fungi kingdom share (at least in some part of their life) a common evolutionary morphology which is characterized by elongated multi-genomic structures commonly known as hyphae or mycelium. The morphology of these microorganisms is commonly defined as “filamentous”. Opposite to the single-cell microorganisms, the morphology of

actinomycetes and fungi allows a continuous communication within the hyphae determining a complex organization of the growing colonies. In particular, expanding colony 'arms' explore and modify new territories and return messages to the other members of the colony largely anticipating future challenges. Typically, filamentous microorganism colonies arise following the germination of a single spore which gives rise to a multi-genomic vegetative mycelium [21,22]. These multicellular organisms forage on complex organic materials that are converted into small molecules using secreted proteases, cellulases, and chitinases [21,23] and at the same time secrete metabolites which can confer competitive advantage to the growing colonies and to the future of the species (i.e., antimicrobial compounds). Upon nutrient depletion behind an actively growing colony front, a developmental program is initiated allowing these microorganisms to escape harsh environmental conditions [21,23,24] through the formation of aerial hyphae that differentiate into spores. The energetic burden associated with the formation of spores is supported by the partial degradation of the vegetative mycelium [25,26]. The vegetative and reproductive growth phases in filamentous fungi and actinomycetes represent an outstanding example of division of labor. Indeed, the vegetative hyphae are programmed to "feed and protect", while the reproductive hyphae lead to stable spores that can persist through starvation and potentially migrate to colonize new niches [21,22] (Figure 2). Spore formation and concomitant secondary metabolite production is metabolically costly, and it was evidenced that production and secretion of secondary metabolites by only a fraction of the hyphae occurs [7]. This mechanism offers resource savings providing benefits to the entire colony [7]. Concomitantly, the secondary metabolite non-producing hyphae could continue foraging while transporting nutrients to other parts of the colony. In the actinomycete *Streptomyces coelicolor,* the division of labor involved extensive

genome modifications, which was proved by whole genome sequencing and by PFGE [18,24]. The analysis of the different phenotypes originating by the process of division of labor, evidenced that genome modifications conferred significantly reduced fitness, thus suggesting that these deletion-derived phenotypes behave like a "sterile caste" that provide direct benefits to the rest of the colony (the non-sterile caste) and receive little in return [7]. Indeed, the sterile caste was not represented in spores and was eventually reestablished independently and differently in the subsequent growth cycles. It was suggested that this mechanism may help to maximize the diversity and amount of secreted secondary metabolites, in particular antibiotics.

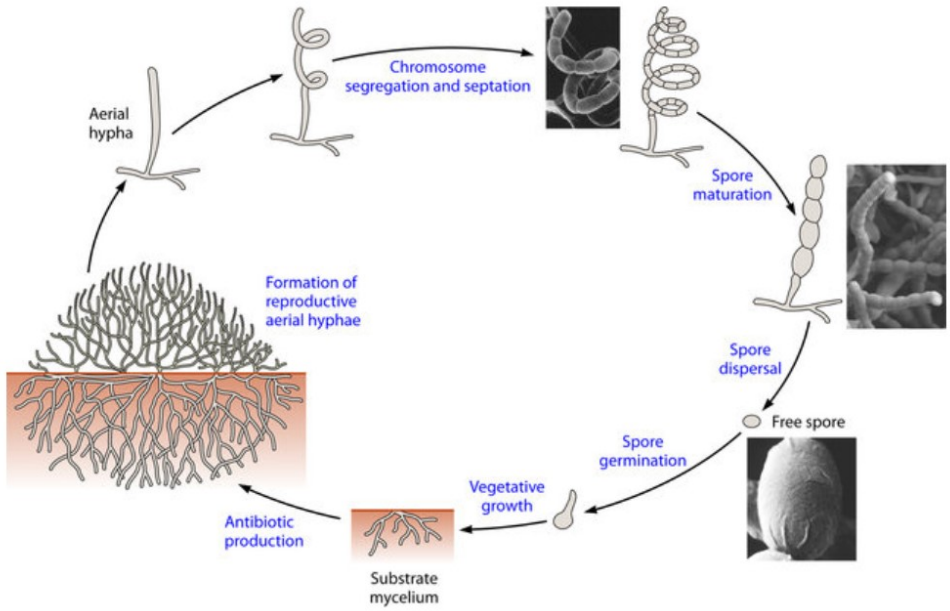

Figure 2. Schematic representation of the typical actinomycete life cycle. Division of labor occurs mainly between the substrate mycelium and the reproductive aerial hyphae (image adapted from [24]).

A broadened spectrum of produced antibiotics could be an evolutionary advantage as competing bacteria are unlikely to be resistant to all of them. It is

also interesting to note that many antibiotics are secreted, so the entire colony, but not susceptible competitors, can benefit from the protection they provide [27]. Thus, in addition to the canonical divisions of labor between, e.g., reproductive and vegetative tasks, we may also anticipate divisions of labor to evolve to reduce the costly production of secondary metabolites and/or secreted enzymes, ideas we consider in the context of actinomycetes and fungi.

In the interest of space, we have only briefly discussed the advantages of a division of labor in actinomycetes and fungi. Readers can refer to the numerous reviews on the topic in multicellular organisms or microbes [9,11,28].

The division of labor in filamentous microorganisms. Practical outcomes

In the model actinomycete *Streptomyces coelicolor*, it was reported that the metabolically costly antibiotic production and secretion, trades-off with growth, and is performed by only a fraction of the hyphae (the "sterile caste") [29,30]. This labor-division-determined resource savings is beneficial to the entire colony by ensuring the spread of the species even if the 60–80% of cells (the sterile caste) are fated to die [29]. This division of labor in *S. coelicolor* was shown to involve both the expression of specific genes and the rearrangement of the whole genome [29], the latter originating potentially stable laboratory lineages.

General remarks on strain improvement

Strain improvement can be defined as the science and technology of designing, manipulating and improving the performance of microbial strains for biotechnological applications. The understanding of the microbial biochemistry and physiology, coupled with advances in fermentation technologies and genetic engineering are the pillars for strain improvement.

The economy of industrial fermentation is mainly determined by the cost of production per unit of product produced and by the cost associated with the construction and management of the production plant. While production and capital costs related to fermenter engineering design can be predicted and are rather fixed along the whole process, the bare fermentation costs can be reduced by acting on fermentation media, fermentation process and strain. Increasing the productivity of the producer microorganisms (strain improvement) to obtain a greater amount of product in less time using the same quantity of raw materials, can lead to a significant reduction in production costs [31] (Figure 3).

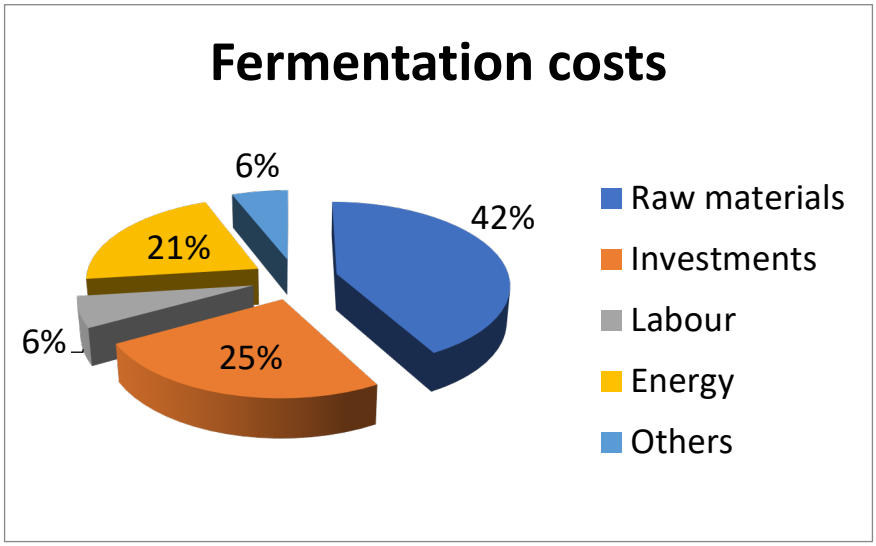

Figure 3. Distribution of costs for microbial based fermentations. Strain improvement can indirectly reduce raw material costs by increasing the product yield per unit of raw material and can directly reduce energy costs by reducing fermentation downtime.

The "traditional" strain improvement approaches (random mutagenesis and selection) still represent a valid and successful industrial yield increase strategy and are rationally combined with media and fermentation process optimization (Figure 4). However, being the above a random approach, positive mutations are

often surrounded by unknown and uncontrolled genomic backgrounds. An alternative and integrated approach for the improvement of filamentous microorganisms for industrial purposes is the manipulation and fusion of protoplasts, (alternative forms of bacterial or fungal cells in which the cell wall has been partially or completely removed) accompanied by direct genetic manipulation whenever strategies for the specific microorganisms have been developed (Figure 4).

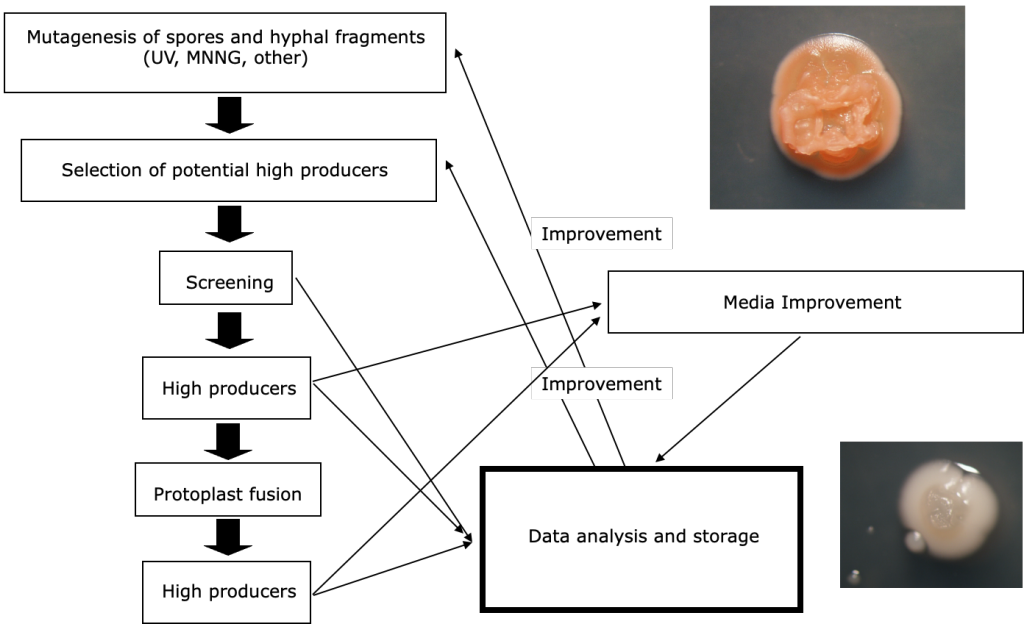

Figure 4. Schematic representation of the strain improvement process integrated with media and fermentation development. Pictures represent the wild type A40926 *Nonomuraea gerenzanensis* ATCC 39727 strain (upper right) and a typical strain improved for A40926 production (lower right).

To obtain viable protoplasts, it is necessary to promote the digestion of the cell wall using lytic enzymes: by carrying out digestion in the presence of an isotonic buffer, it is possible to obtain viable cells without cell wall. The outer layer of the protoplasts is therefore the cytoplasmic membrane.

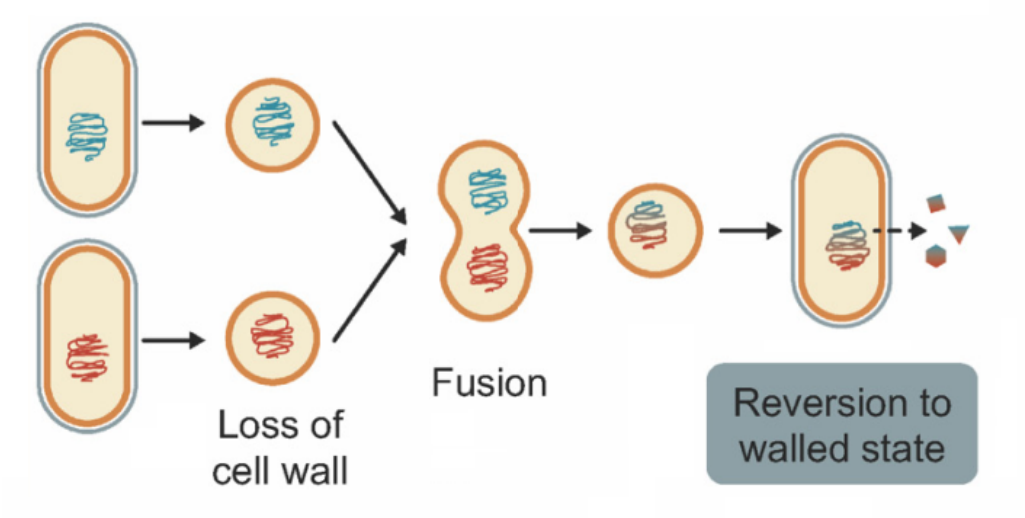

Figure 5. Summary diagram of the main steps in generation and fusion of protoplasts (adapted from [32]).

The mechanical resistance of the membrane is insufficient to preserve the integrity of the protoplast unless the lysis is performed in the presence of osmotic stabilizers. Protoplasts can then be reverted to the mycelium form whenever required [33]. Protoplast preparation was mainly used to introduce exogenous DNA into bacterial cells [34]. In addition to transformation with plasmid DNA, protoplasts can also be used in genetic recombination techniques to obtain improved strains. Furthermore, protoplast fusion is a versatile technique that combines the entire genetic material (including the positive mutations carried by each lineage) of two or more cells to generate recombinants that cannot be obtained through a single mutation event. This approach, known as genome

shuffling, can be extremely useful for accelerating strain improvement [32,35] (Figure 5).

Besides being a strategy for genetic transformation and genome recombination, protoplast formation and regeneration offers the possibility to produce uni-genomic cells from filamentous microorganisms (eventually unable to produce spores, like the above-described sterile caste). Therefore, protoplasts can be used to generate clonal population from genetically heterogenous substrate mycelia potentially un-digging recessive industrially relevant genealogies (Figure 6).

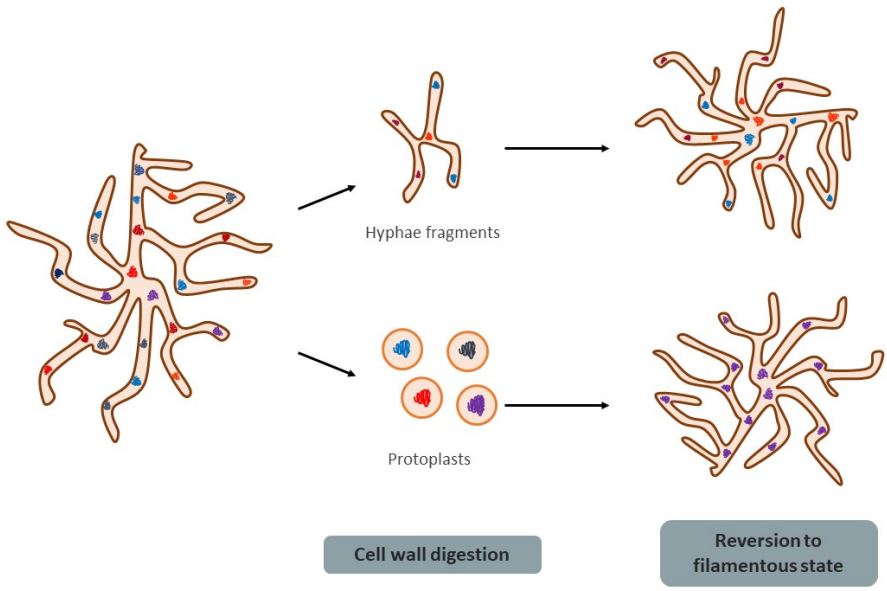

Figure 6. Schematic representation of genome separation by hyphal fragmentation and by protoplast generation and reversion to the hyphal morphology.

General remarks on strain maintenance

If the strain improvement strategies allow the isolation of microorganisms with optimized characteristics for industrial production, the obtained strains with their valuable characteristics must be properly maintained through a process (or a series of microbiological techniques or processes) known as “Strain Maintenance”. Strain maintenance processes are designed to preserve strains so

that they remain viable and stable over time. Despite freezing and lyophilizing for long-term storage [36], continuous plating and fermenting of the microbial strain increases the probability of back-mutations that can lead to loss of the positive industrial characteristics, at least in those cases in which selective pressure (such as growth in the presence of antibiotics specifically selecting for the mutations of interest) cannot be applied. Furthermore, in the case of polynucleated microorganisms (in which clonal populations are not easy to obtain), such as actinomycetes and filamentous fungi, the propagation of the producer strain can easily determine the selection of fast-growing phenotypes within the population which could potentially overwhelm the industrial producer. The strain maintenance processes, which involve the isolation and maintenance of the producer microorganism, are therefore extremely important for this type of bacteria and rely on the ability to correctly separate the different genomes to avoid arising of mixed populations. The preparation of protoplasts from non-spore producing actinomycetes and fungi, is an excellent method for the separation of the single genomes and provides a valid alternative to spores (usually the only single-genome cells in actinomycetes).

Goals of this study

The divisions of labor in filamentous microorganisms have the evolutionary outcome of giving a selective advantage to the growing colony and finally to the fertile progeny. The fertile progeny has the task to preserve the species within the limits of natural evolution and colonization of new niches. A considerable part of the population assembled in a fungus or actinomycete colony (up to 80% as suggested in [7]) is fated to dye since it cannot embed its genome in spores. This part of the population (the sterile caste) is however a rich reservoir of high producers of secondary metabolites (and even of new and yet undiscovered

metabolites), can be theoretically immortalized in laboratories, and can be the starting material for industrial production thanks to its high productivity and novelty of products. The ability to select, preserve and ferment different lineages from the sterile caste in actinomycetes and fungi could offer an advantage in maximizing strain improvement and strain maintenance processes. However, since actinomycetes are mycelial and polynuclear microorganisms, simple replication of colonies, or plating by dilution, do not easily allow the separation of genomes.

In this study, we have analyzed the possibility of taking advantage of the division of labor in actinomycetes for the industrial production of valuable metabolites by introducing the sterile caste in routine programs of strain improvement and strain maintenance (Figure 7). For this purpose, we have approached the selection of the sterile caste by use of three different techniques: morphological selection of those phenotypes having lost the spore-producing ability (study performed on fungi and actinomycetes with some consideration also for one *Bacillus subtilis* strain), the selection of the sterile caste by use of selective agents (applied to fungi and actinomycetes) and the separation of genomes by massive screening of clones produced with the protoplast technique. Our analysis evidenced that the incidence of mutants/variants for the specific traits analyzed (enzymes and secondary metabolites) was above any expected random mutation result. In this study, we suggest that the division of labor in microorganisms should be the first source of improved strains and therefore the initial strategy to be followed in every strain improvement program. Furthermore, hunting for the sterile caste, could result in the production of new and unexpected metabolites as it will be described below.

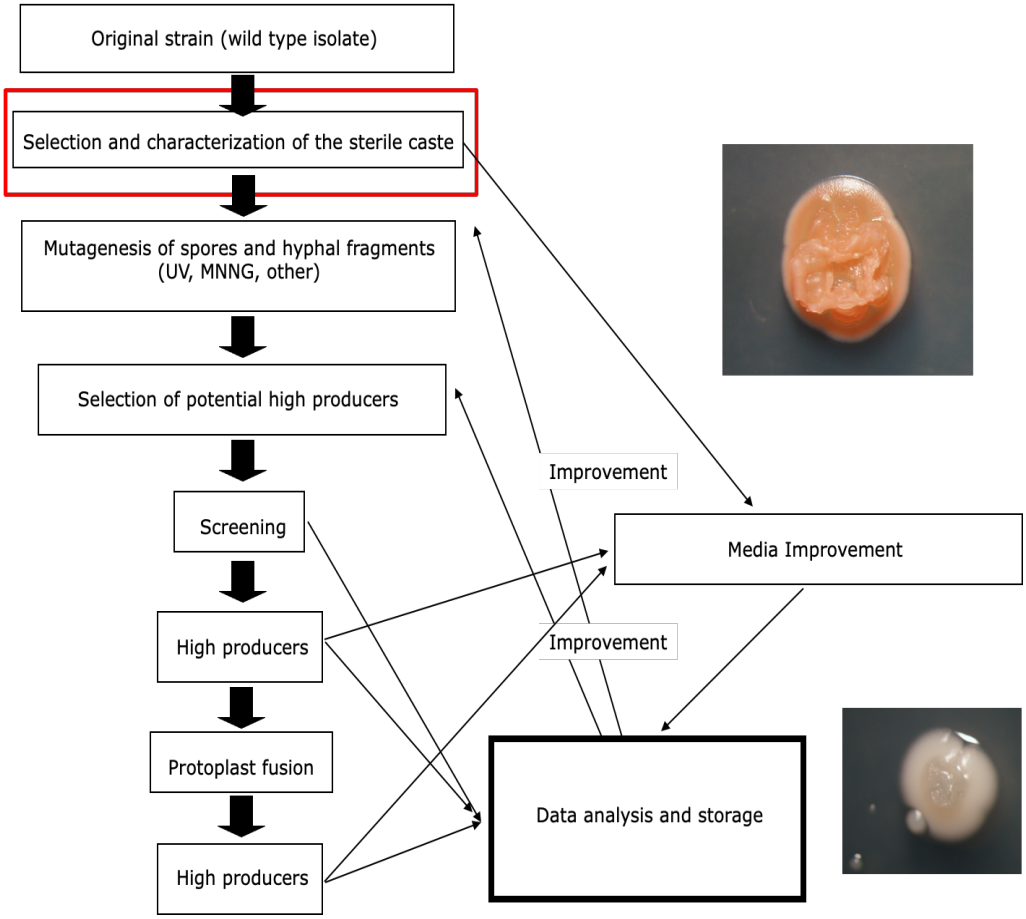

Figure 7. Schematic representation of the strain improvement process integrated with media and fermentation development. The step of isolation and identification of the "sterile caste" proposed in this study is evidenced in red. Pictures represent the wild type A40926 *Nonomuraea gerenzanensis* ATCC 39727 strain (upper right) and a typical strain improved for A40926 production (lower right).

