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REVIEW

Current approaches to exploit actinomycetes as a source of novel natural products

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Abstract For decades, microbial natural products have been one of the major sources of novel drugs for pharmaceutical companies, and today all evidence suggests that novel molecules with potential therapeutic applications are still waiting to be discovered from these natural sources, especially from actinomycetes. Any appropriate exploitation of the chemical diversity of these microbial sources relies on proper understanding of their biological diversity and other related key factors that maximize the possibility of successful identification of novel molecules. Without doubt, the discovery of platensimycin has shown that microbial natural products can continue to deliver novel scaffolds if appropriate tools are put in place to reveal them in a cost-effective manner. Whereas today innovative technologies involving exploitation of uncultivated environmental diversity, together with chemical biology and in silico approaches, are seeing rapid development in natural products research, maximization of the chances of exploiting chemical diversity from microbial collections is still essential for novel drug discovery. This work provides an overview of the integrated approaches developed at the former Basic Research Center of Merck Sharp and Dohme in Spain to exploit the diversity and biosynthetic potential of actinomycetes, and includes some examples of those that were successfully applied to the discovery of novel antibiotics.

Keywords Natural products · Actinomycetes · Microfermentation · Dereplication · Drug discovery

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Introduction

In the last decade, screening of microbial natural products (NPs) as sources of novel lead candidates for the development of new drugs has been vanishing from industrial laboratories to find refuge in academic and small biotechnology companies. It is well known that strong competition from synthetic and combinatorial libraries associated with ultrahigh-throughput screening platforms has represented a serious challenge to the natural products drug discovery area. This challenge has not been addressed in terms of technology innovation, compatibility with new screening technologies, productivity, and development times as required by large pharmaceutical companies [41, 55, 60]. Nevertheless, in spite of this loss of expertise, new microbial natural products have continued to be described at a low rate by smaller biotechnology companies and academic research groups [52], and today NPs remain a continuing source of inspiration for synthetic chemists, with some signs of renewed interest in their potential [6, 32, 46].

For more than 50 years, the natural products drug discovery group of the former Basic Research Center of Merck Sharp & Dohme (MSD) in Spain has been working in natural products drug discovery, with a long track record of identifying novel molecules, including several drugs on the market (cephamycin, thienamycin, lovastatin, and caspofungin) [65]. In the last 10 years this group contributed

significantly to the discovery of novel molecules and the development of different approaches to cope with the new challenges in the field to exploit microbial resources as producers of novel molecules [11, 77, 78, 84]. This effort has found continuation today in the Fundación Medina, a new nonprofit research organization established as a public–private partnership among MSD, the Andalusian government, and the University of Granada, with a primary focus on the discovery of novel molecules to be developed as potential drug candidates.

Drug discovery from natural products in industrial and academic laboratories has traditionally been focused on empirical exploitation of the most prolific microbial groups: actinomycetes and filamentous fungi. Historically, actinomycetes have been the origin of the largest number of new antibiotic drug candidates and lead molecules with applications in many other therapeutic areas [9, 62]. Today, traditional approaches based exclusively on screening of huge numbers of strains have been exhausted and proven to be totally inefficient for delivery of novel molecules in a cost-effective manner. New approaches have had to be developed to respond to the lack of new molecules as sources of novel privileged scaffolds and to improve the possibility of success of finding new chemical entities as leads for the development of drug candidates.

Novel genomic approaches aimed at exploring the biosynthetic potential of actinomycetes revealed in molecular genomics studies and focused on novel bioengineering and metagenomic tools have seen extraordinary development by many research groups [7, 13, 17, 18, 51, 58], who have shown from different perspectives the cryptic biosynthetic capabilities of these filamentous bacteria and other microorganisms in the environment, and the possibilities of starting to learn how to harness the bacterial genome. In spite of the success of these approaches in revealing examples of novel chemistry, most of these techniques have not yet been tested on an industrial scale. Nevertheless, they demonstrate the challenge that lies in developing these tools to produce new drugs from microbial NPs.

Success in drug discovery from microbial products still relies on the proper combination of some key factors that need to be met at some point in the screening process and that have been valid for decades. From the microbiology point of view these include the selection of appropriate individual strains, which can also be translated into genomic approaches able to access new gene pools involved in production of potential novel metabolites, and second, identification of adequate cultivation conditions or use of recombinant clones in appropriate surrogate hosts, switching on expression and ensuring production of novel chemistry. Both factors, in conjunction with selection of druggable biological targets, are still valid elements today in the drug discovery equation, but they need to be approached from a different perspective and with the help of the new analytical technology that has rapidly evolved during the last decade.

Our research in natural products took advantage of some of these technology developments to explore from a different perspective how to improve the process and bring novelty to NP drug discovery. In recent years we benefited among others from developments in high-throughput DNA sequencing that has ensured rapid and cheap identification of producing organisms, commercially available miniaturized microfermentation devices that enabled a change of scale in exploring new nutritional conditions for production of novel compounds, or improved analytical and chemical isolation methods that ensured rapid detection and characterization of compounds; together, these have yielded new insights into microbial diversity and production of bioactive molecules that would have been inaccessible in such a short period of time with classical screening programs.

Access to biodiversity: mining for novel actinomycetes species in the environment

Access to the microbial diversity of actinomycetes in the environment has traditionally been focused on intensive sampling from a wide diversity of geographical locations and habitats. Large numbers of samples were processed empirically by general isolation methods, ending in most cases with the recurrent isolation of the predominant species in these habitats in spite of the biogeography of the samples used. Millions of strains have been isolated and screened over the decades in industrial laboratories, and the probability of isolating novel compounds from common and frequently found related species is today too low to be worth the effort. In spite of the estimates regarding the potential production of unknown novel molecules by *Streptomyces* [79], a large number of species are widespread across many different environments, and they frequently produce well-known and structurally related molecules that have to be identified and discarded as early as possible in drug discovery programs. Contrary to the theory that proposes that everything is everywhere, specific environmental conditions are strong selecting factors for specific microbial assemblages, and the distribution of some microbial species, even in widely occurring taxa, exhibits biogeographic patterns mostly determined by these microenvironmental conditions that may translate into novel chemistry [26, 48, 64, 70]. The evidence strongly suggests that minor species or genetically distinct strains of actinomycetes that have not yet been cultured under laboratory conditions still occur in most environments, and new species are being systematically described that might be the source of novel chemistry with potential new

activities. On the basis of this working hypothesis, we directed exploration towards a large variety of sources, including specific terrestrial niches, plant host associations with unique characteristics, and marine environments. These approaches have emphasized exploration of untapped actinomycetes communities that might be associated with rhizospheres, plant endophytes, lichens, and endolithic bacteria [29, 57], as well as marine sediments and invertebrate-associated actinomycetes, which might guide isolation of novel microbial communities potentially producing novel chemical scaffolds. Our results were confirmed by the work of other groups that have also shown that targeting the right microbial space can deliver novel microbial diversity [14, 28, 36, 49, 54, 72].

This sourcing approach, combined with novel isolation methods developed in house or by different groups targeting cultivation of species underrepresented or previously not cultivated under laboratory conditions [20, 31, 37, 66–68], ensured the isolation of novel or less frequently occurring species within the actinomycetales that are today common members of our screening collections. Most of these methods were focused on selective isolation of members of these taxa through use of poor nutritional media devoid of carbon sources, and including in some cases subinhibitory concentrations of antibiotics that might favor development of slow-growing representatives of these microbial communities after weeks of incubation. Whereas some specific enrichments in certain taxa have been described in the last 15 years [33, 40, 83], the most productive approaches have resulted from systematic isolation of distinct morphological colonies occurring in nutrient-poor and slow-growing isolation plates [37], independently of their filamenting appearance. A good example of experimental data that validate this approach was obtained from the systematic isolation and partial 16S rDNA sequencing of a group of 790 actinomycetes colonies obtained from three randomly selected soil samples that were tested following these methods. Whereas 57% of the strains studied could be assigned to the genera *Streptomyces* and *Micromonospora*, 43% of the isolates were distributed in as many as 39 minor genera distributed into 111 species that could only be recognized by sequence analysis (Fig. 1). In addition to the huge diversity of minor occurring genera, we identified more than 76 species among the 246 *Streptomyces* strains, highlighting the effectiveness of these methods for retrieval of additional microbial diversity from these soils.

These strategies have been applied to ensure the construction of one of the most diverse microbial collections in industry, with more than 58,000 actinomycetes strains currently at Fundación Medina. Less than half of this collection has been identified at genus level, of which 55%