Disclaimer on the "Sterile caste" definition

In the above discussion we have defined as the sterile caste that part of the population which is unable to produce durable forms (spores or similar). However, we must point out that in some of the case studies we were unable to unequivocally prove that the progeny was unable to produce spores. Therefore,

for sterile caste we intend progeny that strictly derives from mycelium or vegetative cells produced in the cultivation conditions applied.

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Results and discussion

This study was performed on different microorganisms and projects of strategical interest for the companies Actygea and BioC-CheM Solutions. Fungi and bacteria were analyzed for their peculiarities in the division of labor. Some of the studies were abandoned for lack of funding or limited interest of the experimental outcomes, and others were supported by private companies at a certain point of their development. The support of the projects by private companies, determined their exit from further communicable development. A short description of the studies which are out of an in-depth disclosure is also reported. Most of the fruitful efforts which are reported in this work were dedicated to the division of labor in actinomycetes.

Chapter 1.

The production of biosurfactants by fungi and bacteria. Division of labor as a strategy to explore new environmental niches
(confidential project sold to a big player of the biosurfactant sector)

The microbial world is a network of interdependencies between species [1] which involves both fungi and bacteria and the division of labor has recently been also documented between genetically different strains or species [2,3]. Given the high promiscuity between cells, even if belonging to different species, specialization is likely beneficial for the group as a whole [4], with individuals gaining an inclusive fitness benefit from helping their surrounding mates [5,6].
In one of our studies, we were able to isolate, from a polluted soil, fungi and bacteria which produced biosurfactants. In the soil environment, cells phenotypically differentiated into surfactant producers and matrix producers where the role of the former cell type is to reduce surface tension, while the latter allowed presumably expanding colony 'arms' to form and explore new territories [7]. Divisions of labor in a society or a single organism require the coexistence of multiple types, or subpopulations, that interact and are specialized to carry out complementary tasks [8]. In microbial colonies, each sub-type can be derived from a single parental cell in response to environmental change (e.g., starvation) via deterministic or stochastic processes [9]. Our novel isolate *Bacillus subtilis* LB/8 strain was able to produce a potentially new surfactin-like biosurfactant (named BAX) and (even if it was not a filamentous microorganism) was a good example of occurrence of a stochastic differentiation of phenotypes due to a change in colour of the colonies. In *Bacillus subtilis* LB/8, several morphological variants were indeed isolated and have shown different abilities (qualitative and quantitative) to produce biosurfactants. Mixed colonies displaying clearly distinguishable pink and white sections (Figure 1) gave rise to the hypothesis of

the presence of a recurrent mutation capable of reverting to the original phenotype (visible from the color of the colony reverting to white and vice-versa). These colonies have been isolated and will be evaluated for future industrial development to obtain a stable phenotype. Indeed, this reversion of phenotypes strongly influenced BAX productivity.

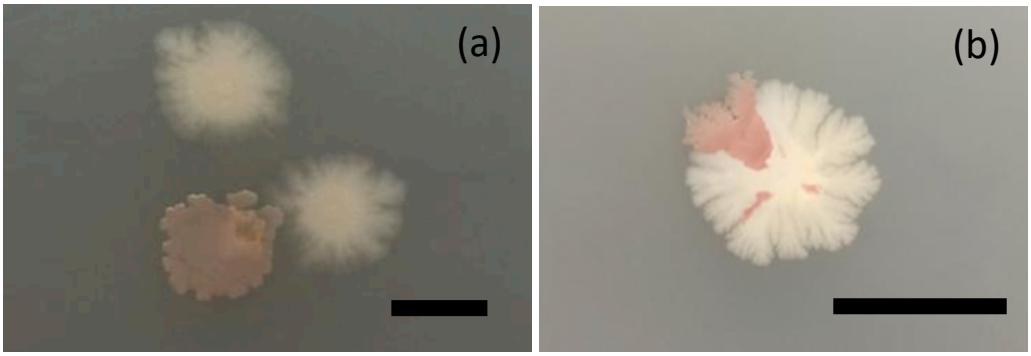

Figure 1. Segregation of different morphologies in *B. subtilis* produce*r* of the BAX biosurfactant (a), and mixed colony evidencing the reversion from the pink to the white phenotype (b). Bars: 5mm

In parallel, the BAX biosurfactant produced was analyzed by HPLC and TLC. The BAX biosurfactant was monitored by HPLC analysis and was present as an extracellular product. For the analysis, the common analytical method for lipopeptide biosurfactants was used and from the chromatographic profile it was observed that the BAX biosurfactant appears as a complex consisting of six different components, one of which is predominant. The chromatographic profile was partially overlapping with that of the commercial surfactin (Sigma-Aldrich), but with novel features still to be evaluated. At present, it was not possible to carry out a quantitative analysis and to calculate the precise concentration of the biosurfactant produced by *B. subtilis* LB /8. From the colors of the TLC analyses, performed with the use of different reagents such as ninhydrin and Ehrlich's reagent, the compound was indicatively classified within the class of

lipopeptides. It is also worth to note that some of the colonies produced the typical surfactin (with a profile fully overlapping with the Sigma-Aldrich standard). This supported our idea of a division of labor in which stochastically originated clones producing alternative surfactins originated in the population.
An extraction protocol based on mixed ethanol and salting out technique was developed. Following the addition of ammonium sulphate in a water ethanol solution containing the biosurfactant, precipitation of the compound did not occur, but the water and ethanol separated in two distinct phases in which the ethanolic phase contained BAX. The increase in salt concentration has in fact favored the separation of the solvent phase from the aqueous one (which can be mixed in the absence of salt), with the consequent recovery of the product in the ethanolic phase with a yield of 77%. The raw extract obtained had an oily caramel-colored appearance that will require further purification process that will allow us to obtain a product suitable for more reliable analyses. NMR analyses will in fact be necessary to verify the actual novelty of the isolated compound. In its physical appearance, BAX was very different from surfactin (which appeared as a white solid after purification), indicating that some evolutive advantage could be associated to the production of this new biosurfactant.

In the same polluted soil, a strain of *Acremonium persicinum* was identified as the producer of an unknown biosurfactant. This strain segregated different colony morphologies of which one was unable to produce spores and able to produce considerable amounts of biosurfactants (Figure 2). These morphological variants showed different characteristics of growth, consumption of carbon sources and production of biosurfactants. Based on the above data, it was possible to select the non-spore producer morphological variant as the best for industrial production. The inability to produce spores, supported the hypothesis

that also in fungi a sterile caste can be isolated for the efficient production of metabolites of relevance for the survival of the species. The biosurfactant was detected by TLC analysis, and development with different reagents indicated it to belong to the lipopeptides class. HPLC showed the generation of a majority peak which allowed a relative quantification of production. The chemical characteristics of the product were further investigated by LC-MS: the chromatogram obtained showed the presence of a main peak corresponding to a compound of mass 724 m/z. Further analyses, such as H^{1}-NMR and C^{13}-NMR, will be necessary to verify the novelty of the isolated product.

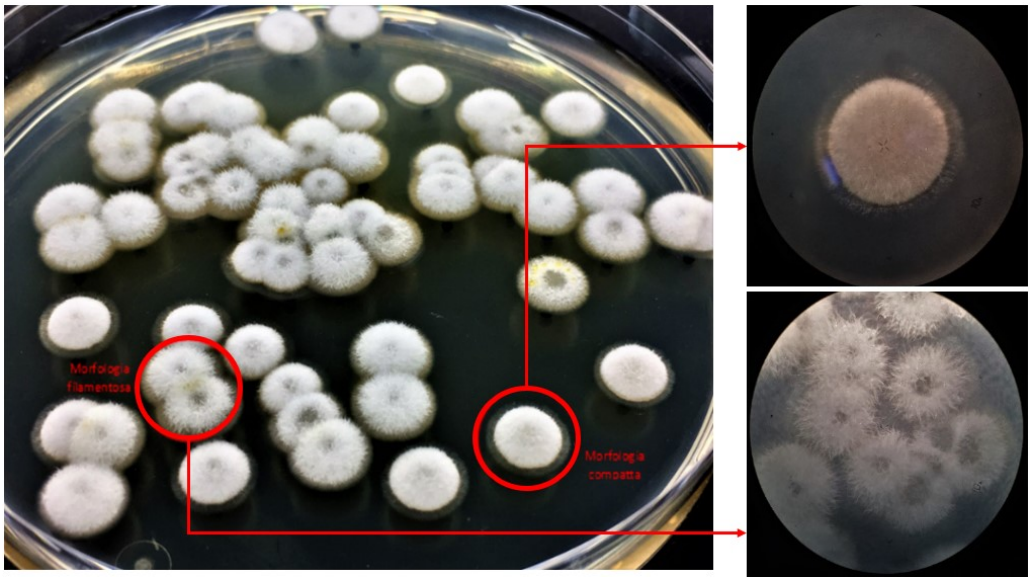

Figure 2. Typical morphologies of *A. persicinum* colonies. The non-spore forming colony (up right) is the producer of the biosurfactant.

The functional characterization of the product allowed to highlight a good surfactant activity, with a decrease of the surface tension of 38 mN/m, a fair emulsifying activity in *n*-hexadecane (EI24 62%) and an antimicrobial activity against gram positive bacteria. However, the product was not active against fungi and yeasts. The biosurfactant has shown interesting characteristics from the point

of view of applicability to cosmetic, pharmaceutical and food products (good lowering of surface tension, good emulsification index, possibility of purification with solvents compatible with cosmetic applications).

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Chapter 2.

Coriolopsis gallica MUT 3329 and the production of laccases isoforms. Preliminary studies of division of labor

(Abandoned due to lack of funding)

The accumulation of wood in anoxic environments started to be limited at the end of the Carboniferous period. The duplication and diversification of the lignin and cellulose acting enzymes was indeed taking place at that time in fungi and was the key to a rapid increase in their degrading ability. The diversification of wood degrading activity was finally able to influence substantially the history of Earth [1]. From that time on, fungi have acquired an enormous potential of modification and degradation of the vegetable biomass structure (Figure 1) and have insured CO_2 cycle.

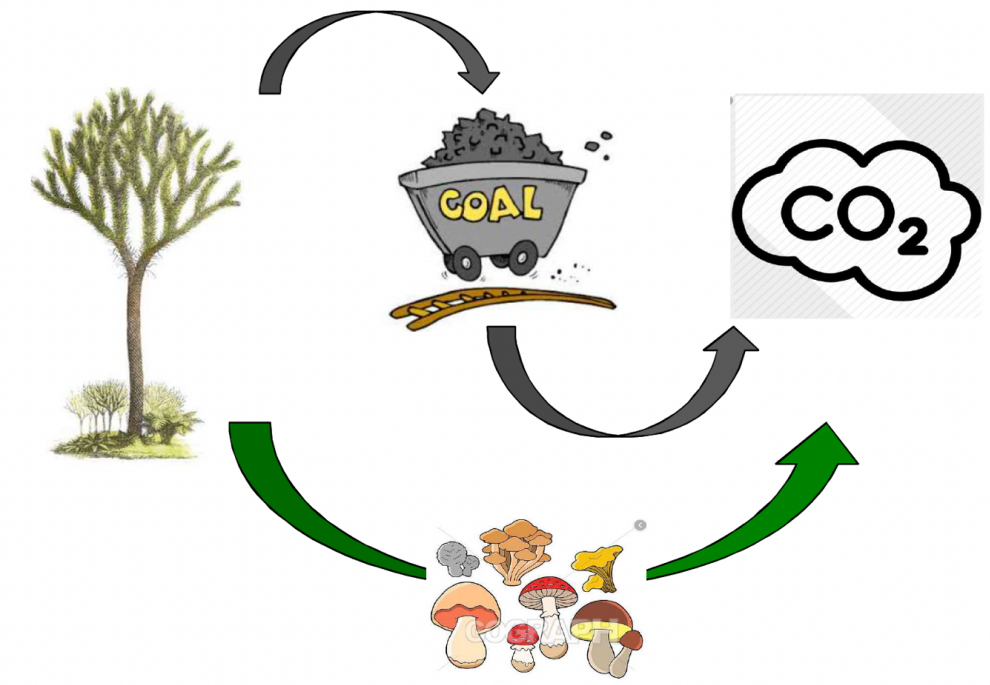

Figure 1. The accumulation of fossil carbon was limited by wood degrading fungi. Wood degrading fungi shortened the cycle of carbon on earth and changed radically the environment.

Wood is the main depository of photosynthetically fixed carbon on earth [2], and represents a plentiful, renewable supply of organic carbon. Wood is therefore particularly attractive as a feedstock for different industrial uses, especially because the depletion of fossil fuels urges for the identification of novel sources of organic carbon. The degradation of wood is a complex spatial and temporal process, which in nature involves the interaction with the wood substrate and with the different microorganisms participating in the revolving of carbon to the atmosphere [3]. White rot fungi are the most effective in the degradation of wood [4], thanks to their oxidative enzymes. White rot fungi can produce a complex of lignin degrading enzymes (enzyme cocktails, mainly isoforms of laccases and peroxidases) and are the key mechanism of CO_2 recycling in the biosphere. These enzymes derive from the expression of individual genes, alternative splicing, or both [5]. However, complex regulatory mechanisms which direct the synthesis of wood degrading enzyme cocktails have not been completely elucidated.

The general objective of this project was to investigate the gaps in the expression of suitable combination of enzymes (enzyme cocktails) for the degradation of different kinds of wood. The specific objective of the project were:

- identification of new enzymes/enzyme cocktails that could improve the efficacy of lignocellulosic bio-chemical processing on a wood-kind specific way;
- Understanding which of the enzymes are more useful for a specific wood application;
- Producing in liquid culture the enzymes for the specific applications.

The work herein reported, suggests that is in principle feasible to identify specific cocktails of enzymes from the same fungal strain. In consideration of the general purpose of this dissertation, we also propose that, in wood degrading fungi, a

mechanism of rearrangement of the enzyme set could also be determined by the reorganization of genomes as already reported for actinomycetes [6].

Materials and methods

Biotechnologically speaking, oxidative enzymes (mainly laccases and peroxidases) from fungi are structurally simple, are the main component of the fungal secretome, and their activity can be quantified with easy to perform spectrophotometric assays. Furthermore, oxidative enzymes are easily detected on native PAGE by colorimetric assays. Many of these enzymes are expressed at high levels in rather standard cultivation conditions, which makes relatively easy to purify them. The fungal strains from the Mycotheca Universitatis Taurinensis *Coriolopsis gallica* MUT 3329 (Figure 2) was used in this study.

Figure 2. *C. gallica* fruiting bodies (https://www.biolib.cz)

The expression of the fungal secretomes was performed on sterile milled wood inoculated with the fungus of interest and amended with the suitable amount of water. After visible growth, the milled wood was diluted in water and the soluble proteins were quantified using the Pierce® Thermo Scientific's BCA Protein Assay Reagent Kit, (Rockford, Illinois, USA). The laccase activity in the supernatants of the cultures was determined spectrophotometrically using the 2,2'-azino-bis (3-ethylbenzothiazolin-6-sulfonic) acid (ABTS) as substrate as suggested by Li *et al.* [7].

The SDS-PAGE analysis was carried out with the method described by Laemmli [8] using a 3% pH 6.9 stacking gel and a 12% pH 8.9 separative gel. The running buffer used was Tris-Glycine-SDS at pH 8.3. The proteins were visualized on the gel by staining with Comassie Blue.

Protein electrophoresis in native conditions (zymogram assay) was carried out using the same conditions described above, depriving all reagents of SDS. In this case, the gels were treated with 2 mM guaiacol in sodium acetate buffer at pH 5, to visualize native laccases. Alternatively, the gels were stained with Coomassie Blue [9].

Results

We have grown *C. gallica* MT 3329 on different kinds of milled wood with the aim of identifying different oxidative secretomes. Only laccase activity was tested to date. Data show that the laccase activity differs on the different kinds of wood (Table 1). As shown in Figure 3, in most of the wood kinds, the secreted laccase activities mapped in the same position on the zymograms, suggesting that a common lignin degradative pathway is used in most cases. However, when *C. gallica* MUT 3329 colonizes Oak (lane 2A) and Wine tree (lane 7A) wood, there is the predominance of an alternative laccase activity. On Palm tree (lane 2B),

both signals show a comparable level of activity and other signals are visible. In conclusion, growth on different woods stimulates the expression of different secretomes. It was reasonable to think that the different secretomes arise in response to the different stimuli received from the different kinds of wood. The quantitative/qualitative difference in the cross-comparison were the proof of concept of the approach.

Since growth of *C. gallica* MUT 3329 occurs in myceliar form, it is possible that the different secretomes are the result of differential induction response but also of the selection of different genotypes operated by the different wood types, or, eventually, by rearrangement of the genomes.

Table 1. Laccase activity normalized for gram of milled wood

Number	Wood Type	Laccase activity U/ml (normalized)
1A	Chestnut	0.5
2A	Oak	2.4
3A	Larch	1.5
4A	Red Larch	2.5
5A	Ash tree	10.8
6A	Cherry tree	2.5
7A	Wine tree	5.0
8A	Control	60
1B	Spruce	2.0
2B	Palm tree	24.6
3B	Lime	4.1
4B	Laurel	2.1
5B	Boxwood	3.6
6B	Ailanthus	1.4
10B	Control	60

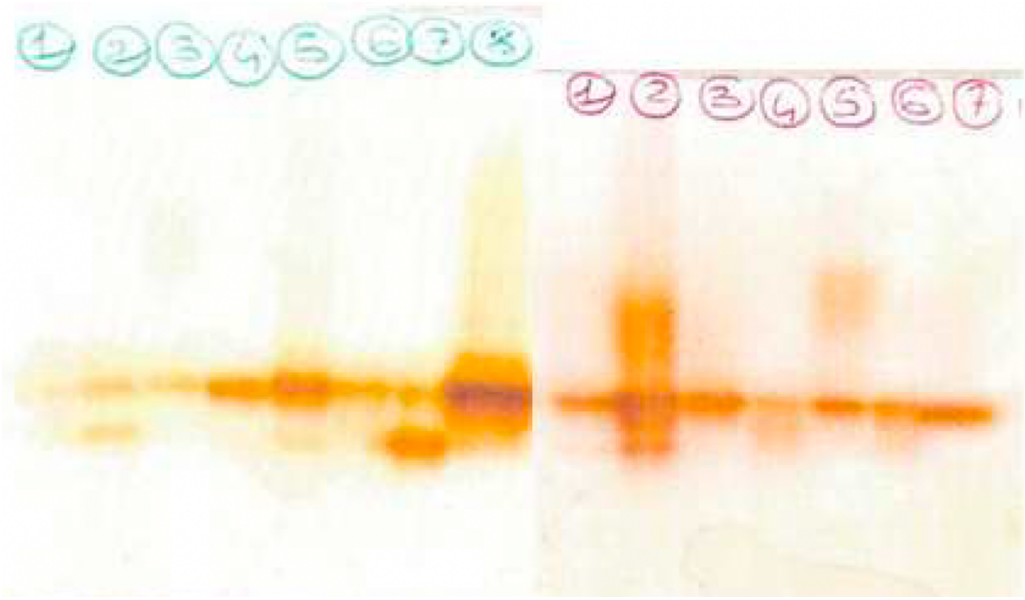

Figure 3. Zymograms of the secretomes of *C. gallica* MUT 3329 grown on different milled woods. Samples as in Table 1 (A, left gel, B, right gel).

Discussion

C. gallica MUT 3329 can produce a broad range of oxidative enzymes. These enzymes can be produced and/or modulated in response to the different wood substrates that the fungi must degrade. The nutritional adaptation could derive from differential gene expression or from the nutrient induced selection of adapted mutants, or by the presence of a "sterile caste". From the industrial point of view, standard cocktails of enzymes are available for lignin treatment [10]. However, the low activity of those cocktails of enzymes limits the results during application, and chemico-physical treatments are still preferred [11]. Furthermore, the possibility to produce a specific oxidizing enzyme at levels of economic interest for low-income industrial applications, is far to come. The possibility to have the correct cocktail of enzymes for each specific biomass at a reasonable price remains a gap to be filled.

In our approach, we tried to identify the specific production of laccase enzymes in response to different wood substrates by growing on different milled woods

(solid state) and analyzing by PAGE and SDS-PAGE the laccase production. Particular attention was dedicated to those milled wood types, which allowed the quantitative production of distinct oxidative enzymes as these could be the selective condition for the identification of stable lineages producing a single laccase isotype (in case of stable mutations) or the starting point for selecting fermentation substrates for the production and characterization of the specific laccase isotype (in case of differential gene expression). This could be the launching pad to produce industrially relevant oxidative enzymes for low-income applications.

Conclusions

We have evidenced that the production of specific laccases isoforms could have some meaning in adaptation to different nutritional conditions which the fungus *C. gallica* MUT 3329 could encounter in the environment. Therefore, it is theoretically feasible to design and develop a system for the industrial production of enzymes/secretomes which best suit the specific application to a biomass/wood/lignin degradation or modification. To date we have identified at least two secretomes which are expressed only on a specific wood type. This is the proof of concept that it is possible to produce secretomes which suit a specific wood type. The protocol is in principle applicable to any kind of fungus for the identification of optimal cocktails. The question if this differential expression is due to specific genetic lineages remains opened for future studies.

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Chapter 3

Nonomuraea genzanensis ATCC 39727 and the identification of improved progeny by use of selective agents

Nonomuraea is a genus of "rare" or "uncommon" filamentous actinomycetes (to distinguish them from the most easy-to-isolate and -to-cultivate *Streptomyces* spp.), whose capability to produce specialized (secondary) metabolites is poorly explored [1–3]. *Nonomuraea gerenzanensis* ATCC 39727 is a filamentous actinomycete producing the glycopeptide antibiotic (GPA) A40926 [4], which is the natural precursor of the semi-synthetic derivative dalbavancin [5] (Figure 1). Dalbavancin is a second-generation GPA, which is in clinical practice to treat severe infections caused by multidrug-resistant Gram-positive pathogens [6]. Dalbavancin is today marketed in Europe and USA under the trade names Xydalba and Dalvance respectively, and it has been the first antibiotic designated as a Qualified Infection Diseases Product by FDA because of its potency, extended dosing interval, and unique dose regimen (once-a-week).

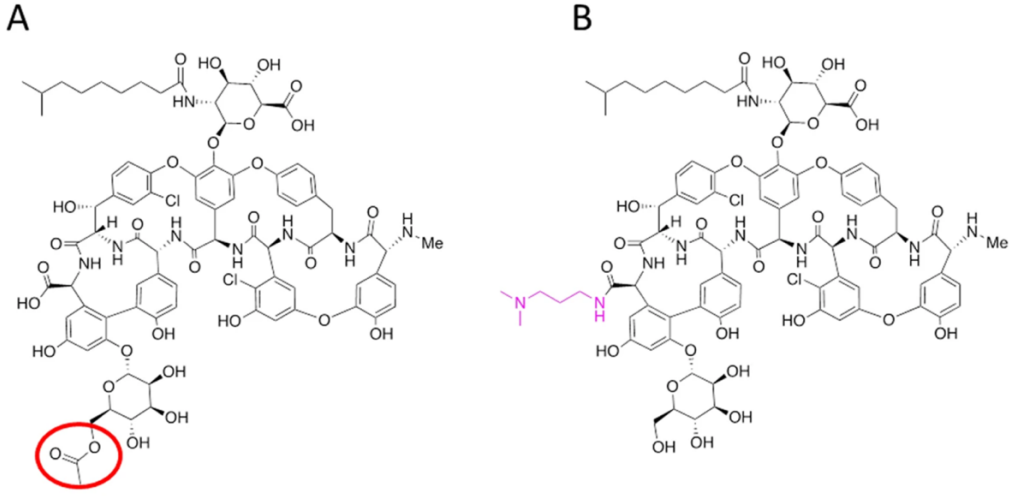

Figure 1. Chemical structure of the A40926 molecule (A) and dalbavancin (B). The red circle evidenced the acetyl group which is removed during purification of the molecule (see text). The violet substituent is the syntetic part of the dalbavancin molecule (images adapted from [7]).

Although many efforts have been devoted in the last two decades to the improvement of A40926 production process [8], its cost still largely exceeds that of first-generation GPAs, i.e., vancomycin and teicoplanin. Therefore, following the sequencing of the A40926 biosynthetic gene cluster (BGC), named *dbv*, in 2003 [9], multiple aspects of A40926 biosynthesis and its regulation were investigated with the aim to improve its production [10–12]. It was recently clarified that *N. gerenzanensis* ATCC 39727 produces the GPA in the form of O-acetyl-A40926 (with an O-acetylated mannose residue) (Figure 1), but the acetyl group is lost during the alkaline extraction of the antibiotic [7,13]. Since it was this deacetylated GPA that was initially named A40926, we will continue to refer to it as A40926 hereafter.

Self-resistance in the producer strain appeared co-regulated with A40926 antibiotic production and it increased during the antibiotic biosynthesis [14,15]. Self-resistance was induced by the A40926 (the available antibiotic) but apparently repressed by its acetyl-form (the real end product of the biosynthesis), although through a regulatory circuit that it is still not completely understood [15,16].

In this study, we focused our attention on *N. gerenzanensis* ATCC 39727 wild type population which interestingly shows a heterogeneous self-resistance profile to the antibiotic A40926. Exposing *N. gerenzanensis* ATCC 39727 to sub-inhibitory concentrations of A40926 led to the isolation of differently resistant phenotypes to which a diverse A40926 productivity was associated, paving the way for a rational strain improvement based on the selection of increasingly resistant colonies.

Materials and Methods

Strains and cultivation conditions

Nonomuraea gerenzanensis ATCC 39727 was maintained as a lyophilized master cell bank (MCB). A working cell bank (WCB) was prepared from a first-generation slant originating from the MCB as previously described [17,18]. 1.5 mL cryo-vials were stored at –80 °C for up to six months without influence on the A40926 production during fermentation. Mycelium for plating and for the isolation of colonies was prepared as follows. Cryo-vials of the WCB were thawed at room temperature and 2 mL were used to inoculate 100 mL of SM medium [19]. Strains were grown to the exponential phase (approximately 72 hours) at 28 °C with shaking. Mycelium was then harvested by centrifugation, resuspended in 0.9% (wt/vol) NaCl, and fragmented by sonication with Vibracell Albra sonicator 400 W model at an intensity of 6 kHz, pulse on, 5 seconds and pulse off, 3 seconds, for a total interval sufficient to give single unbranched hyphae with size ranging from 1 to 5 μm (checked with microscope), as previously described [14–16]. The resulting cell suspension was filtered through a 5 μm Durapore membrane filter (Millipore), harvested by centrifugation, resuspended in fresh SM medium and incubated for 3 hours with shaking to revitalize the mycelium. The mycelium was either stored in 1 mL aliquots at –80 °C or immediately processed.

Fermentation for A40926 production was performed as follows. Cryo-vials of the WCB were thawed at room temperature and 2 mL were used to inoculate 100 mL of E25 vegetative medium in 500 mL baffled flasks and grown for 72-96 hours on a rotary shaker at 200 rpm and 28 °C. E25 medium components were in g/L: yeast autolysate (Costantino) 4; soy bean meal (Cargill) 20; dextrose (Cerestar) 25; NaCl (Carlo Erba) 1.25; $CaCO_3$ (Baslini) 5; antifoam (Hodag) 0.3; pH of the medium prior to autoclaving adjusted to 7.5 with NaOH [14,15]. Fermentation was started by adding a 10% (vol/vol) inoculum from the

vegetative medium flask into the FM2 production medium in 500 mL baffled flasks or in a 2 L working volume P-100 Applikon glass reactor (height, 25 cm; diameter, 13 cm) equipped with an AD1030 Biocontroller and AD1032 motor. FM2 components were in g/L: bacto-yeast extract (Costantino) 8; soybean flour (Sigma-Aldrich) 30; dextrose (A.D.E.A) 30; malt extract (Costantino) 15; $CaCO_3$ (Sigma-Aldrich) 4; L-valine (Sigma-Aldrich) 1; pH of the medium prior to autoclaving adjusted to 7.4 with NaOH. Cultivations in bioreactors were carried out at 30 °C, with stirring at 500 to 700 rpm (corresponding to 1.17 to 1.64 m/sec of tip speed) and 2 L/min aeration rate. Foam production was controlled by the addition of Hodag antifoam through an antifoam sensor. A total of 25 mL of each culture was extracted every day for 1 week of fermentation [14,15,18].

A40926 extraction and analysis

A40926 was extracted by mixing 1 volume of mycelium and 3 volumes of borate buffer (100 mM H_3BO_3, 100 mM NaOH, pH 12). Samples were then centrifuged (16,000 × g for 15 min) and incubated for 1 hour at 50 °C. The glycopeptide-containing supernatant was filtered through a Durapore membrane filter (0.45 μm) (Millipore). Glycopeptide production was estimated by High Performance Liquid Chromatography (HPLC) per-formed on a 5 μm-particle-size Ultrasphere ODS (Beckman) column (4.6 by 250 mm) eluted at a flow rate of 1 mL/min with a 26-min linear gradient from 25% to 37% of phase B. Phase A was 20 mM $HCOONH_4$ (pH 4.5)-CH_3CN (95:5 [vol/vol]), and Phase B was 20 mM $HCOONH_4$ (pH 4.5)-CH_3CN (5:95 [vol/vol]) mixture. Chromatography was per-formed with a VWR Hitachi diode array L-2455 HPLC system with detection at 254 nm. A40926 purchased from Sigma-Aldrich (purity of > 95%) was used as an internal standard [14,15]. 10 mL of culture were collected from a parallel set

of flasks for Packed Mycelium Volume (PMV %) and pH determination. Biomass production was also estimated as dry weight after 24 hours incubation in an 80 °C oven and glucose consumption by using Diastix sticks (Bayer).

Minimal Inhibitory Concentrations (MICs) and Population analysis profile (PAP)

MICs and PAP were determined as follows. Hyphae were sonicated as above described in order to disperse as much as possible the mycelial bacterial population, and then seeded on Medium V0.1 agar (concentrations in g/L: soluble starch 2.4; glucose 0.1; meat extract 0.3; yeast extract 0.5; triptose 0.5; agar, 15; pH 7.2) [17] supplemented with different glycopeptide concentrations. Up to 10^7 colony forming units (cfu) were spread per each agar plate and incubated at 28 °C for 15 days. The MIC values were determined as the lowest antibiotic concentrations that inhibited visible growth after 10 days of incubation. The PAPs were determined as the surviving fraction of the population for each antibiotic concentration tested. The following A40926 (Sigma Aldrich) concentrations were used: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 , 6, 7, 8 µg/mL [14].

VanYn-related activity measurement

D,D-carboxypeptidase activity was measured in VSP medium [18] at different time points from inoculum. Mycelial lysates were prepared as described previously [20]. The enzyme activity releasing D-Ala from the tripeptide N-Acetyl-L-Lys-D-Ala-D-Ala (10 mM) was followed spectrophotometrically by a D-amino acid oxidase/peroxidase coupled reaction that oxidizes the colorimetric substrate 4-aminoantipyrine to chinonemine [21]. D,D-carboxypeptidase activity was normalized to dry biomass weight, as previously reported [16,21]. One unit is defined as the amount of enzyme that is able to convert one micromole of substrate in one minute.

Results

Heterogeneous A40926 resistance profile in *Nonomuraea gerenzanensis* ATCC 39727 population

As previously reported [14–16], in mycelial actinomycetes, due to their complex life cycle, the standard method used in unicellular bacteria to determine MICs and to select resistant mutants is compromised by the formation of multicellular aggregates and by the coexistence of cells in different physiological states (e.g., vegetative mycelium, aerial mycelium, and spores). A sonication/filtration procedure was thus needed to prepare a more homogeneous population represented by viable unbranched hyphae with sizes ranging from 1 to 5 μm. Sonicated hyphae of *N. gerenzanensis* ATCC 39727 were then plated in Medium V0.1 in the presence of increasing concentrations of A40926. The strain morphology appeared uniform in Medium V0.1 agar plates without antibiotic addition (non-selective condition, Figure 2a) and in the presence of very low concentrations of A40926 (0.5, 1, 1.5 μg/mL). MIC of A40926 under these experimental conditions was 4 μg/mL. Using the sub-inhibitory concentration of 2 μg/mL (so far identified as selective condition), two colony phenotypes were easily distinguished within the microbial population (Figure 2b). The same phenotypes were also observed in plates containing 3 or 3,5 μg/mL A40926, but in those cases the number of colonies per plate tended to be significantly reduced. In non-selective condition, colonies were generally regular, round shaped, orange pigmented, 2-3 mm in diameter, with a typical irregular convolute surface in the centre (Figure 2a). In the selective condition, two different sized-colonies were detectable: in the so-called large colonies (G, from grand), the diameter size was 4-5-fold that of the small ones (named petit, P). Their morphology, both of G and P colonies, was different than in non-selective condition, being more pinkish, round shaped, rather flat but with a protruding centre.

Once isolated, these colonies were sub-cultivated in V0.1 agar medium in the presence of 2 µg/mL of A40926. G phenotype appeared to be stable when sub-cultured in selective condition, whereas the P population, when sub-cultivated, continued to segregate G colonies with a frequency of 10 to 20% at each generation, indicating an intrinsic instability of this phenotype.

PAPs of the stable G population and of the wild type indicated that A40926 MICs, which in these experimental conditions is considered the antibiotic concentration that inhibits the growth of 99.9% of the population, were *ca.* 6 versus 3 µg/mL, respectively (Figure 3). In the unstable P population, the MIC was lower than in the wild type (around 2.5 µg/mL) (Figure 3). These data confirm that *N. gerenzanesis* ATCC 39727 wild type population has a heterogeneous resistance profile which can probably vary according to the prevalence of a more or less resistant phenotype under the pressure of sub-inhibitory concentrations of A40926.

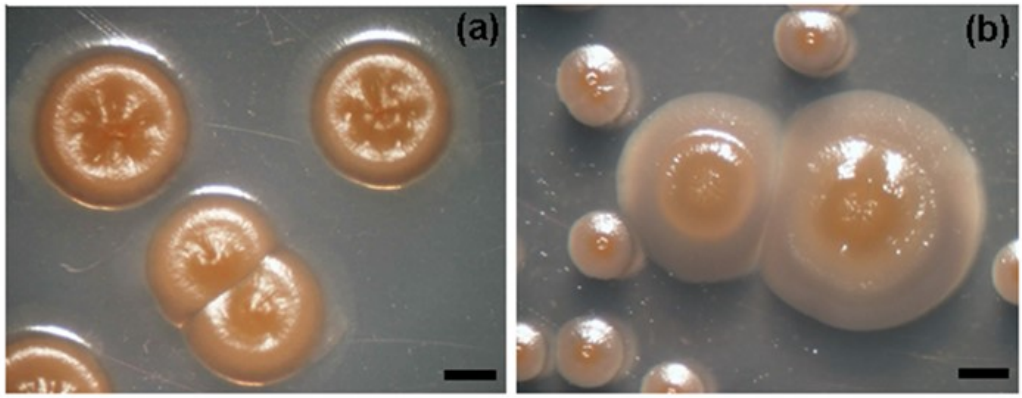

Figure 2. (a) Typical morphology of *N. gerenzanensis* ATCC 39727 colonies growing on Medium V0.1 agar without antibiotic addition: colonies appeared uniform in color, size, and shape. (b) Colonies growing in the presence of 2 µg/mL of A40926 showed a different morphology and dimensions: on the basis of their diameter, it was possible to distinguish P (petit, small) from G (grand, big) colonies. Bars are 0.8 mm.

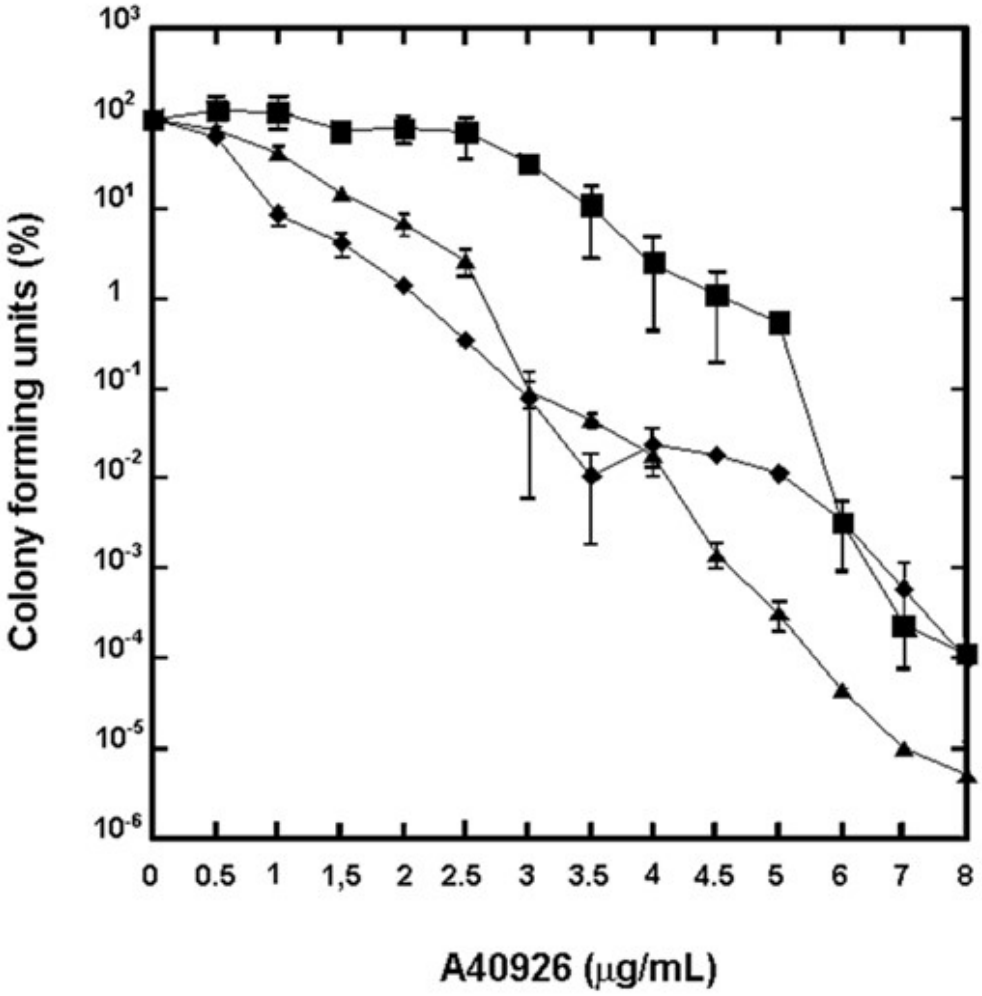

Figure 3. Population analysis profiles of *N. gerenzanensis* ATCC 39727. Colonies were grown in the presence of increasing concentrations of A40926 in the wild type (filled triangles) and in the two selected populations G (filled rectangles) and P (filled rhombi). Three colonies for each population were tested in parallel and results are the average of three independent experiments. Bars indicate ± standard deviation.

Since self-resistance in *N. gerenzanensis* ATCC 39727 was reported to be due to the action of a transmembrane D,D-carboxypeptidase encoded by the cluster-situated gene dbv7, which removes the last D-Alanine from the peptidoglycan precursors [20,21], we measured the D,D-carboxypeptidase activity in the

membrane extracts of *N. gerenzanensis* ATCC 39727 wild type and of its more resistant G population. The colorimetric-coupled assay detecting the amount of D-Ala released by the hydrolysis of N-acetyl-L-Lys-D-Ala-D-Ala tripeptide indicated that VanYn activity was two-fold higher in G cells than in the wild type (respectively 32 ± 2.5 vs 15 ± 1.6 nmol/min*mg of total proteins, after 24 hours from the induction by 2 µg/mL of A40926). These results confirm that VanYn expression correlates with the resistance phenotypes of G population and wild type.

A40926 production in *N. gerenzanensis* ATCC 39727 and in its G and P subpopulations

Ten colonies for each population (G and P) were cultivated in parallel with the wild type at flask level, and A40926 production was estimated by HPLC as reported in Material and Methods. Results reported in Figure 4 indicate that the more resistant colonies G produce significantly more antibiotic than the wild type (ca. 300 vs 180 mg/L), whereas the less resistant P colonies produced slightly less (150 mg/L). This evidence corroborates the existence in the wild type populations of a correlation between antibiotic A40926 production and self-resistance, which was previously demonstrated generating recombinant strains [15].

Upscaling the fermentation process at 2 L working volume bioreactor scale, the growth curve and the production time course of the best producer G variant were compared with the wild-type profile (Figure 5a, 5b). In the G population, the dry biomass production was higher than in the wild type, reaching the maximum value of 300 g/L after 120 hours from inoculum versus the 200 g/L produced by the wild type in the same cultivation time. Coherently, glucose consumption was faster in G population, being completely exhausted within the first 96 hours of

fermentation, whereas 144 hours were needed to the wild type to completely consume it.

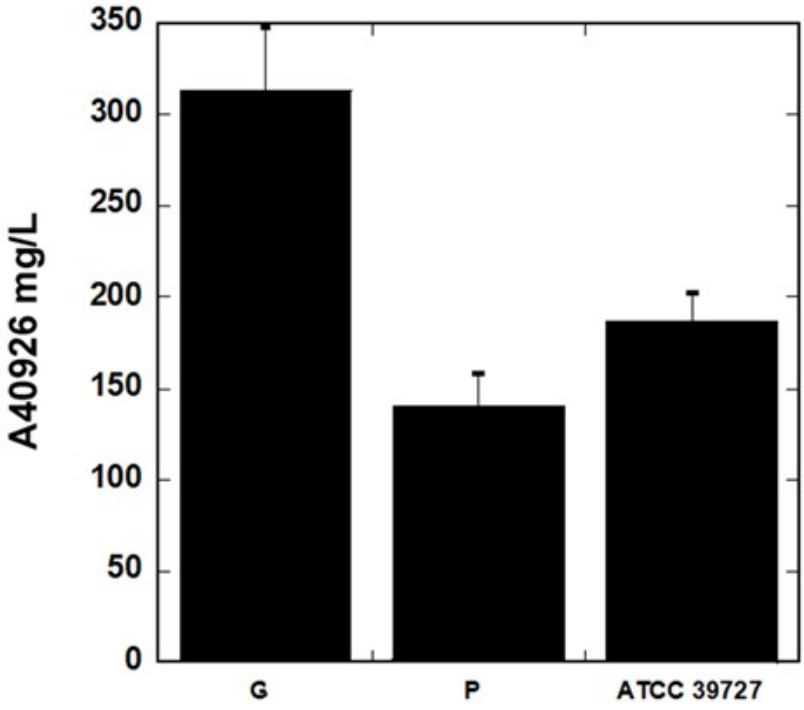

Figure 4. A40926 production, expressed in mg/L, of *N. gerenzanensis* ATCC 39727 and of its G and P variants grown in FM2 for 144 hours after the inoculum in 500 mL Erlenmeyer flasks. Results given are the mean values from the cultivation and extraction of ten independent colonies per each condition. Bars indicate ± 1 standard deviation.

In the G population, A40926 production reached its maximum of 400 mg/L after 120 hours from the inoculum versus the 150 mg/L produced in the wild type after 144 hours of cultivation. Thus, the G variant was con-firmed at bioreactor scale to produce more than two-fold A40926 than the wild type. It is worthy to note that the G variant conserved the same productivity in fermentation without the need to cultivate it in selective conditions. After twenty cycles of replication on

non-selective conditions, no relevant change in morphology or antibiotic productivity was observed (data not shown).

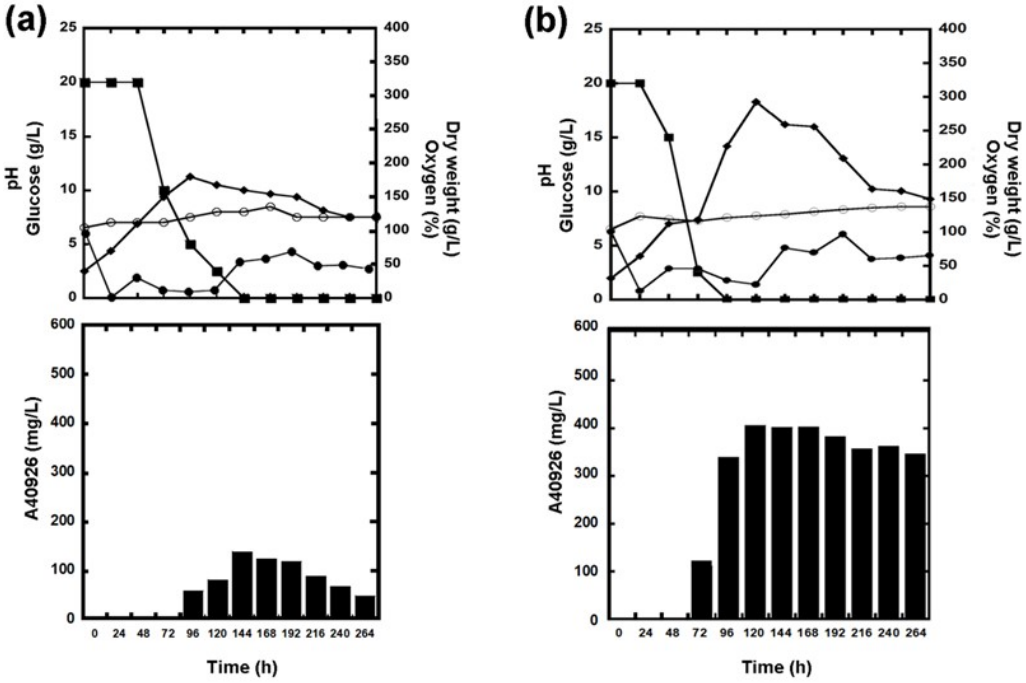

Figure 5. Time courses of *N. gerenzanensis* ATCC 397272 (a) and its more A40926-resistant G variant (b) cultivated in FM2 medium at 2 L working volume bioreactor scale. Glucose consumption (filled rectangles), biomass accumulation (filled rhombi), pH (circles), O2 level (filled circles), and A40926 production (filled bars) were monitored every 24 hours.

Discussion.

Nonomuraea is a genus of so-called rare or uncommon filamentous actinomycetes (to distinguish them from the most easy-to-isolate and to-cultivate *Streptomyces* spp.), whose capability to produce specialized (secondary) metabolites is still rather poorly explored [1,3,22]. Probably, the most known *Nonomuraea* species is the producer of the antibiotic A40926, which was discovered in the late eighties [4]. Notwithstanding its pharmaceutical relevance, the A40926 producer strain was properly classified at the species level three decades later [23]. At the same time, the complete annotation of its genome became available [24]. The final genome is ca. 12 Mb in size, organized in one

main circular chromosome and three extra-chromosomal elements, and its main feature is the paralogous gene expansion, being many genes duplicated or expanded [24]. Another peculiar trait that was reported on *N. gerenzanenis* ATCC 39727 genome is that a large percentage of genome (13.5%) is devoted to regulation, particularly transcription regulation. Additionally, many genes appear involved in microbial adaptation under stressful conditions [24,25]. In this light, it is not surprising that we could distinguish different morphological phenotypes within the wild-type population, when it was subjected to stress conditions. The large genome of this filamentous actinomycete has some degree of intrinsic instability/plasticity and its versatile specialized (secondary) metabolism is under the control of sophisticated regulatory circuits that allow differential gene expression in response to varying environmental and cultivation conditions [24–26].

It is known that the chromosome of the mostly investigated *Streptomyces* spp. is very unstable and it undergoes very large deletions spontaneously at rates higher than 0.1% of spores [26]. High-copy-number tandem amplifications of specific chromosomal regions are frequently associated with the deletions, and RecA seems to be involved in the amplification mechanism and in the control of genetic instability. It is likely that a similar molecular mechanism occurs in *Nonomuraea* spp. (so far only three complete assemblies of *Nonomuraea* spp. genomes are available [1]), although this aspect has not been investigated yet. Further studies are thus needed to unveil the molecular mechanisms underlaying the genetic instability we observed in *N. gerenzanensis* ATCC 39727 wild type population and in its P variant.

What is promising from the practical point of the strain improvement of *N. gerenzanensis* ATCC 39727 is that, under the pressure of the sub-inhibitory concentrations of the glycopeptide antibiotic that it produces, we could select

morphologically diverse phenotypes to whom a different level of antibiotic production was associated. These results confirm the co-regulation of A40926 biosynthesis and self-resistance towards the produced glycopeptide, as suggested by the organization of the A40926 biosynthetic gene cluster, named *dbv* [9,14,15]. A40926 is a potent antibiotic active towards Gram-positive bacteria, and its producing microorganism belongs to the Gram-positive group. Consequently, *N. gerenzanensis* ATCC 39727 needs to protect itself during antibiotic production in order to avoid suicide [14–16]. In the light of the recent report that the real final biosynthesis product seems to be the acetyl-form of A40926 [7,13], it would have been interesting to test this last molecule, which unfortunately is not commercially available, in parallel with A40926. It is indeed known that A40926 and its acetyl-form are equipotent in antibacterial activity against most of the bacterial strains tested [4].

The only determinant of self-resistance in *N. gerenzanensis* ATCC 39727 so far reported is the expression of the vanYn gene (*dbv7*, clustered with the biosynthetic genes), encoding a D,D-carboxypeptidase, which converts the glycopeptide-sensitive peptidoglycan pre-cursor of cell wall biosynthesis into its resistant counterpart [14,20,21]. It was described [7] that the *dbv7* expression is induced by A40926 and it is repressed by its acetyl biosynthetic derivate, in a feed forward mechanism that reveals a complex regulatory circuit. Here, we reported that in the more abundantly growing and stable phenotype G, which produces nearly twice A40926 than its parental strain, VanYn expression was about the double than in the wild type, suggesting that the increased self-resistance is associated to the improved antibiotic productivity, as recently highlighted in engineered recombinant mutants [14,15]. Other authors previously reported that selecting for self-resistance leads to an improved productivity in antibiotic producing actinomycetes [27]. Amplification of DNA segments

including antibiotic biosynthetic genes clustered with self-resistance genes might represent a common molecular mechanism leading to an increased production in industrial strains selected in the presence of the produced antibiotics. For instance, in *Streptomyces kanamyceticus*, the level of kanamycin production depended on the copy number of its biosynthetic gene cluster, suggesting that DNA amplification occurred during strain improvement because of selection for increased kanamycin resistance [28].

In industrial programs of strain and fermentation improvement, it is routinely applied the so called "strain maintenance" protocol, based on the selection of the best clonal populations growing on agar media to then scale up them in large scale submerged culture-based fermentations. Selection at this stage is often based on phenotypic traits (colony morphology, colour, or simply increased productivity) which are basically applied blindly [29]. In the present work, we have found a simple and rational way to discriminate high producing colonies from *N. gerenzanensis* ATCC 39727 wild type mixed population, by using sub-inhibitory concentrations of the commercially available antibiotic A40926. A different colony morphology in plate was associated to the increased resistance and antibiotic productivity both at flask and at bioreactor scale, confirming that the empirical practice based on colony morphology observation still represents a valid tool, especially if applied to the original wild type isolates, which exhibit some intrinsic heterogeneity. Although recombinant engineering is increasingly attracting industrial interest allowing specific genetic modifications in the wild-type background, a preliminary analysis of the wild type population is recommended to avoid misinterpretation of the results. In the case of *N. gerenzanensis* ATCC 39727, we suggest that any further step of genetic manipulation should be applied to the stable selected G variant, which produces twice more than the wild type. Finally, the fact that this higher producing variant

is more resistant to the produced glycopeptide antibiotic suggests that a possible way to further improve A40926 production process could be the selection/construction of increasingly resistant A40926 mutants and/or the continuous removal of the antibiotic product during the fermentation process. Increasing the yield of the A40926 production might significantly contribute to the cost reduction of its clinically relevant semisynthetic derivative dalbavancin.

Conclusions

Heterogeneous A40926 self-resistance profile in *N. gerenzanensis* ATCC 39727 population has permitted the isolation of two morphologically distinct populations, to which a different pattern of A40926 production is associated. Under the experimental conditions used here, the more resistant variant produces more biomass and more antibiotic, reaching the maximum productivity of 400 mg/L A40926 at bioreactor scale. These results might contribute to developing a more sustainable process for producing A40926 and its semi-synthetic derivative dalbavancin.

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Chapter 4.

Digging the heterogeneity of *Actinoplanes teichomyceticus* ATCC 31121 using protoplasts. Strain improvement, strain maintenance and antibiotic complex modulation applications

Division of labor is a common feature in natural systems and can be found at different levels of biological organization, from the individuals of a shared society to the cells of a single multicellular organism. In the microbial world, examples of division of labor among colony subpopulations which specialize to perform different cooperative tasks have been extensively described [1,2]. The allocation of tasks can be achieved either at the phenotypic or at the genotypic level [3]. Filamentous actinomycetes are microorganisms that grow forming complex structures in which the alternation of vegetative and re-productive growth phases represents an example of division of labor. Indeed, the vegetative hyphae are programmed to support and protect the reproductive hyphae and the uni-genomic spores. Spores can then persist and disseminate, to allow the spread of the species [4–6]. In the model actinomycete *Streptomyces coelicolor*, it was reported that the metabolically costly antibiotic production and secretion, trade-off with growth, and are performed by only a fraction of the hyphae, identified as the "sterile caste" [7,8]. On one side the sterile caste loses the possibility to produce spores and therefore to propagate (hence "sterile"), while on the other displays a maximization in the diversity and production of secreted antibiotics [7]. If in a natural environment, the sterile caste is irremediably fated to die, in industrial settings the ability to identify and propagate the sterile caste within a population of an actinomycete of interest, may represent an opportunity for selecting industrially relevant lineages (i.e., high producers or producers of new antibiotics).

Actinoplanes teichomyceticus ATCC 31121 (Figure 1) is a "rare" or "uncommon" actinomycete (a group of filamentous actinomycetes other than *Streptomyces* spp., which are quite difficult to isolate, cultivate and genetically manipulate [9]), producer of the clinically relevant lipoglycopeptide antibiotic teicoplanin (Figure 2).

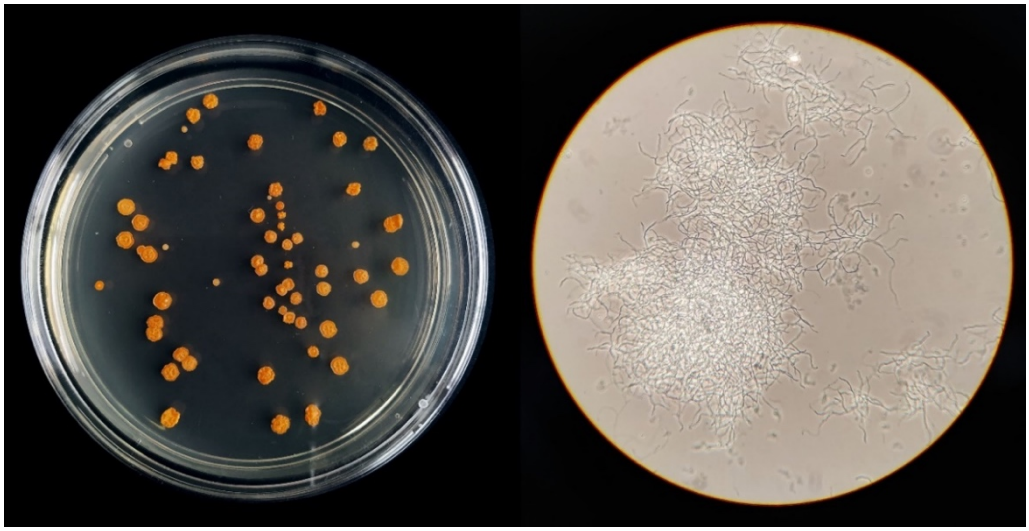

Figure 1. *A. teichomyceticus* colonies grown on Petri dish (right) and phase contrast microscope image (left).

Teicoplanin has bactericidal activity against Gram-positive aerobic and anaerobic bacteria including Methicillin Resistant Staphylococcus aureus (MRSA) [10] and was recently shown to target the main protease (MPro) in Sars-CoV-2 virus [11,12]. Teicoplanin was first approved for marketing in Italy as Targocid, with an International Birth Date (IBD) of 30 July 1987 and is actually a mixture of five closely related main compounds (T-A2-1, T-A2-2, T-A2-3, T-A2 -4 and T-A2-5) which differ in the length and branching of an aliphatic chain. The sixth, more polar active compound, T-A3, is not found in the fermentation broth but is found in raw or purified extracts as a hydrolytic product of T-A2.

The T-A2 components of teicoplanin each have comparable in vitro antibacterial activity, however, the proportions of each component of the mixture ensures the correct biological activity of the final pharmaceutical ingredient as defined in the European Pharmacopoeia (Ph. Eur.) monograph for teicoplanin [13].

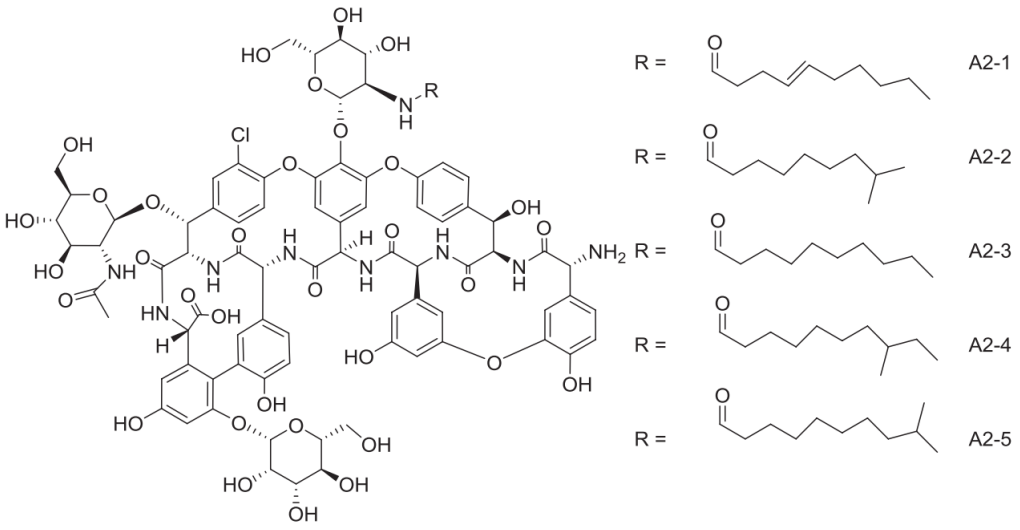

Figure 2. Chemical structure of teicoplanin molecules. The core aglycone is a linear heptapeptide consisting of seven aromatic amino acids. The core aglycone is modified by the addition of three sugars (N-acetyl(acyl)glucosamine and α-D-mannose) and is bound to different aliphatic chains (indicated with R) which characterize the different components of the teicoplanin complex.

Despite being on the market for more than 30 years, teicoplanin is still a drug of last resort used with success in clinical settings due to its antimicrobial activity and clinical safety. For the above reasons, our laboratories are actively working on teicoplanin production by *A. teichomyceticus* ATCC 31121 and we are continuously running strain improvement and strain maintenance programs. When dealing with strain maintenance, we reasoned about the potential advantages of identifying and preserving genetically stable high teicoplanin producers within the "sterile caste" lineages. Furthermore, the complexity of the teicoplanin complex of related molecules (or factors), allows also to investigate

the identification of lineage-dependent complex composition. The study of bacterial cell variants has been facilitated by growing interest in bacterial multicellularity [14] and by technical upturn in single-cell analysis technologies [15–18]. However, the single cell analysis technology is complicated in multicellular microorganisms like actinomycetes and fungi. In the study of *A. teichomyceticus* ATCC 31121, we proposed protoplasts as a strategy to analyze the single genome fate in this complex microorganism.

We have evidenced that, the underlying genetic variability which occurs in the substrate-mycelium hyphae of *A. teichomyceticus* ATCC 31121, can be separated by use of the protoplast generation technique. The protoplast technique allowed the separation of single genomes even for those genotypes which have lost the ability to produce spores (the "sterile caste"). The analysis of the generated lineages has shown an underlying metabolic complexity which influenced productivity, complex composition and occurrence of new antibiotics in *A. teichomyceticus* ATCC 31121. Protoplasts are therefore an instrument for strain improvement and identification of new molecules.

Materials and Methods

Strains and cultural conditions

Actinoplanes teichomyceticus ATCC 31121 [19] was obtained from the ATCC public collection. The strain was maintained as a lyophilised Master Cell Bank (MCB). A Working Cell Bank (WCB) was prepared from the first-generation slant originating from the MCB as already described [20]. Cryo-vials from the WCB were thawed at room temperature and 1 ml of the WCB were used to inoculate 10 ml of Medium V (soluble starch [BD, Franklin Lakes, US] 24 g/l, dextrose [Roquette, Lestrem, France] 1 g/l, meat extract [Costantino & C, Favria, Italy] 3 g/l, yeast extract [Costantino & C, Favria, Italy] 5 g/l, Bacto-tryptose [BD, Franklin Lakes, US] 5 g/l, $CaCO_3$ [Gamaco, Imerys, Paris, France] 4 g/l)

in 50-ml baffled flasks containing 5-10 glass beads of 3-mm of diameter. 1% w/v agar (Merck KGaA, Darmstadt, Germany) was added to the growth medium as suggested by Hobbs et al. [21] to obtain a better dispersed growth.

The preparation of hyphal fragments for the generation of hyphae-derived clones was performed as follows. Strains were grown in Medium V to the exponential phase (approximately 72 hours) at 28 °C with shaking. Mycelium was then harvested by cen-trifugation, resuspended in 0.9% (w/v) NaCl, and fragmented by sonication with Vibracell sonicator 400 W model (AL.BRA S.r.l, Milano, Italy) as previously described [22]. The mycelium was either stored in 1 mL aliquots at –80 °C or immediately processed by plating on Medium V plates solidified with 20 g/L agar [HiMedia, Schenzhen, China].

For protoplast preparation, 10% v/v of the culture grown for 5 days at 28°C and 180 rpm was inoculated in 100 ml of Medium VSP (soluble starch [BD, Franklin Lakes, US] 24 g/l, dextrose [Roquette, Lestrem, France] 1 g/l, meat extract [Costantino & C, Favria, Italy] 3 g/l, yeast extract [Costantino & C, Favria, Italy] 5 g/l, Bacto-tryptose [BD, Franklin Lakes, US] 5 g/l, $CaCO_3$ [Gamaco, Imerys, Paris, France] 4 g/l, sucrose [Merck KGaA, Darmstadt, Germany] 103 g/l, L-proline [Merck KGaA, Darmstadt, Germany] 3,5 g/l) [23], and growth was allowed for further 48-72 hours at the same temperature and agitation conditions.

Protoplast preparation

A. teichomyceticus ATCC 31121 protoplasts were prepared by modifying the method described in [23], to reduce residual contaminating hyphae and increase protoplast number and regeneration efficiency. In brief, ca. 100 ml of growth culture were centrifuged at 3250 x g. The mycelium was washed once in P medium [24], then 10 g (fresh weight) were suspended in 50 ml of P medium. For cell wall digestion, Hen egg white lysozyme (HEWL) (Merck KGaA,

Darmstadt, Germany) and *Candida antarctica* Lipase B (Merck KGaA, Darmstadt, Germany) were added at a final concentration of 10 mg/ml and 0.1 mg/ml, respectively. The non-ionic detergent Pluronic (Merck KGaA, Darmstadt, Germany) was supplemented at the final concentration of 0.1 mg/ml. The digestion solution was then incubated at 28°C with gentle shaking at 50 rpm, for 24 hours. Protoplasts were detached from residual mycelium clumps by thoroughly pipetting up and down, then separated from residual hyphal fragments by filtration through 5-µm durapore membrane filters (Merck Millipore, Burlington, US). The protoplasts suspension was then centrifuged at 16,200 x g, and finally re-suspended in fresh P medium. The formation of protoplasts was monitored by microscopic observation (Zeiss Axioscope, Carl Zeiss, Jena, Germany) at 400x magnification. Total protoplast number was determined by using a Petroff-Hausser counting chamber.

Regeneration of mycelium from protoplasts

Mycelium regeneration from protoplasts was performed using the overlay technique suggested by Shirahama *et al.* [25], already applied to *A. teichomyceticus* ATCC 31121 [23]. Plates were seeded by pouring 0.5 ml of protoplast suspension on hypertonic M3 medium, then overlaid with VMS0.1 Medium [23]. To assess residual hyphal contamination of protoplast suspensions, control plates, with V0.1 Medium as under layer and VM0.1 Medium as the upper layer, were seeded [23]. In these media devoid of sucrose, hyphal cells but not protoplasts were able to grow.

DAPI staining of mycelium and protoplasts

Unfiltered protoplasts (containing also residual mycelium) were treated with 4',6-diamidine-2-phenylindole (DAPI) dye, solubilized in isotonic P medium at a concentration of 1 mg/ml and incubated at room temperature in dark conditions.

After 5-minutes incubation, fresh samples were observed using an optical fluorescence microscope Zeiss Axioscope (Carl Zeiss, Jena, Germany) at 254 nm.

Teicoplanin production and analysis

Tests for teicoplanin production were performed starting from WCBs produced from colonies generated from hyphae or protoplasts. 1 ml for each individual WCB was inoculated in 30 ml of vegetative medium (meat extract [Lab M Ltd, Heywood, UK] 4 g/l, meat peptone [Costantino & C, Favria, Italy] 4 g/l, autolysed yeast [HiMedia, Schenzhen, China] 1 g/l, NaCl [Carlo Erba Reagents Srl, Cornaredo, Italy] 2.5 g/l, soybean meal [Cargill Srl, Wayzata, US] 10 g/l, $CaCO_3$ [Baslini Spa, Milan, Italy] 5 g/l, glucose [Roquette, Lestrem, France] 27.5 g/l) in 250 ml DIN ISO 24450 certified unbaffled flasks, and grown for 48 hours at 28°C on a rotary shaker at 240 rpm. 3 ml of the vegetative culture were then transferred to 30 ml of productive medium (glucose [Roquette, Lestrem, France] 12 g/l, autolysed yeast [HiMedia, Schenzhen, China] 4 g/l, malt extract [Costantino & C, Favria, Italy] 35 g/l, cotton meal [Mucedola Srl, Settimo Milanese, Italy] 11 g/l) in 250 ml DIN ISO 24450 certified unbaffled flasks. Flasks were incubated at 28°C and 240 rpm. At regular time intervals total teicoplanin was extracted by mixing 1 volume of productive culture broth and 1 volume of acetone. Samples were then centrifuged (16200 x g for 10 minutes) and the teicoplanin-containing supernatant was filtered through a Durapore membrane filter (pore size, 0.45 mm; Merck Millipore, Burlington, US).

HPLC analyses for quantifying teicoplanin production were performed with the method described in the Ph. Eur. [13], on a 5 μm-particle-size Hypersil ODS (Thermo Fisher Scientific, Waltham, US) column (4.6 by 250 mm) with elution at a flow rate of 2.3 ml/min with a 30-min linear gradient from 50% v/v to 90%

v/v phase B. Phase A was 6.89 g/l NaH_2PO_4 (Merck KGaA, Darmstadt, Germany) pH 6 / CH_3CN (Carlo Erba Reagents Srl, Cornaredo, Italy) 9:1 [v/v] and phase B was 6.89 g/l NaH_2PO_4 Merck KGaA, Darmstadt, Germany) pH 6 / CH_3CN (Carlo Erba Reagents Srl, Cornaredo, Italy) 3:7 [v/v]. Chromatography was performed with a 1100 HPLC system (Hewlett-Packard, Palo Alto, US), and UV detection at 254 nm. Pure samples of teicoplanin were used as the reference standard (EDQM, Strasbourg, France). Along this paper, we refer to teicoplanin as the sum of the related molecules as defined in the European Pharmacopoeia (Ph. Eur.) document [13] as well as to the single factors or group of factors. For the estimation of compliance of the isolated clones with a potential industrial application, the reference limits for each teicoplanin complex component are those described by the Ph. Eur. [13].

Statistical analysis was performed with the R statistical package [26].

Results

A. teichomyceticus ATCC 31121 protoplast production and regeneration as the base for genome separation

The possibility to separate genomes (and the related phenotypes) within the complex population of *A. teichomyceticus* ATCC 31121 can be of paramount importance for industrial purposes, to select only those members of the population actively devoted to teicoplanin production. However, the complex structure of hyphae causes the accumulation of genomes in clumps that are difficult to separate. Therefore, the productivity data collected are usually (quantitatively and qualitatively) determined by the average performance induced by the concurrence of different lineages. To efficiently separate (potentially) non-spore forming single genomes from the mycelium of *A. teichomyceticus* ATCC 31121, the production and regeneration of protoplasts was selected as the most promising method.

The filamentous *A. teichomyceticus* ATCC 31121, growing in liquid media as tough pellets consisting of aggregating hyphae, presents several cell wall modifications that can result in great resistance to lysozyme [27]. Because of this peculiar growth, when treated with standard procedures for protoplast generation, cell wall is poorly accessible to enzymatic hydrolysis, and mycelium is scarcely converted into protoplasts, thus resulting in high contamination by hyphal fragments [23]. To improve protoplast production and to reduce hyphal contamination, *A. teichomyceticus* ATCC 31121 mycelium was treated with a mixture of lysozyme and lipase as described in Materials and Methods section. The efficiency of protoplast formation was assayed by microscopic enumeration at different intervals of incubation in the digestion solution. Maximum protoplast yield ($10^8/10^9$ protoplasts from 100 ml of culture) was achieved after incubation times ranging from 24 to 48 hours (Figure 3a).

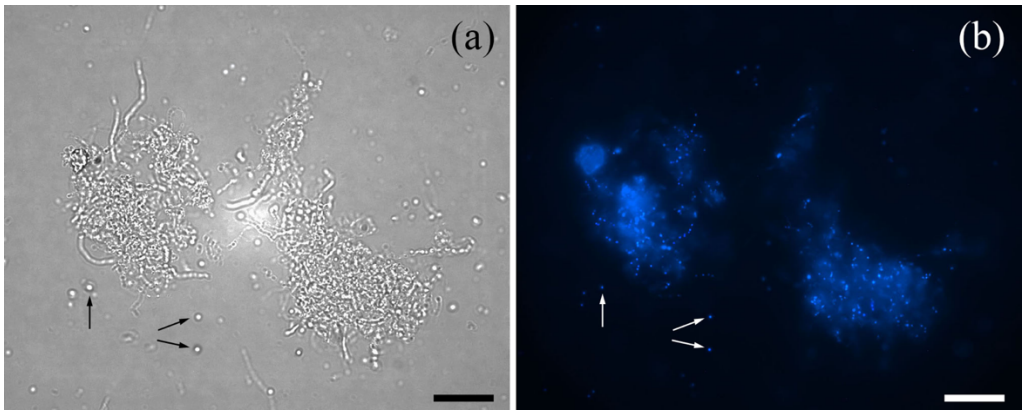

Figure 3. Mycelium clumps and protoplasts (examples of the latter are indicated by arrows), observed with optical fluorescence microscope (Zeiss Axioscope) at 400x magnification (a). DAPI staining (b) evidenced the complexity of the multinucleated mycelium clumps in comparison to protoplasts. Bar dimension: 10 μm.

In the model actinomycete *S. coelicolor*, mycelium development has been associated to a progressive movement of replicated chromosomes towards

hyphae tips and new branching, according to a mechanism known as nucleoid migration [28,29]. The result is a homogeneous distribution of nucleoids along the whole length of the sporogenic hyphae, followed by segregation of single genomes within the spores, a process regulated by the ParA-ParB protein complex [30]. When the unfiltered protoplasts of *A. teichomyceticus* ATCC 31121 (containing also residual mycelium) were treated with 4',6-diamidine-2-phenylindole (DAPI), a fluorescent organic dye that strongly binds DNA regions rich in AT sequences, fluorescent signals confirmed also for *A. teichomyceticus* ATCC 31121 such organization of the genetic material. Nucleoids appeared well defined and evenly distributed along the vegetative hyphae, as occurs in *S. coelicolor*. On the contrary, according to the fluorescence intensity and to the limited dimension of the protoplasts, it was reasonable to attribute a single genome to the regenerating protoplasts (Figure 3b). The absence of fluorescence in some protoplasts could indicate absence of genetic material and therefore the impossibility to revert to mycelium.

Macroscopic and microscopic analysis of hyphae-derived and protoplast-derived clones

Clones regenerated from protoplasts and clones derived from simple plating of mycelium clumps, did not show relevant differences in growth or in other morphological characteristics when replicated on agar plates. Similarly, when inoculated in vegetative and/or production media, hyphae-derived clones showed no variability in the phenotype: presumably, the presence of a consistent number of genomes concurred in all cases in determining an overall similar morphology (Figure 4b). On the opposite, when each independent protoplast-derived clone was inoculated in liquid media, differences in colour (Figure 4a), mycelium clump dimension (Figure 4cd), foaming, and overall growth were observed. This result suggested that protoplasts were an efficient tool to separate phenotypes and

that the separation was able to uncover an underlying genetically heterogeneous population.

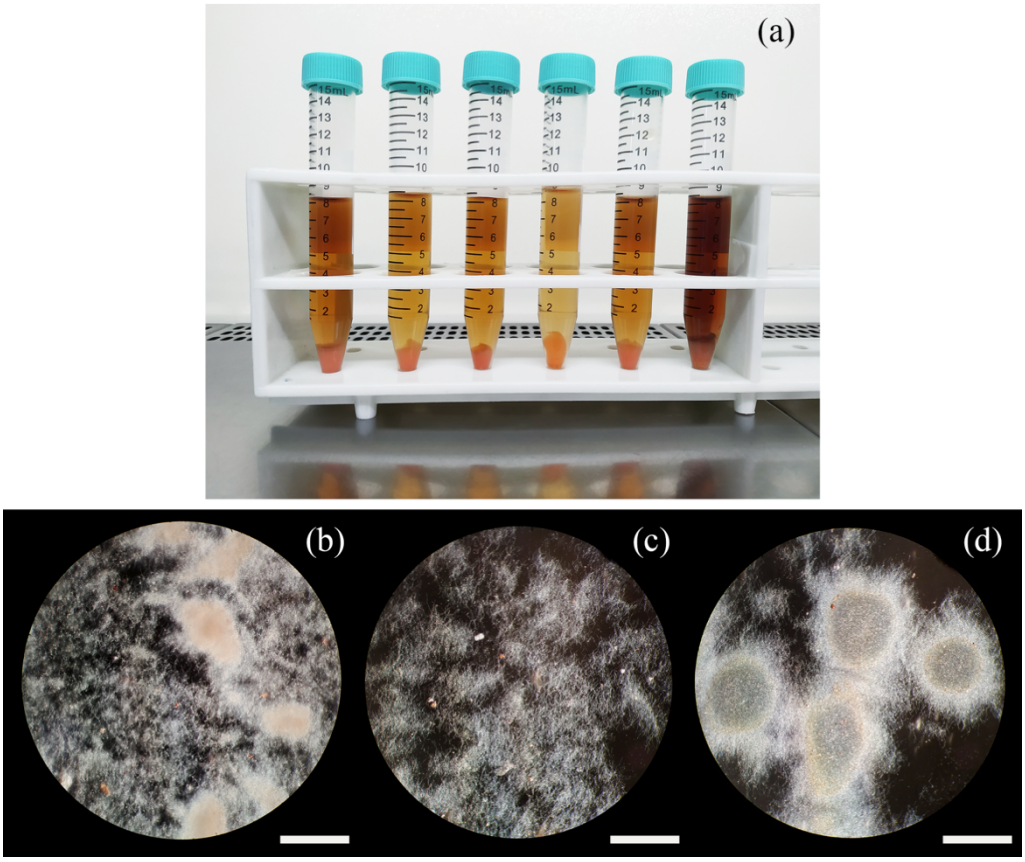

Figure 4. Macroscopic details of cultures derived from protoplast-regenerated clones (a). At the microscopic level (b-d), protoplast-regenerated clones displayed a different degree of mycelium aggregation and clump dimension (exemplified by the extremes in c and d), while hyphae-derived clones were invariably growing in a mixed dispersed-clumped situation (b). Bar dimension: 40 μm.

Analysis of teicoplanin production and complex composition

Teicoplanin is produced by *A. teichomyceticus* ATCC 31121 as a mixture of related molecules, differing in their alkyl side chain [31]. For commercializing the pharmaceutical product, two different specifications exist. The first one is

described in the European Pharmacopoeia (Ph. Eur.) [13], the second one is reported in the Japanese Pharmacopoeia (J. P.) [32] (Tables 1 and 2). The bottleneck in the industrial production of teicoplanin, lies in keeping the balance between the different related molecules (complex of factors) to adhere to the below reported specifications.

Table 1. Elution order and relative retention time of the teicoplanin product according to Ph. Eur.

Name of component	Elution order (Ph. Eur.)	Relative retention time (Ph. Eur.)*
Teicoplanin A3-1	1	0.43
Teicoplanin A2-1	2	0.93
Teicoplanin A2-2	3	1.00
Teicoplanin A2-3	4	1.04
Teicoplanin A2-4	5	1.12
Teicoplanin A2-5	6	1.14

*the time of elution is relative to Teicoplanin A2-2

Table 2. Specifications of the teicoplanin product according to J. P. and Ph. Eur.

Name of component	Limits adopted by J.P. for content of each component of the complex	Limits adopted by Ph. Eur. for content of each component of the complex
Teicoplanin A3	NMT 15.0%	NMT 15.0%
Teicoplanin A2 group*	NLT 80.0%	NLT 80%
Teicoplanin A2-1	NA	NMT 20%
Teicoplanin A2-2	NA	NLT 35% and NMT 55%
Teicoplanin A2-3	NA	NMT 20%
Teicoplanin A2-4	NA	NMT 20%
Teicoplanin A2-5	NA	NMT 20%
impurities	NMT 5%	NMT 5%

* As group the sum of all the A2 factors is indicated
NLT: Not Less Than
NMT: Not More Than
NA: Not Applicable

The characteristics of the complex of factors are strictly dependent, as reported to date, on the composition of the fermentation medium and on the presence of the precursors of the different components of the complex [33]. The fermentation studies performed in this work were aimed at achieving a teicoplanin product in line with the requirements of the Ph. Eur. (an example of an HPLC profile out of the specifications and one in compliance with the Ph. Eur., are shown in Figure 5 a and b, respectively).

In the analysis of teicoplanin production, 49 independent clones derived from hyphal fragments and 49 independent clones derived from protoplast regeneration, were fermented according to the protocol described in the Materials and Methods section. The overall production of teicoplanin (calculated as the sum of the main Ph. Eur. relevant components of the complex, excluding TA3 which derives from downstream processing of the product during industrial purification), and the proportion of the different related complex components were quantified. In fermentations of hyphae-generated clones, the mean of teicoplanin productivity was 219.1 mg/l with a standard deviation (SD) of + 65.9 mg/l (Table 3). Teicoplanin productivities ranged from a minimum of 111.0 mg/l to a maximum of 373.7 mg/l (Table 3 and Table 4). Teicoplanin production in protoplast-derived clones, ranged from 12.6 mg/l to 508.0 mg/l with a mean of 282.6 mg/l and a SD of + 110 mg/l (Table 3 and Table 5). Hence, compared to what observed in hyphae-derived clones, the distribution of teicoplanin productivity in protoplast-derived clones was skewed towards both the extremes, with the appearance of both low and high producers (Figure 6, “Complex Sum” panel). According to the analysis of teicoplanin complex composition (Figure 6 and Figure 7 a and b), clones producing a teicoplanin complex compliant with the Ph. Eur. represented the 22.4% of the analysed hyphae-derived samples and the 12.5% of the analysed protoplast-derived clones. The different factors of the

Table 3. Summary statistics of fermentations to produce teicoplanin performed using hyphae- and protoplasts-derived clones

Teicoplanin complex Factors in production from Hyphae	N	Mean	St. Dev.	Min	Pctl(25)	Pctl(75)	Max
TA2-1	49	5.242	1.372	2.239	4.251	6.416	8.287
TA2-2	49	88.130	36.214	22.084	68.754	114.649	161.687
TA2-3	49	43.424	19.839	11.953	28.195	58.074	96.498
TA2-4	49	43.439	16.152	8.900	30.176	51.000	85.040
TA2-5	49	38.766	14.025	4.914	28.676	46.366	75.849
Complex Sum	49	219.111	65.898	110.983	169.705	274.221	373.695
Teicoplanin complex Factors in production from Protoplasts	**N**	**Mean**	**St. Dev.**	**Min**	**Pctl(25)**	**Pctl(75)**	**Max**
TA2-1	49	8.876	8.385	0	4.8	8.7	32
TA2-2	49	135.479	60.614	8.695	110.038	151.891	331.047
TA2-3	49	50.974	29.381	0	23.7	78.4	103
TA2-4	49	46.723	23.268	0	32.9	66.5	89
TA2-5	49	40.567	19.119	0	28.0	52.0	87
Complex Sum	49	282.619	110.026	12.562	215.429	348.970	507.852

teicoplanin complex were produced in variable amounts with increased variability in protoplast-derived clones (Figure 6 and Figure 7 b). The analysis of correlation of the production of the different teicoplanin complex factors, evidenced that TA2-1 and TA2-2 abundance in the different hyphae-generated clones, were not cross-correlated and were not correlated neither with the abundance of the TA2-3, TA2-4, and TA2-5 factors (for details see Table 4 and 5). However, TA2-2, being the most abundant component of the teicoplanin complex, correlated with the overall teicoplanin production ($r=0,73$; $p<0.001$). On the other side, TA2-3, TA2-4, and TA2-5 factors production was interconnected, and also correlated with the overall teicoplanin production (Table 6). A similar outcome was observed in protoplast-generated clones (Table 7). Notably, teicoplanin minor factors, usually expressed at low level, were uncovered by the separation of protoplasts (for an example see Figure 5b the "uncharacterized" peak).

Table 4. Teicoplanin production and complex composition in hyphae-derived clones

Clone	TA2-1 (%)	TA2-2 (%)	TA2-3 (%)	TA2-4 (%)	TA2-5 (%)	Teicoplanin (mg/L)
Ph. Eur.	**< 20**	**> 35 and < 55**	**< 20**	**< 20**	**< 20**	**Not applicable**
H1	4,2	16,7	30,3	30,2	18,7	138,4
H2	3,4	10,8	32,2	37,6	16,0	226,2
H3	4,4	13,3	34,2	31,5	16,5	169,7
H4	3,8	15,4	32,0	31,8	16,9	169,2
H5	4,0	13,8	34,1	31,3	16,7	160,4
H6	2,1	41,7	14,5	21,3	20,4	201,1
H7	2,0	41,8	18,5	22,2	15,4	327,9
H8	1,9	39,5	18,6	21,2	18,8	218,5
H9	1,9	41,1	14,2	21,6	21,2	199,2
H10	2,2	37,7	19,8	20,1	20,2	197,3
H11	2,2	39,2	17,9	20,7	20,2	274,2
H12	2,3	39,0	17,4	19,7	21,5	258,3
H13	2,9	46,6	12,6	15,7	22,2	172,2
H14	2,0	44,5	14,9	19,7	18,8	128,6
H15	2,1	46,3	18,7	13,8	19,2	185,7
H16	2,1	49,2	16,5	16,2	16,1	312,0
H17	1,6	51,0	20,8	11,9	14,6	316,9
H18	2,1	57,9	9,2	14,3	16,5	240,0
H19	1,8	28,8	21,8	21,9	25,7	238,4
H20	2,8	56,3	8,8	14,2	18,0	178,2
H21	1,6	38,1	24,1	19,4	16,8	295,7
H22	4,3	71,8	10,8	8,7	4,4	111,0
H23	2,7	52,4	11,9	18,3	14,8	160,9
H24	2,6	43,1	19,8	18,1	16,3	251,1
H25	1,7	44,1	21,7	17,1	15,3	289,4
H26	1,7	44,1	21,7	17,1	15,3	289,3
H27	2,5	43,5	19,0	18,9	16,1	202,5
H28	2,9	45,8	12,4	20,7	18,2	189,7
H29	2,9	54,3	20,5	12,7	9,6	224,4
H30	3,5	51,4	9,6	19,0	16,5	188,7
H31	2,3	47,9	15,1	18,3	16,4	242,1
H32	1,8	41,1	24,3	18,0	14,8	334,1
H33	1,8	42,8	22,7	17,6	15,2	280,9
H34	2,2	45,5	20,7	17,6	14,0	263,8
H35	5,0	67,6	14,7	6,5	6,3	137,9
H36	3,5	36,6	16,1	23,7	20,1	188,6
H37	2,8	36,5	20,4	20,2	20,0	196,8
H38	2,4	46,6	14,5	18,8	17,8	259,5
H39	1,6	30,7	25,8	21,6	20,3	373,7
H40	2,4	38,3	19,0	21,9	18,3	170,3
H41	1,9	40,7	20,5	19,6	17,3	125,9
H42	3,6	36,2	17,2	22,1	20,9	133,2
H43	2,8	36,5	17,8	22,2	20,7	135,9
H44	2,5	37,3	21,3	20,2	18,7	132,7
H45	2,1	34,5	17,0	24,3	22,2	191,0
H46	2,8	23,8	25,2	25,4	22,8	294,8
H48	1,4	44,8	18,1	17,4	18,3	158,0
H49	2,6	24,0	25,3	25,2	22,8	294,8
H50	1,3	51,3	19,4	12,4	15,5	307,5

Table 5. Teicoplanin production and complex composition in protoplast-derived clones

Clone	TA2-1 (%)	TA2-2 (%)	TA2-3 (%)	TA2-4 (%)	TA2-5 (%)	Teicoplanin (mg/L)
Ph. Eur.	**< 20**	**> 35 and < 55**	**< 20**	**< 20**	**< 20**	**Not applicable**
P31	4,1	42,0	21,6	17,0	15,2	280,9
P32	0,0	61,1	10,0	15,1	13,7	218,2
P33	9,5	39,8	19,5	18,2	13,0	296,7
P34	0,0	100,0	0,0	0,0	0,0	12,6
P35	0,0	57,4	12,2	16,9	13,6	215,4
P36	0,0	40,7	18,6	23,2	17,5	268,9
P37	0,0	55,8	12,3	17,7	14,2	285,8
P38	2,8	44,9	15,2	21,0	16,1	188,8
P39	3,3	76,2	8,8	5,5	6,3	233,8
P40	0,0	53,9	16,9	17,8	11,5	209,1
P41	2,1	36,0	25,2	20,1	16,6	349,0
P42	7,3	42,7	24,9	13,9	11,1	320,2
P43	7,7	50,5	17,2	14,2	10,3	271,5
P44	3,3	42,3	23,0	17,7	13,8	359,1
P45	7,7	49,2	19,4	14,5	9,1	234,1
P46	6,5	35,0	26,5	17,5	14,4	387,2
P47	7,9	45,9	21,0	14,1	11,1	403,3
P48	11,5	45,9	18,8	14,4	9,4	265,3
P49	0,7	60,8	16,8	10,5	11,2	463,9
P50	7,7	42,7	22,3	15,8	11,6	382,6
P51	2,8	57,4	18,4	9,2	12,2	401,8
P52	2,9	65,6	10,0	9,7	11,8	238,1
P53	3,6	70,6	7,9	7,6	10,3	188,4
P54	4,0	63,5	8,1	14,4	9,9	68,7
P55	2,8	52,2	9,9	14,8	20,3	192,4
P56	2,1	67,8	7,0	10,4	12,7	297,8
P57	2,5	53,2	3,3	21,9	19,0	302,5
P58	1,8	39,1	25,3	18,7	15,1	388,8
P60	1,4	37,0	20,9	19,4	21,2	410,0
P61	1,8	35,0	26,5	21,2	15,5	314,0
P62	1,6	36,1	18,8	22,3	21,1	398,6
P63	1,8	36,1	25,0	20,8	16,3	321,8
P64	2,4	40,4	21,0	19,8	16,5	198,3
P65	2,0	39,9	25,4	18,2	14,5	346,0
P66	1,4	34,4	17,9	24,6	21,8	345,8
P67	3,0	35,4	22,6	22,0	17,0	319,9
P68	4,6	66,9	12,1	7,6	8,8	46,9
P69	1,6	39,2	17,5	22,4	19,3	340,3
P70	4,1	67,1	9,4	7,8	11,6	183,0
P71	4,0	61,6	7,5	11,0	15,9	165,8
P72	1,8	60,1	13,4	11,1	13,7	199,7
P73	1,3	65,2	12,5	12,0	9,0	507,9
P74	1,4	55,4	18,7	14,6	9,8	485,6
P75	3,0	44,4	18,8	19,3	14,5	293,2
P76	2,5	35,5	15,9	26,4	19,7	281,4
P77	2,7	40,1	20,4	18,6	18,2	246,5
P78	2,4	31,2	24,9	22,1	19,4	314,5
P79	1,6	62,5	14,8	8,8	12,4	380,2
P80	6,4	36,2	14,7	19,6	23,1	24,0

Table 6. Correlation of teicoplanin factors production in hyphae-derived clones

Parameter1	Parameter2	r	95% CI	t (47)	p
TA2-1	TA2-2	0.02	[-0.26, 0.30]	0.16	> .999
TA2-1	TA2-3	0.48	[0.23, 0.67]	3.72	0.004**
TA2-1	TA2-4	0.55	[0.31, 0.72]	4.46	< .001***
TA2-1	TA2-5	0.25	[-0.03, 0.50]	1.79	0.282
TA2-1	Complex Sum	0.37	[0.10, 0.59]	2.71	0.047*
TA2-2	TA2-3	0.26	[-0.02, 0.50]	1.85	0.282
TA2-2	TA2-4	0.06	[-0.23, 0.33]	0.39	> .999
TA2-2	TA2-5	0.39	[0.13, 0.61]	2.93	0.031*
TA2-2	Complex Sum	0.73	[0.56, 0.84]	7.22	< .001***
TA2-3	TA2-4	0.83	[0.72, 0.90]	10.20	< .001***
TA2-3	TA2-5	0.68	[0.50, 0.81]	6.44	< .001***
TA2-3	Complex Sum	0.81	[0.68, 0.89]	9.33	< .001***
TA2-4	TA2-5	0.77	[0.62, 0.86]	8.17	< .001***
TA2-4	Complex Sum	0.70	[0.53, 0.82]	6.78	< .001***
TA2-5	Complex Sum	0.83	[0.72, 0.90]	10.26	< .001***

p-value adjustment method: Holm (1979)
Observations: 49

Table 7. Correlation of teicoplanin factors production in protoplast-derived clones

Parameter1	Parameter2	r	95% CI	t(47)	p
TA2-1	TA2-2	0.15	[-0.13, 0.42]	1.06	0.657
TA2-1	TA2-3	0.42	[0.15, 0.63]	3.15	0.020*
TA2-1	TA2-4	0.18	[-0.11, 0.44]	1.25	0.657
TA2-1	TA2-5	0.07	[-0.21, 0.35]	0.49	0.657
TA2-1	Complex.Sum	0.32	[0.04, 0.55]	2.33	0.105
TA2-2	TA2.3	0.44	[0.18, 0.64]	3.34	0.013*
TA2-2	TA2-4	0.33	[0.05, 0.56]	2.39	0.105
TA2-2	TA2-5	0.40	[0.13, 0.61]	2.98	0.027*
TA2-2	Complex.Sum	0.82	[0.70, 0.89]	9.76	< .001***
TA2-3	TA2-4	0.79	[0.66, 0.88]	8.96	< .001***
TA2-3	TA2-5	0.73	[0.56, 0.84]	7.29	< .001***
TA2-3	Complex.Sum	0.83	[0.72, 0.90]	10.39	< .001***
TA2-4	TA2-5	0.92	[0.87, 0.96]	16.42	< .001***
TA2-4	Complex.Sum	0.78	[0.64, 0.87]	8.51	< .001***
TA2-5	Complex.Sum	0.79	[0.65, 0.88]	8.80	< .001***

p-value adjustment method: Holm (1979)
Observations: 49

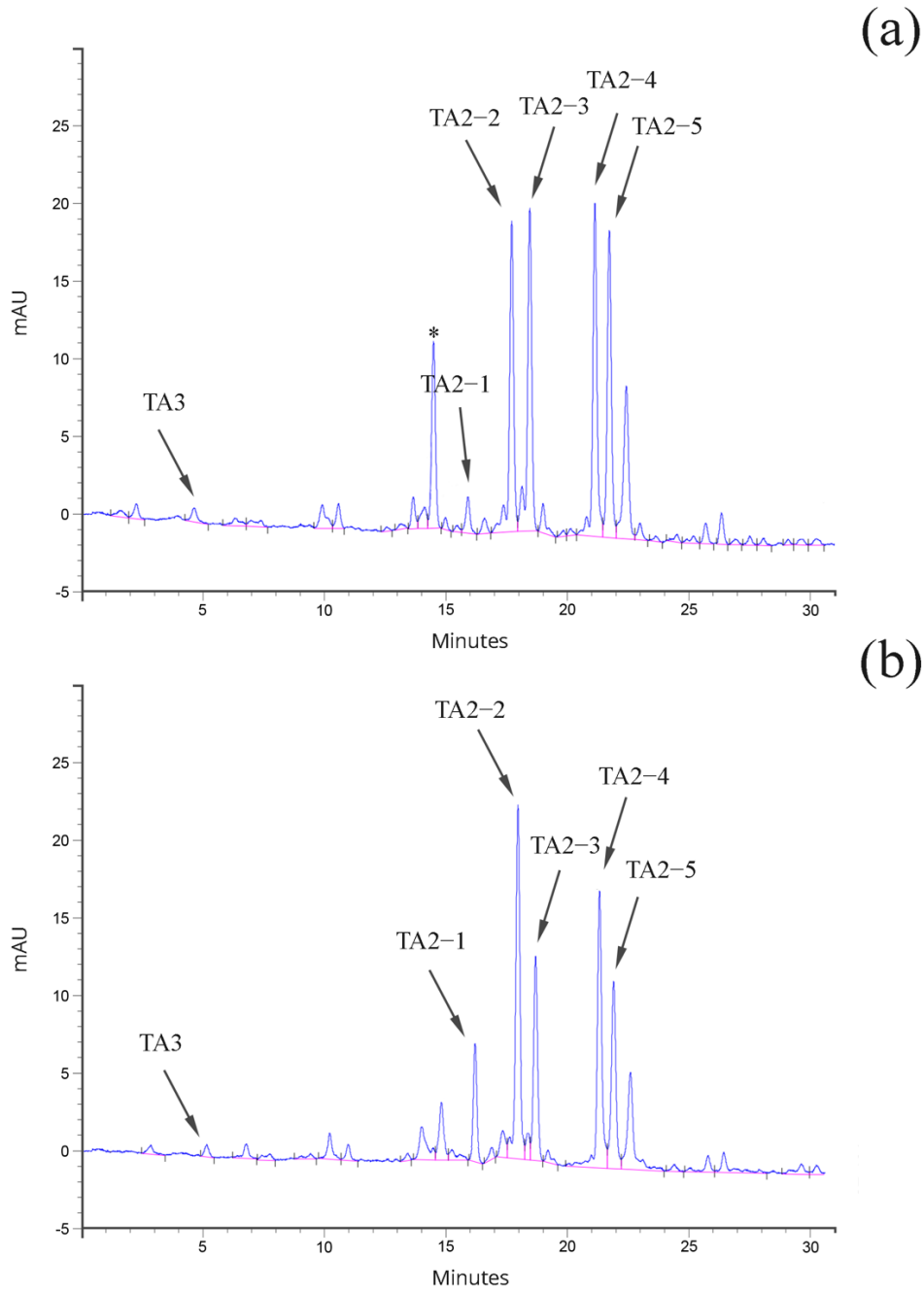

Figure 5. HPLC profile of Ph. Eur. out of specification teicoplanin (from the hyphae derived clone H46) (a) and Ph. Eur. compliant teicoplanin (from the protoplast derived clone P75) (b). Worth of note is the increase in uncharacterized peak marked by asterisk in panel (a).

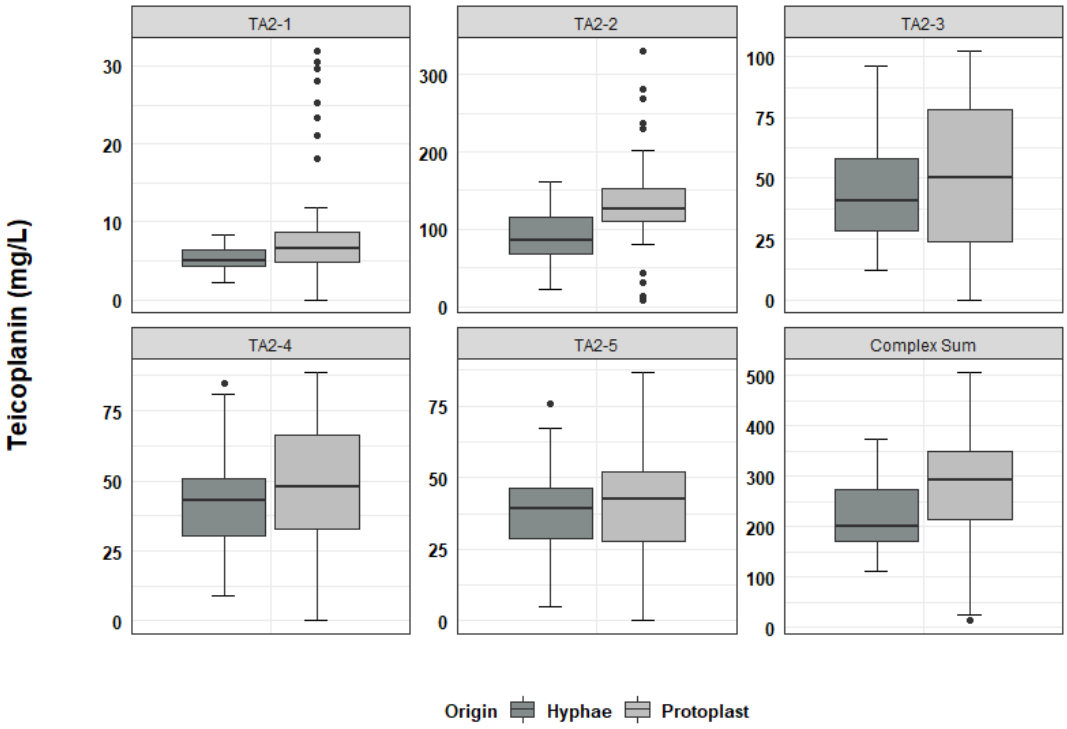

Figure 6. Boxplot distribution of teicoplanin production from fermentation of hyphae-derived clones (Origin: Hyphae) and protoplast-derived clones (Origin: Protoplast). Dispersion of productivity both in overall production and in single complex factor production was increased in protoplast-derived clones.

Finally, four colonies obtained by plating clone P73 (see Figure 7 b and Table 5) on Medium V0.1 agar, were independently fermented. Data achieved showed good consistency among the four colonies, in terms of growth, teicoplanin overall productivity and teicoplanin complex distribution, thus proving the genetic stability of protoplast-derived clones.

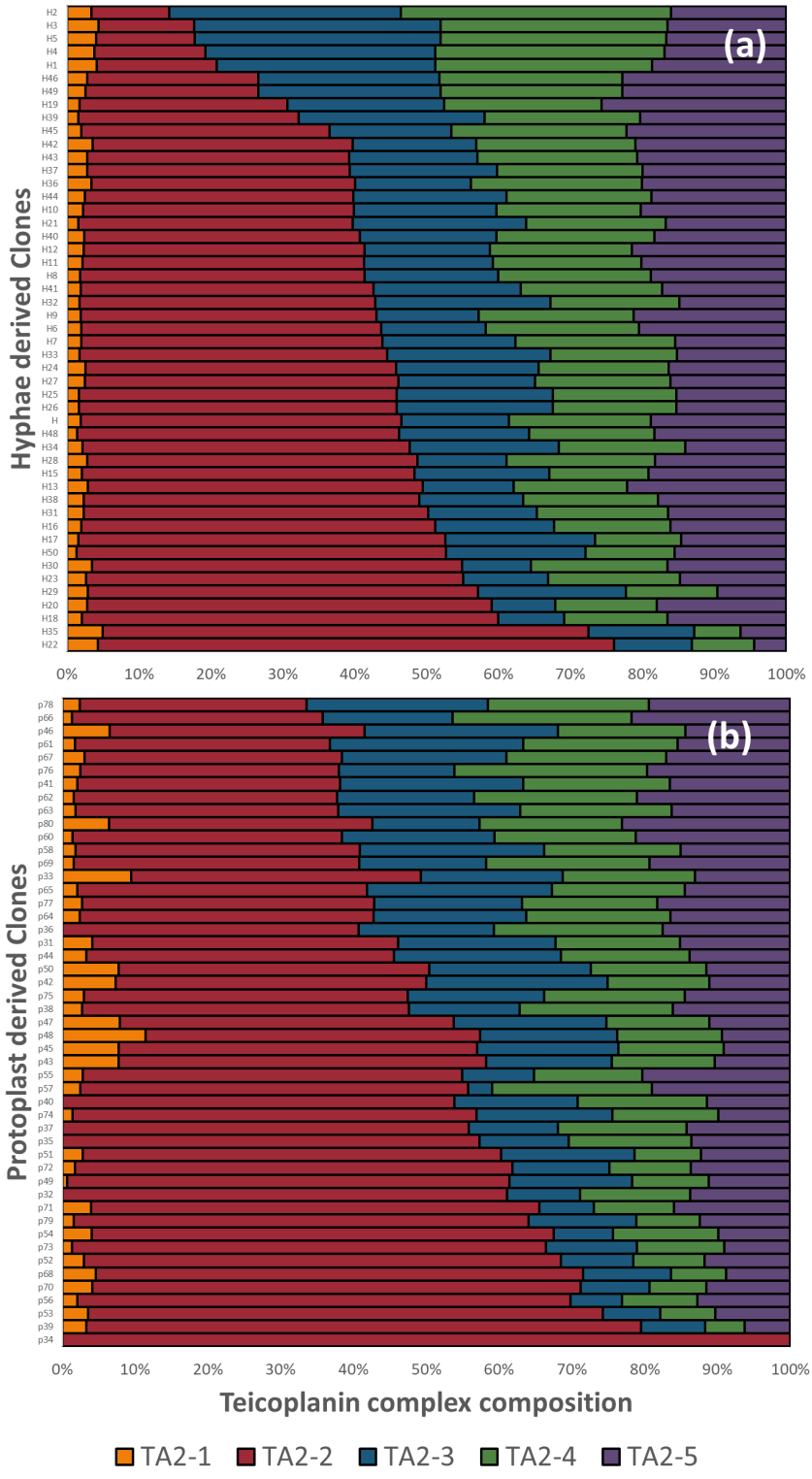

Figure 7. Distribution of teicoplanin complex factors in hyphae-derived (a) and protoplast-derived (b) fermentations

Discussion

Hunting for high producers of pharmaceutically relevant metabolites (or, more generically, of secondary or specialized metabolites), from actinomycetes, still strongly relies on protocols of random mutagenesis and selection. More recently, and where applicable, protoplast fusion and whole genome shuffling [34] were applied to improve the production rates of clinically relevant molecules, with the most known and successful results being achieved for tylosin production [35]). Rational genetic manipulation of producer actinomycetes is possible but restricted by the still limited availability of ad hoc genetic tools for these bacteria [36–38].

In the quest for industrial production candidates, only a little effort has been dedicated to the understanding of the intrinsic phenotypic variability of multinucleate microbes [39] with the drawback that variability in industrial production still occurs with a high incidence and the loss of high producers is the inevitable destiny of those companies which do not apply rigorous strain maintenance protocols. Antibiotic production is a costly process involving a direct energy trade-off between production and reproductive capacity [40]. It was recently demonstrated that *S. coelicolor*, when forming colonies on solid substrates, has evolved an elegant mechanism of growth based on a division of labor that limits antibiotic production to a fraction of the colony. The delegation of production of antibiotics to a "sterile caste" (non-spore producing) of the microbial population, reduces the overall costs of biosynthesis, maximizes the magnitude and diversity of the produced antibiotics and increases the reproduction efficiency by the "non-sterile caste" (spore producing) [8]. The process that predisposes to the division of labor is based on differential gene expression and on genomic instability, the latter creating phenotypically

heterogeneous subpopulations of cells, mainly by means of large and irreversible deletions or amplifications at the chromosomal termini [41–43].

Although heterogeneity is a beneficial trait in natural biological systems since it is at the origin of adaptation and evolution, it is generally considered a production pitfall in industry where the reproducibility of microbial-based processes is of fundamental im-portance. In this study, we reversed this vision and demonstrated that, for industrial applications, the understanding, identification, and preservation of the heterogeneous sterile caste of a microbial population could be of paramount importance for strain improvement and strain maintenance. The study was based on embedding single genomes of *A. teichomyceticus* ATCC 31121 in protoplasts and on the analysis of the derived protoplast-generated clones. Considerable differences in the colour of the cultures (ascribable to the expression of silenced metabolic pathways and/or to mutations in metabolic pathways which determine the accumulation of intermediates), foam formation, and growth were observed. However, no general rule was identified that could be predictive of the presence of high productivity. By separating the different genomes that are represented in *A. teichomyceticus* ATCC 31121 mycelial clumps, and by measuring teicoplanin production and the proportion of the different factors of its complex, we observed that a situation like that observed in *S. coelicolor* was reproduced also in *A. teichomyceticus* ATCC 31121. Indeed, when compared to standard hyphae-derived clones, protoplast-derived fermentations displayed an extended range of teicoplanin productivity, with both low and high producers (the latter being extremely interesting from an industrial point of view). Furthermore, in protoplast-derived clones the production of a complex of factors distinct from those produced by clones derived from multinucleated hyphae was observed. Such heterogeneous distribution in the production of the different teicoplanin complex factors, could be interpreted as

an adaptative response which may help to maximize the diversity of the secreted antibiotics. Indeed, it was reported that the different teicoplanin factors have a variable antimicrobial activity [31] and therefore, their diversity could help in the struggle against competing bacteria attacking the *A. teichomyceticus* ATCC 31121 growing colony. Not surprisingly, the highest variability in production was observed in the teicoplanin complex factors TA2-1 and TA2-2, which display the highest antimicrobial activity [31]. Their production appeared instead unrelated to the synthesis of the less active TA2-3, TA2-4 and TA2-5, thus suggesting the possibility of an attack-emergency response specifically based on the most active antimicrobials. These results also suggested that, besides being influenced by fermentation medium composition as previously determined [33,44], teicoplanin complex variability has also a genetic base. In the light of the present results, chemical mutagenesis applied to strain improvement should be re-analyzed. It is indeed accepted that a chemical or physical mutagenic treatment has the result of killing from 90.0% to 99.9% of the treated microorganisms [34]. This implies that, when the mutagenic treatment is applied to mycelia of non-spore forming microorganisms or of sterile industrial strains, most of the genomes within a hyphal fragment are killed. This results in a treatment which is very similar to the one that we have depicted in this work, i.e., the generation of single genome cells. Therefore, improved mutants could in some cases derive from genome separation, mimicked by genome destruction, rather than from random mutations. After all, the fact that the identification of improved mutants is very low in classical strain improvement treatments, could derive by the fact that most of the induced mutations have a negative outcome on productivity, therefore competing with the improvements potentially uncovered by the simple separation of genomes.

Conclusions

In this study, we demonstrated that modifications of the phenotype naturally occur in cultures of the teicoplanin producer *A. teichomyceticus* ATCC 31121, as result of a division of labor. By producing and regenerating protoplasts, this underlying genetic variability can be uncovered: hence, genome separation might be used as tool to select high teicoplanin producers, with complex profiles eventually in line with the Ph. Eur. requirements.

Based on the analysis performed, and on the assumptions already reported for *S. coelicolor*, the use of protoplasts could replace that of hyphal fragments or spores in strain maintenance protocols. This is valid not only for *A. teichomyceticus* ATCC 31121, but it is virtually applicable to any filamentous actinomycete of industrial interest for which protoplasts can be produced. With our approach, we could simplify the strain maintenance work, at least to the extent in which mutations conferring the high productivity traits are sufficiently stable. This paves the way to a completely new approach to the protocols of strain improvement and strain maintenance.

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Chapter 5

The unpredictable fate of the "biosynthetic dark matter". *Amycolatopsis coloradensis* ATCC 53629 and the production of a novel avoparcin complex of antibiotics

Actinomycetes are important producers of bioactive secondary metabolites of industrial interest which include antibacterials, anticancer, antifungals, immunosuppressants, and herbicides. Despite the description of over 10,000 bioactive secondary metabolites produced by actinomycetes (representing more than 45% of all bioactive microbial metabolites discovered) [1], most of the potential of each single producer actinomycete remains hidden within their genomes. Indeed, genome sequencing revealed that actinomycetes, and in general microbes with large genomes, have the genetic information to potentially produce up to 30-50 secondary metabolites [2]. The evolutionary meaning of this "biosynthetic dark matter" is currently being explored in relation to the division of labor which, besides higher organisms, occurs in actinomycetes and in other multicellular or colony-forming microorganisms. In actinomycetes, which are typically solid-substrate-growing microorganisms displaying a life cycle characterized by a complex morphological and biochemical differentiation [3], the genetic potential could be activated/deactivated in a part of the microbial population according to function, spatial position in the growing colony, and temporal requirements [4]. Of particular importance is the expression of the hidden genomic potential by the vegetative hyphae (or substrate mycelium). The substrate mycelium (in some cases defined as the "sterile caste") has indeed the task to surround, nourish and protect spore formation. The possibility for the substrate mycelium to diversify the production of secondary metabolites could offer selective advantages over competing microorganisms [5]. The potential for production of different secondary metabolites can be activated by the mere expression of specific genes and by point mutation and/or irreversible

rearrangements of the genome [6]. Point mutation and irreversible rearrangements of the genome could result in lineages which display specific characteristics including the modulation of production of secondary metabolites (high or low producers) or the synthesis of different secondary metabolites [4]. These characters are not necessarily inherited by spores and can be preserved artificially in laboratory or production plants and exploited for the industrial production of metabolites.

Actinomycetes of the genus *Amycolatopsis* have a high genetic potential to produce secondary metabolites and bioactive molecules [7]. Strains belonging to this genus are in fact the producers of some of the most widely used antibiotics: rifampicin A, vancomycin, balhimycin, nogabecin and ristocetin produced respectively by *A. mediterranei* or *A. rifamycinica*, *A. orientalis*, *A. balhimycina*, *A. keratiniphila* and *A. lurida* [8]. Among these, the most important are rifampicin and vancomycin, used as broad-spectrum antibiotics, respectively for the treatment of tuberculosis and as a last line of defense for the treatment of infections from multi-resistant Gram-positive microorganisms [8].

The interest in *A. coloradensis* ATCC 53629 is linked to the production of the glycopeptide antibiotic avoparcin, also known as LL-AV290 [9,10]. Avoparcin administration was performed in sub-therapeutic doses for prolonged periods of time in intensive farming to prevent disease (prophylaxis) and promote growth [11]. Due to the misuse of this antibiotic, avoparcin is commonly considered as one of the causes of the onset of glycopeptide resistance in pathogenic bacteria (in particular Glycopeptide-Resistant *Enterococcus*, GRE) [12,13]. For this reason, avoparcin was banned in 1995 but its illegal use is still a health issue and needs monitoring [14]. Unfortunately, monitoring is hampered by the difficulty of finding on the market analytical standards of α-avoparcin and β-avoparcin, the major components of the complex product. With the purpose of obtaining an

analytical standard, we isolated morphological variants, we developed a fermentation medium and a purification method for the quantitative production of avoparcin analytical standards. Surprisingly, while producing avoparcin, we were able to identify new avoparcin-like glycopeptides produced by the avoparcin producer *A. coloradensis* ATCC 53629.

Materials and Methods

Strains, Cultivation Conditions and identification of morphological variants

The original avoparcin producer strain *Amycolatopsis coloradensis* ATCC 53629 was purchased from the DSMZ strain collection in 2018. The strain was routinely preserved as a frozen master cell bank (MCB). Working cell banks (WCBs) were prepared from agar plates or slants of Medium V0.1 [15] originating from the MCB as already described for other actinomycetes [16]. Cryo-vials of 1.0 mL were stored at –80 °C for up to two years without influence on the avoparcin and rystosamynil-avoparcin production. Mycelium for colony isolation was prepared as already described [17,18]. Colonies were selected based on the different morphology, with the support of a Carl Zeiss Stemi SV 6 stereomicroscope (Carl Zeiss, Jena, Germany). Agar media for selection were ISP2 (composition in g/L: Yeast extract [Costantino & C SpA, Favria, Italy] 4.0 g; Malt extract [Costantino & C SpA, Favria, Italy] 10.0; Dextrose [A.D.E.A Srl, Busto Arsizio, Italy] 4.0; Agar [HiMedia, Schenzhen, China] 20.0; pH 7.2 with NaOH/HCl [Carlo Erba Reagents Srl, Cornaredo, Italy]), BTT-P (composition in g/L: Yeast extract [Costantino & C SpA, Favria, Italy] 1.0; Meat extract [Costantino & C SpA, Favria, Italy] 1.0; Casein hydrolysate [Costantino & C SpA, Favria, Italy] 2.0; Dextrose [A.D.E.A Srl, Busto Arsizio, Italy] 10; Agar [HiMedia, Schenzhen, China] 15; pH 7.2 with K_2HPO_4 [Carlo Erba Reagents Srl, Cornaredo, Italy]) and

BTT-0.25 (composition as for BTT-P but diluted ¼ - besides agar- with ultrapure water).

Fermentation for avoparcin and rystosamynil-avoparcin production was performed as follows. Cryo-vials of the WCB were thawed at room temperature, and 2 mL were used to inoculate 50 mL of Medium 3-9 [19] in 500 mL baffled flasks and grown for 48 hours on a rotary shaker at 240 rpm and 28 °C. Medium 3-9 components were in g/L: Cane Molasses (Kivinat, Saint-Marcel-Lès-Valence, France) 20; N-Z amine type A (Merck KGaA, Darmstadt, Germany) 10; pH of the medium prior to autoclaving was adjusted to 7.5 with NaOH/HCl (Merck KGaA, Darmstadt, Germany). Fermentation was started by adding a 10% (vol/vol) inoculum from the vegetative medium flask into 50 ml of BCS360 production medium in 500 mL flasks. BCS360 components were in g/L: bacto-yeast extract (Costantino & C SpA, Favria, Italy) 9; soybean flour (Costantino & C SpA, Favria, Italy) 10; dextrose (A.D.E.A Srl, Busto Arsizio, Italy) 15; soluble starch (Carlo Erba Reagents Srl, Cornaredo, Italy) 10; $CaCO_3$ (Merck KGaA, Darmstadt, Germany) 4; pH of the medium prior to autoclaving was adjusted to 7.5 with NaOH. Foam production was controlled by dropping antifoam (Hodag; Vantage, Deerfield, IL, USA) whenever required. 0.5 ml culture samples were used for daily monitoring of pH, glucose and glycerol consumption, and for the production of avoparcin and ristosamynil-avoparcin. A total of 10 mL of culture was collected from a parallel set of flasks for Packed Mycelium Volume (PMV %).

For glycerol and glucose analysis, samples were mixed with 10% (v/v) of a 35% $HClO_4$ (v/v) solution, incubated at -20°C for 10 min and then neutralized with 7M KOH (w/v) (both reagents from Carlo Erba Reagents Srl, Cornaredo, Italy). Monitoring of glycerol and glucose was performed by High Performance Liquid Chromatography (HPLC) on a Aminex HPX-87H cationic exchange column

(300*7.8 mm) (BioRad, Hercules (CA), USA) eluted at 30°C at a flow rate of 0.6 mL/min with 5 mM H_2SO_4 in water (Carlo Erba Reagents Srl, Cornaredo, Italy) for 20 min. Chromatography was performed with a HPLC Agilent 1260 Infinity system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a Refraction Index Detector (RID) and with an UV detector set at 210 nm. Glucose and glycerol were quantified by comparison with real standards (purity of > 95%) (Merck KGaA, Darmstadt, Germany).

Avoparcin complex extraction, analysis and purification

The Avoparcin complex of molecules was extracted by mixing 1 volume of whole fermentation broth and 1 volume of distilled water. Samples were then vortexed 1 min and finally centrifuged (16,000 × *g* for 5 min). The glycopeptide-containing supernatant was filtered through a Durapore membrane filter (0.22 µm) (Merck Millipore, Burlington, MA, USA). Glycopeptide production was estimated by High Performance Liquid Chromatography (HPLC) performed on a Inertsil ODS-3 column (250*4,6 mm, 5 µm) (GL Sciences, Tokyo, Japan) eluted at 40°C at a flow rate of 1.4 mL/min with a 35-min gradient as follows: 0 min = 3% Phase B; 30 min = 50% Phase B; 30.1 min = 95% Phase B; 35 min = 95% Phase B; 35.1 min = 97% Phase B. Phase A was 0.3 % HCOOH (Merck KGaA, Darmstadt, Germany) -water (Carlo Erba Reagents Srl, Cornaredo, Italy), and Phase B was 0.3 % HCOOH – Methanol (Carlo Erba Reagents Srl, Cornaredo, Italy) mixture. Chromatography was performed with a HPLC diode array Agilent 1260 Infinity system (Agilent Technologies Inc., Santa Clara, CA, USA) with detection at 280 nm. DAD spectra were acquired between 190 and 400 nm and the acquired spectra were compared with those described in [20]. Avoparcin standard produced at BioC-CheM Solutions (purity of > 95%) was used as an internal standard.

Assignment of the structure of the avoparcin complex component was obtained by use of Tandem Liquid Chromatography – Mass Spectroscopy (LC/MS) experiments operating in positive-ion mode (ESI), and NMR studies. The mass spectrometric detection was realized using a single quadrupole LC/MSD system (Agilent Technologies Inc., Santa Clara, CA, USA) operating in positive-ion mode (ESI). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker (Billerica, MA, USA) Avance 400 (400 and 100.6 MHz, respectively); chemical shifts are indicated in parts per million (ppm) downfield from $SiMe_4$, with residual proton [$(CH_3)_2SO$ = 2.50 ppm, HOD = 4.80 ppm] and carbon [$(CD_3)_2SO$ = 40.45 ppm] solvent resonances as internal reference. Protons and carbon investigations were achieved by ^{13}C attached proton test (APT), $^{1}H-^{1}H$ correlation spectroscopy (COSY), and $^{1}H-^{13}C$ heteronuclear correlation experiments.

Quantitative recovery and purification of the glycopeptide antibiotic from the fermentation broth was performed by use of the affinity resin Sepharose-D-alanyl-D-alanine [21]. For the processing of 2 liters of fermentation broth containing *ca.* 15 grams of avoparcin complex, approximately 700 ml of resin were loaded on a 7 cm wide glass column. The purification procedure was as follows. Two liters of whole fermentation broth were centrifuged to separate the supernatant from the mycelium (residual supernatant volume 1700 mL). Mycelium was washed with 700 ml of deionized water to recover residual avoparcin. Approximately 2.4 L of avoparcin enriched broth were then corrected at pH 7.5 with HCl 1M (Carlo Erba Reagents Srl, Cornaredo, Italy) and loaded on the column at a speed of 10 mL/min. After loading, the column was washed with 2 L of deionized water, 2 L of 0.1M Acetic Acid (Carlo Erba Reagents Srl, Cornaredo, Italy) and 2 L of 0.1M NH_4OH (Carlo Erba Reagents Srl, Cornaredo, Italy). Avoparcin was then eluted with a linear gradient from 0.3M to 0.5M NH_4OH. The avoparcin enriched fractions were then pooled, corrected at pH 4

with 5% H_2SO_4 (Carlo Erba Reagents Srl, Cornaredo, Italy) and avoparcin was precipitated by dropping the above solution into 4 volumes of Acetone (Carlo Erba Reagents Srl, Cornaredo, Italy).

Results

Identification of an heterogeneous population in *A. coloradensis* ATCC 53629

It is known that the chromosome of the model actinomycetes belonging to the genus *Streptomyces* undergoes large spontaneous deletions at rates higher than 0.1% of seeded spores [22]. Tandem amplifications of specific chromosomal regions and associated deletions occur, and RecA seems to be involved in the control of this genetic instability. These genome modifications could result in modification in the productivity of secondary metabolites and even in the production of new metabolites [4]. A similar molecular mechanism could be the rule in other actinomycetes as increasing evidence of phenotypic variation are piling up [17]. Based on the above statements, we started our study with the analysis of the morphology of the avoparcin producer strain. From the seeding of the original lyovial purchased from the DSMZ strain collection on three different agar media (ISP2, BTT-P and BTT-0.25), we were able to distinguish 12 distinct colony morphologies (identified as C1, C6, C8, C10, C14, C17, C18, C22, C23, C24, C29, and C30 (Figure 8 and Table 1). The preliminary analysis of these morphologies revealed that all were able to produce different amounts (ranging from 2 to 9 g/L) of the avoparcin complex with C14 being the highest producer (used in the subsequent experiments). The isolated morphologies were, in general, characterized by a limited production of aerial mycelium and spores. *A. coloradensis* C14 typical colony was around 2.5 mm in diameter, with a fluffy white/light yellow aerial mycelium and no evidence of spores. The colony was typically protruding in the center and breaks appeared with aging.

Surprisingly, all the variants were able to produce an alternative avoparcin complex composed mainly of α-avoparcin, β-avoparcin and their respective ristosamylated analogues (see below).

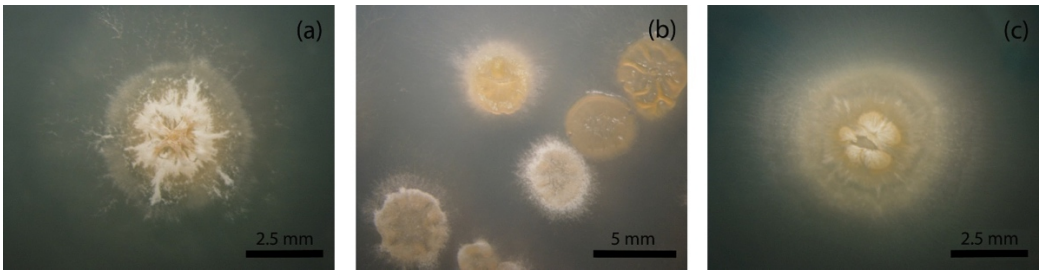
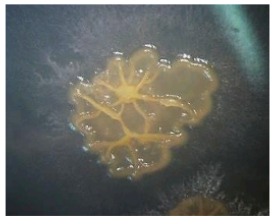


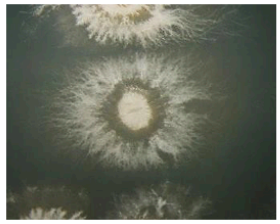
Figure 8. (**a**) Typical morphology of *A. coloradensis* ATCC 53629 growing on Medium V 0.1, (b) different morphologies observed on agar medium, and (c) high producer *A. coloradensis* C14 grown on Medium V 0.1 agar.

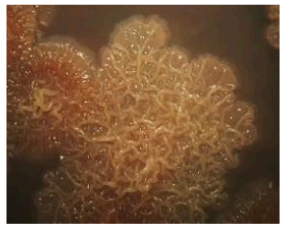
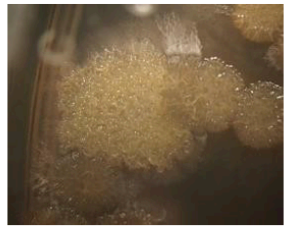
Fermentation of *A. coloradensis* C14 for the production of avoparcin

The screening of the best producer medium for avoparcin, was performed with the database BCSMedDat, a proprietary database of industrial media for the production of secondary metabolites of interest. The selected medium, BCS360, was characterized by a neutral pH and by the presence of both glucose and glycerol as carbon sources. The fermentation of *A. coloradensis* C14 occurred with the concomitant utilization of glucose and glycerol. The onset of avoparcin production initiated roughly with glucose depletion and proceeded till glycerol decreased below 10 g/L. Production reached values above 9 g/L in 120-144 hours of fermentation and biomass production reached a maximum PMV level of 25% with a SD of $\pm$ 5%. The pH during fermentation was in the range 7.2-7.6 (Figure 2).

Table 1. Description of the morphological variants isolated from *A. coloradensis* ATCC 53629. Each independent clone was cross-checked for purity and stability of the phenotype on different agar media.

Colony number	Macroscopic and Microscopic appearance	Morphology description	Identification medium
1		Shape: irregular, surrounded by radial mycelium growth Margins: lobate Color: okra Surface: rough	BTT-P
6		Shape: irregular Margins: lobate, surrounded by radial mycelium growth Color: orange Surface: rough	ISP2
8		Shape: round Margins: irregular, surrounded by vigorous radial mycelium growth Color: okra Surface: rough	BTT-P
10		Shape: round Margins: irregular, surrounded by radial mycelium growth Color: white to light yellow Surface: vertically protruding at the margins and depressed in the center, rough	ISP2
14		Shape: round Margins: regular and fluffy, surrounded by radial mycelium growth with brownish color Color: okra to light brown Surface: rough	BTT-P

Colony number	Macroscopic and Microscopic appearance	Morphology description	Identification medium
17		Shape: round Margins: regular, surrounded by radial mycelium growth Color: white Surface: dry and fragmented	BTT-P
18		Shape: round Margins: irregular, surrounded by fluffy and vigorous radial mycelium growth Color: white Surface: rough	ISP2
22		Shape: round Margins: irregular, surrounded by radial mycelium growth Color: white Surface: fluffy	ISP2
23		Shape: round Margins: regular Color: light brown/pinkish Surface: rough, irregular and protruding in the center	BTT-P
24		Shape: irregular Margins: slightly lobate, surrounded by faint radial mycelium growth Color: light brown Surface: rough and heavily protruding in the centre	ISP2

29		Shape: irregular Margins: lobate, fast growth and colonization of neighboring colonies Color: light brown/okra Surface: rough	BTT-P
30		Shape: round Margins: irregular, fast growth and colonization of neighboring colonies, lobate surrounded by scarce radial mycelium growth Color: light yellow Surface: rough	ISP2

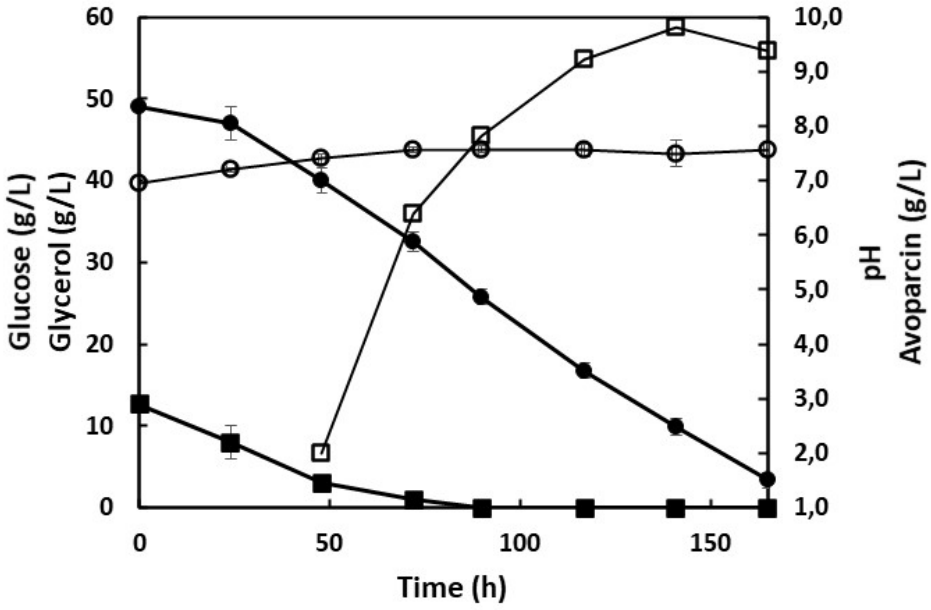


Figure 2. Time course of avoparcin complex production by *A. coloradensis* C14 cultivated in BCS360 medium. Glucose consumption (filled squares), Glycerol consumption (filled circles), pH (circles), and avoparcin complex production (squares) were monitored every 24 hours. Avoparcin complex includes α- and β-Avoparcin, and α- and β-ristosamynil-avoparcin

Purification and chemical characterization of the avoparcin complex

The level of production obtained through the combination of the high producer clone C14 and of the medium BCS360, allowed to obtain a significant amount of avoparcin complex directly from flask fermentation. A set of 20 parallel flasks (100 ml each), was run and harvested at the productivity peak. The product was then purified by use of agarose D-Ala-D-Ala resin [21], as described in the section Materials and Methods. From this experimentation, a quantity of avoparcin (9.27 g from 2 L of broth culture) was obtained in a solid form. This amount was sufficient for a fine characterization of the molecule and to produce a certificate of analysis. The purified product was analysed by NMR and LC-MS as described in the section Materials and Methods. The avoparcin complex consisted mainly of the two structurally related glycopeptides: α-avoparcin and

β-avoparcin as minor and major components, respectively (Figure 3) [20,23–28]. As reported in the literature, together with α- and β-avoparcin various types of epimers or hydrolysis products at the sugar level of the avoparcin aglycones have been detected and identified [20,23–28]. On the contrary, avoparcin fermentation products bearing additional sugar units are up to date not described. The development of the HPLC analytical method allowed us to define the characteristics of the avoparcin fermentation complex (Figure 3). Together with the α- and β-avoparcin signals (retention time (rt) α-avoparcin: 8.050 min; rt β-avoparcin: 8.813 min) the HPLC trace revealed the presence of two additional peaks (rt Unknown (U) 1: 8.289 min; rt U 2: 9.099 min, Figure 3). Interestingly, the UV absorption spectra of U 1 and U 2 signals showed the same profile as that observed for α- and β-avoparcin, indicating a close relation between the chemical structures of compounds U 1 and U 2 and α-, β-avoparcin. High-Resolution Liquid Chromatography-Tandem Mass spectrometry (LC-MS) experiments were also performed to gain additional structural information. The full-scan LC-MS (ESI) spectra of the fermentation complex confirmed the presence of the protonated ions at $[M + 2H]^{2+}$ of α- (*m/z* 956) and β-Avoparcin (*m/z* 973) [20,29]. On the other hand, the LC-MS response related to U1, and U2 could not be traced back to any literature data. In particular, the MS spectra of compounds U1 and U2 are characterized by the presence of cluster ions at *m/z* 1022 and 1039. The fragmentation of these latter ions gave rise to the ions at m/z 956 and 973, corresponding to those of α- and β-avoparcin. Thus, signals at *m/z* 1022 and 1039 are consistent to an increase of 66 to the α- and β-avoparcin *m/z* values. It is worth of note that variation of 66 in the electrospray ionization experiments of avoparcin aglycones is correlated to a variation at the ristosamine sugar [20,23]. These data suggested that the U1 and U2 formation could arise from an additional ristosamine units in α- and β-avoparcin, respectively. Thus,

unknown glicopeptides U1 and U2 were identified as a α-ristosamynil-avoparcin, and β-ristosamynil-avoparcin. NMR analysis conducted to elucidate the conjugation site of the ristosamine residue to the avoparcin aglycones allowed to hypothesize two potential sites of the molecule which could be attached to ristosamine (Figure 4). Due to the low amount of α- and β-ristosamynil-avoparcin together with their high molecular weight (α-ristosamynil-avoparcin relative molecular mass: 2038; β-ristosamynil-avoparcin: 2072) the exact regiochemistry of α- and β-ristosamynil-avoparcin still remains under discussion.

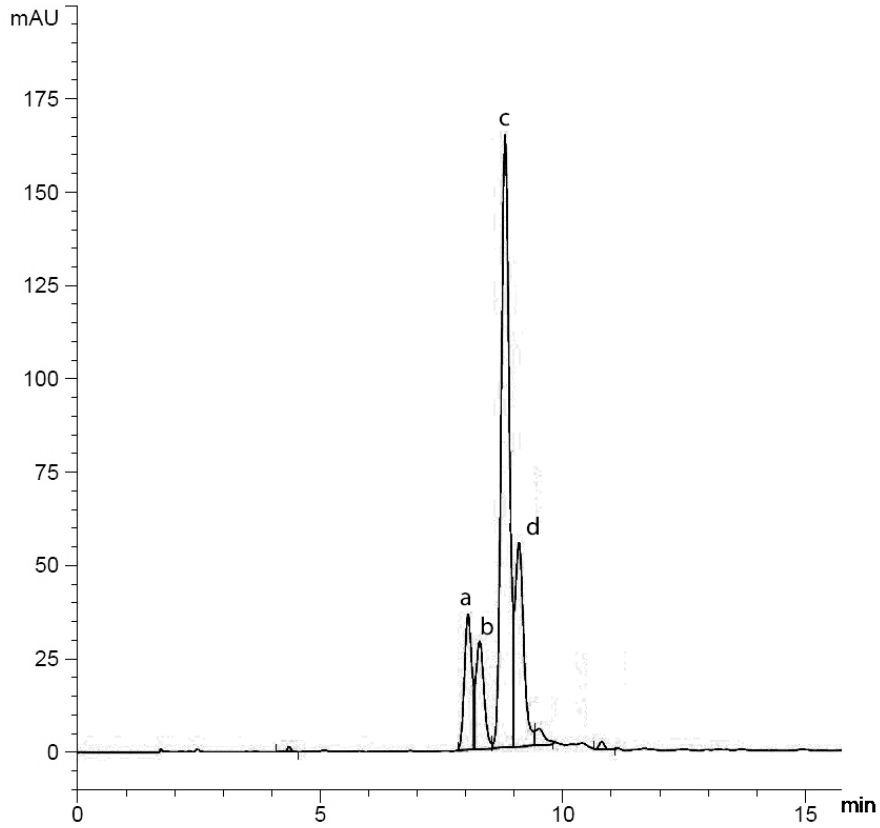


Figure 3. HPLC profile of the agarose D-Ala-D-Ala purified avoparcin complex. (a) α-avoparcin, (c) β-avoparcin (b) α-ristosamynil-avoparcin, and (d) β-ristosamynil-avoparcin.

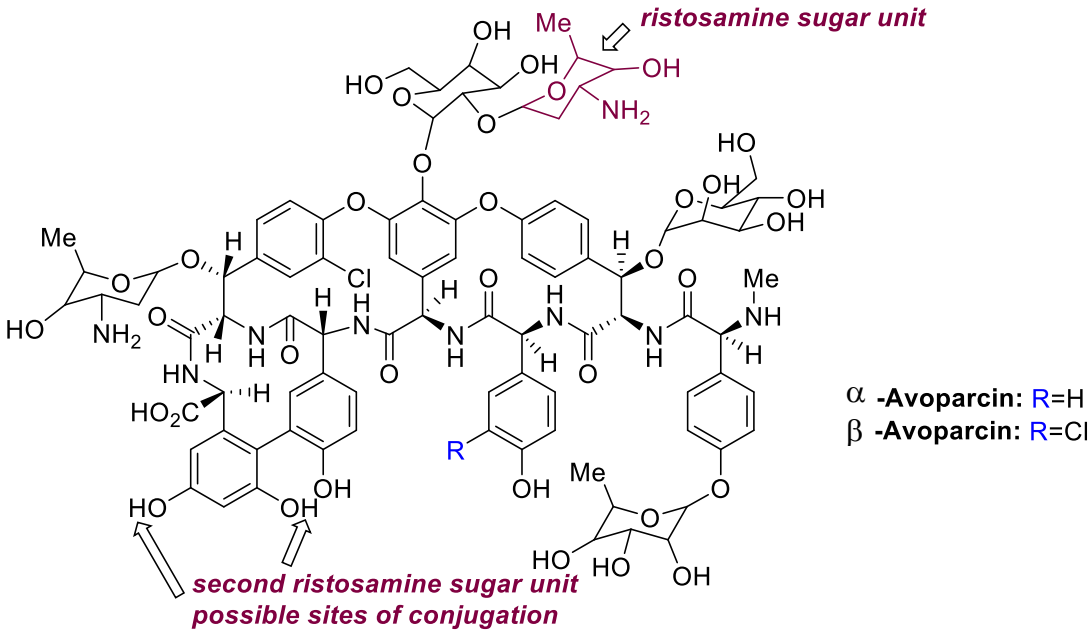


Figure 4. Chemical structure of avoparcin possible sites of attachment of the second ristosamine residue giving rise to the respective rystosamynil-avoparcin are evidenced by arrows.

Discussion.

Examples of bacterial differentiation that result in morphological change have been known for decades. In *A. coloradensis* ATCC 53629, we observed that the population originating from the lyovial directly purchased from the DSMZ strain collection (Braunschweig, Germany), displayed a high number of morphological variants when seeded on different agar media. Different avoparcin complex productivities were also evidenced and, noteworthy, the isolated morphological variants, as well as the most represented population (which we considered as the "wild type") invariably produced a modified avoparcin complex rich in the not yet described glycopeptide ristosamynil-avoparcin. It was reported that the *Amycolatopsis* genus displays relevant genomic plasticity hotspots (defined as quasi-core regions to distinguish them from core regions in which the plasticity

is reduced) [30]. This genomic plasticity was elegantly connected to the production of antibiotics, by the analysis performed by Kumari and co-workers [31]. The authors analyzed twelve strains of rifamycin producing *A. mediterranei.* The strains were independent isolates or mutants with the ability to produce different rifamycins (rifamycin B, SV, P, Q, R, U, W) and it was shown that the identification of new lineages producing rifamycin analogs and/or overproducing a single rifamycin factor occurred with relative ease thus supporting the plasticity of the genome of the strain. The analysis of the genome of *A. mediterranei* has also shown the presence of more than 30 gene clusters for the biosynthesis of secondary metabolites [32] while in *A. orientalis* HCCB10007 (the industrial vancomycin producer strain) twenty-six secondary metabolite biosynthetic gene clusters were predicted [30]. To date, 67 *Amycolatopsis* genomes, including the one from *A. coloradensis* ATCC 53629, have been completely sequenced and they range in size from 5.62 Mbp in *A. granulosa* to 10.94 Mbp for *A. anabasis* (source NCBI datasets: https://www.ncbi.nlm.nih.gov/datasets/). The complete genome sequence of *A. coloradensis* ATCC 53629 has a notable size of 9.05 Mbp and contains 8364 genes (NCBI Reference Sequence: NZ_MQUQ00000000.1). Although for *A. coloradensis* the potential to produce secondary metabolites was not studied in detail, data collected for *A. orientalis*, *A lurida* and *A. mediterranei*, show the presence of 25 to 36 secondary metabolite gene clusters indicating a noteworthy hidden potential for the synthesis of new metabolites [33]. In conclusion, *Amycolatopsis* possess several biosynthetic gene clusters (BGCs) that can be of biological importance, and which can arise during standard programs of strain improvement and strain maintenance. It was therefore not surprising to find a new avoparcin complex during our work. What was surprising, was rather that, upon testing of different fermentation media (including the one described in the

original patent from Murray *et al.* [19]), we were able to invariably produce a complex of molecules with a high amount (up to 40%) of ristosamynil-avoparcin factors. We have reasoned that this result could have different potential origins which will need to be investigated in detail. Among our hypothesis for this effect, the lyophilization process could have been a selection system which drastically reduced the number of regenerating genomes eventually contained within the stored hyphae. In example, survival rates after the drying of *Escherichia coli*, *Pseudomonas putida*, *Serratia marcescens*, and *Alcaligenes faecalis*, decreased for the first 5 years upon lyophilization and then stabilized to around 10% thereafter [34]. We can therefore argue that the surviving 10% of the population was somehow selected by the storage. It was also reported that freeze drying influenced the antibiotic resistance of the preserved strain [35]. Therefore, the selection of mutants in the lyovials of *A. coloradensis* ATCC 53629 could arise from the long-term storage under suboptimal conditions.

In industrial programs of strain and fermentation improvement, the so-called strain maintenance protocol is routinely applied, based on the selection of the best clonal populations growing on agar media to then scale them up in large-scale, submerged, culture-based fermentations. Selection at this stage is often based on phenotypic traits (colony morphology, color, or simply increased productivity) that are basically applied blindly [28]. In addition, bacterial populations contain phenotypic cell variants that lack morphological change. In *A. coloradensis* ATCC 53629, the classical isolation of different morphologies was fruitful in giving rise to potentially industrial producers with high incidence. A different colony morphology in plate was associated with the increased antibiotic productivity, confirming that the empirical practice based on colony morphology observation still represents a valid tool, especially if applied to the original wild-type isolates, which exhibit some intrinsic heterogeneity. Although

recombinant engineering is increasingly attracting industrial interest, allowing specific genetic modifications in the wild-type background, a preliminary analysis of the wild-type population is recommended to avoid misinterpretation of the results.

From the practical point of view, the isolation of morphological variants of *A. coloradensis* ATCC 53629*,* allowed the identification of high producers with relative ease, indicating that the variability associated to the *Amycolatopsis* chromosome could be an evolutive strategy as already described for *Streptomyces* and *Actinoplanes*. It is worth of note that at least 20% of the morphological variants which we have identified in the population, were unable or seriously hampered in the production of aerial mycelium and spores. This suggests that a general concept of division of labor could apply also to *Amycolatopsis* as already reported for S*treptomyces* and *Actinoplanes* and that the sterile caste could play a crucial role in specific aspects of the *Amiycolatopsis* growth.

In the light of the continuous urge in new, cutting-edge antibiotics [36], it will be of absolute interest to test the antimicrobial activity of the newly identified molecules and also to test the possibility to semi-synthetically improve their clinical characteristics. From the industrial point of view, the production of more than 9 g/L of the avoparcin complex will allow a cheap production process.

Conclusions

A. coloradensis ATCC 53629 clonal populations selected based on their colony morphology produced different amounts of avoparcin in fermentation broth. The different morphologies, with no exclusion, were invariably able to produce a complex of molecules which is consistently different from the one described in the literature [26]. The sum of the new identified factors α– and β– ristosamynil

-avoparcin represents indeed *ca.* 30% of the avoparcin complex upon purification with affinity resins. This is the first description of ristosamynil-avoparcin and this result supports the idea of any single actinomycete as a source of several bio-active molecules which are uncovered whenever suitable conditions arise. Concerning the rise of this peculiar avoparcin complex, we speculated that long term storage in freeze dried condition could have selected recessive mutation/s which determined the production of the new molecules. This observation uncovered the risks of a suboptimal storage system which could result in loss of the original phenotypes by microorganisms deposited in strain collections with a consequent loss in biodiversity and, specifically for *A. coloradensis* ATCC 53629, the potential presence in the illegal market of alternative and unknown (and mostly undetectable by standard tests) avoparcin products.

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Conclusions and outlook

Microorganisms with large genomes are frequently organized in small integrated societies (exemplified by colonies) in which a division of labor occurs. From the industrial point of view, actinomycetes and filamentous fungi are microorganisms with large genomes of paramount industrial relevance able to produce enzymes, antibacterials, anticancer, antifungals, immunosuppressants, and herbicides. Indeed, over 10000 bioactive metabolites produced by actinomycetes (representing more than 45% of all bioactive microbial metabolites discovered) have been described. Furthermore, most of the potential of each single producer remains hidden within their genomes as genome sequencing revealed that actinomycetes, have the genetic information (known also as "biosynthetic dark matter") to potentially produce up to 30-50 secondary metabolites.

Along the present work, we have worked on the hypothesis that in filamentous fungi, actinomycetes and in bacteria with large genomes, a common characteristic is to segregate phenotypes in which the production of secondary metabolites and enzymes is maximized in some individuals of the colony. This portion of the colony, is represented by cells which have lost the capacity to preserve themselves in a durable form (as spores or similar), hence are known as the sterile caste, while reproduction is delegated to another part of the population (the non-sterile caste, generally represented by spores or other durable cells). By use of different approaches, we were therefore selecting the sterile caste. The goal of this selection was the identification, characterization and long-term preservation of industrially relevant lineages. By working on 5 different microorganisms (*Coriolopsis gallica, Bacillus subtilis, Nonomuraea gerenzanensis, Actinoplanes teichomyceticus* and *Amycolatopsis coloradensis*) we have revealed that the isolation of high producers and producers of alternative

metabolites among the representatives of the sterile caste is the rule and not the exception.

We are aware that our approaches have some limitations (basically bound to the use of the correct strategy for each organism) and that further investigations are required for a better understanding of the genetic bases of the variability observed among the clones herein produced, for instance, to assess if mutations in biosynthetic and/or in regulatory genes could have been the reasons for the differences observed in production among different clones. However, what is clear from our study is that integrating strain improvement and strain maintenance approaches with the use of protoplasts, colony selection, and by use of selecting agents can be a successful strategy for unravelling the hidden industrial potential of multicellular microbial strains. We could also argue that in the future, micromanipulation and bacterial cell sorting will evolve to be applicable to mycelial microorganisms, allowing a more focused isolation of genomes of interest. Finally, we can speculate that applying recurrent cycles of vegetative mycelium growth and screening to the most promising clones selected, might be a key for identifying clone(s) with an even higher titer of secondary metabolite/enzyme production than those presented in this study.

Although highly speculative, the present results might also be a cue for providing a different explanation for classical mutagenesis applied to strain improvement. It is indeed accepted that a chemical or physical mutagenic treatment has the result of killing between 90.0% to 99.9% of the treated microorganisms. This implies that when the mutagenic treatment is applied to hyphal fragments, most of the genomes present therein are lethally mutated, with an outcome very similar to the one that we have depicted in this work, i.e., the segregation of single genome cells (particularly evident in the work performed with protoplasts of *A. teichomyceticus* ATCC 31121). Therefore, it cannot be excluded that, at least in

some cases, improved mutants might derive from genome separation, mimicked by genome destruction, rather than from random mutations. This is a fascinating aspect that deserves further investigations.

The general conclusion is that the approach of genome separation must be applied as soon as the producer of a novel metabolite is isolated and it must be applied to the very first environmental isolate which still retains the complete potential to produce improved amounts of the metabolites of interest or even new and more active metabolites. This approach is in principle applicable to any filamentous actinomycete of industrial interest for which protoplasts can be produced, including those for which tools and protocols for genetic manipulation are absent or only in their infancy. With our method, we could simplify the strain maintenance work, at least to the extent to which mutations conferring the high productivity traits are sufficiently stable. This paves the way to a new approach to the protocols of strain improvement and strain maintenance.

Finally, in this PhD thesis, we scraped the surface of the evolutionary meaning of the "biosynthetic dark matter" by attributing to it a fitness advantage which is expressed during the division of labor which occurs in actinomycetes and in other multicellular or colony-forming microorganisms.

Prizes

December 2019: Qilu award (Shandong province, China)

Publications during the PhD period

González V, Vargas-Straube MJ, Beys-da-Silva WO, Santi L, Valencia P, **Beltrametti F**, Cámara B. Enzyme Bioprospection of Marine-Derived Actinobacteria from the Chilean Coast and New Insight in the Mechanism of Keratin Degradation in *Streptomyces* sp. G11C. Mar Drugs. 2020 Oct 28;18(11):537.
doi: 10.3390/md18110537
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Binda, E.; Berini, F.;Marinelli, F.; Bava, A.; **Beltrametti, F.**
Heterogeneous A40926 Self-Resistance Profile in *Nonomuraea gerenzanensis* Population Informs Strain Improvement. Fermentation 2021, 7, 140. doi.org/10.3390/fermentation7030140

Ramoni, G.; Capozzoli, C.; Bava, A.; Foschi, F.; Broggini, G.; **Beltrametti, F.** New Avoparcin-Like molecules from the Avoparcin Producer *Amycolatopsis coloradensis* ATCC 53629. Fermentation 2021, 7, x.
doi.org/10.3390/xxxxx
Submitted for publication

Mellere L, Bava A, Capozzoli C, Branduardi P, Berini F, **Beltrametti F.** Strain Improvement and Strain Maintenance Revisited. The Use of Actinoplanes teichomyceticus ATCC 31121 Protoplasts in the Identification of Candidates for Enhanced Teicoplanin Production. Antibiotics. 2022; 11(1):24. https://doi.org/10.3390/antibiotics11010024

Collaborative projects

Life Biorest – Closed
FuturEnzyme – Ongoing
Life 2020 – Rejected

iPEST – iFERM – Rejected
NATURE WATER – REJECTED
Misura Tech Fast Regione Lombardia (Biocosme) – Ongoing

Biosurf unibrescia
Progetto Sito internet (Bando Connessi) – Ongoing
Smart Money - Ongoing

Collaborations with Academic institutions

Università Tecnica Federico Santa Maria, Valparaiso, Chile
Prof. Michael Seeger Pfeifer
Prof. Beatriz Camara

University of Milano Bicocca
Prof. Paola Branduardi

University of Insubria, Varese
Prof. Flavia Marinelli
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Companies

Co-Founder and shareholder of the Company BioC-CheM Solutions srl (from September 2020). Present position in the company: Chief Scientific Officer (CSO)

Tutor of the following Magister Degree Thesis

Candidate	University	Thesis Title	Internal Tutor	External Tutor	Academic Year
Paolo Tosi	Università Degli Studi di Pavia	Messa a punto delle condizioni di produzione di *Photobacterium phosphoreum*, un biosensore per il monitoraggio della qualità delle acque	Prof. Fiorenzo Pastoni	Fabrizio Beltrametti	2018-2019
Carolina Matteucci	Università Degli Studi dell'Insubria	Produzione di biosurfattanti in funghi isolati dal sito di interesse nazionale (SIN) Ex-carbochimica di Fidenza	Prof. Flavia Marinelli	Fabrizio Beltrametti	2018-2019
Chiara Marcolli	Università Degli Studi dell'Insubria	Cosmetica "green" e surfattanti: nuovi impulsi dalla microbiologia del suolo	Prof. Viviana Orlandi	Fabrizio Beltrametti	2019-2020
Gina Ramoni	Università Degli Studi dell'Insubria	Nuove sfide da vecchi prodotti. Produzione e purificazione dell'antibiotico glicopeptidico Avoparcina da	Prof. Luciano Piubelli	Fabrizio Beltrametti	2018-2019

		Amycolatopsis coloradensis			
Giulia Bortolot	Università Degli Studi di Milano-Bicocca	La sfida del controllo qualità nelle fermentazioni industriali. Luci ed ombre di pigmentazioni mutevoli e contaminazioni ricorrenti	Prof. Paola Branduardi	Fabrizio Beltrametti	2019-2020
Fabiana Pistolesi	Università Degli Studi di Milano-Bicocca	Sfide dell'identificazione chimica e della produzione industriale di un nuovo biosurfattante microbico	Prof. Paola Branduardi	Fabrizio Beltrametti	2020-2021
Luca Mellere	Università Degli Studi di Milano-Bicocca	Preparazione ed utilizzo di protoplasti nei processi di strain improvement e maintenance del ceppo produttore di teicoplanina *Actinoplanes teichomyceticus* ATCC 31121	Prof. Paola Branduardi	Fabrizio Beltrametti	2020-2021
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Editorial activities

Guest editor:
Special Issue "Fermentation Processes to Produce Specialized Metabolites"
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