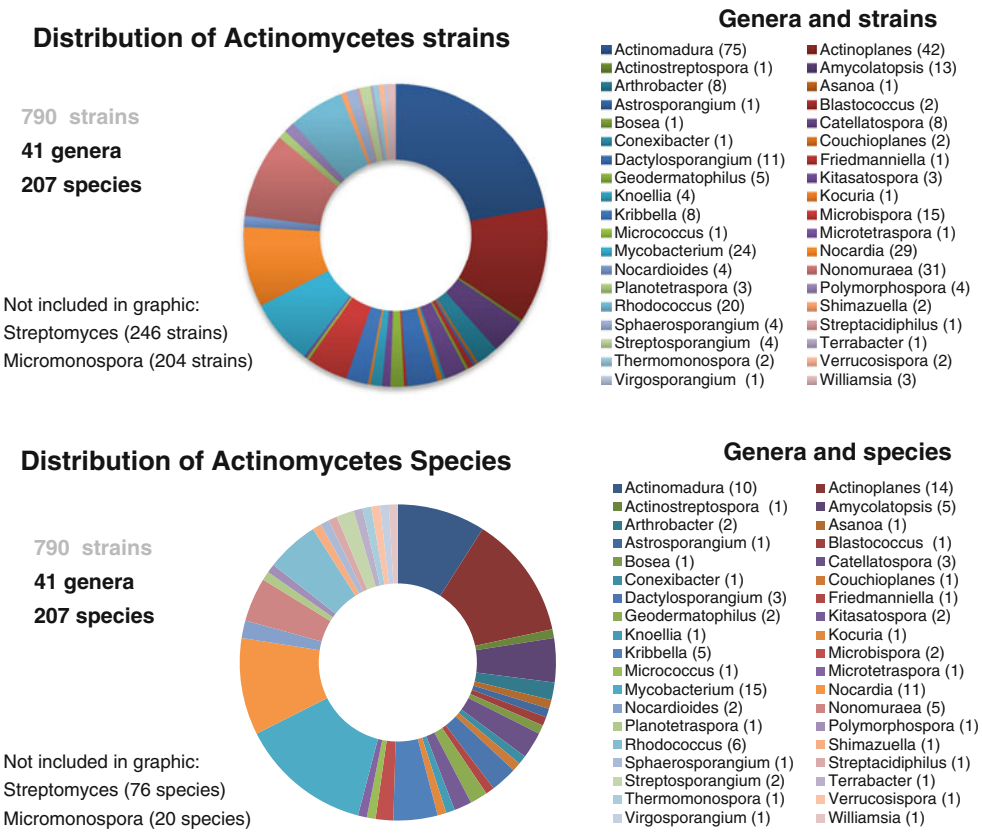

Fig. 1 Distribution of actinomycetes species and strains obtained from systematic isolation and partial 16S rDNA sequencing of colonies obtained from three environmental soils

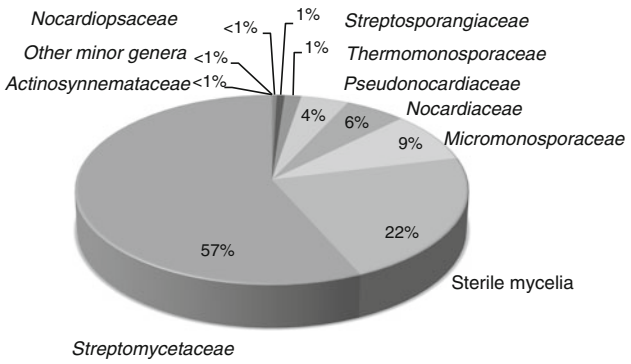

Fig. 2 Taxonomic diversity of the actinomycetes collection. The screening collection contains 53,300 strains, of which 27,400 have been identified at genus level. Sterile mycelia: nonsporulating strains devoid of morphological taxonomic characteristics

correspond to *Streptomyces* species selected from a wide diversity of habitats and geographical origins. More than 45% of the strains in the collection belong to the major lineages of the so-called rare actinomycetes or non-*Streptomyces* (Fig. 2). Whereas the majority of the strains are of terrestrial origin, a marine program initiated in the last decade rapidly contributed to the diversification of the collection with a small group of 5,000 marine isolates obtained from sediments and marine invertebrates sampled at different depths at tropical and temperate Atlantic latitudes.

Partial 16S rDNA sequencing has been carried out to confirm the taxonomic affiliation of a large fraction of this screening collection and to assess molecular data on its microbial diversity and individual phylogenetic relationships. In spite of the high value of this data, this method was rapidly shown to be inefficient to be applied upfront as a selection criterion. The intraspecies heterogeneity existing in actinomycetes taxa could not be resolved in phylogenetic inner branches, requiring alternative dereplication tools to assess the uniqueness of potential new strains. Levels of intraspecies variability among strains from the same species obtained from the same environment have frequently been observed [2, 30] and make it necessary to introduce additional molecular fingerprinting tools for selection. From the different molecular fingerprinting methods available [Amplified Fragment Length Polymorphism (AFLPs), Random Amplified Polymorphic DNA (RAPDS), Repetitive sequence-based PCR (REP-PCR) fingerprinting], random BOX-PCR amplification (PCR based on primers targeting the highly conserved repetitive DNA sequences of the BOX element of *Streptococcus pneumoniae*) was shown to be one of the most useful tools to detect variability within actinomycetes species and to establish the relatedness of isolates [44, 74, 75, 80]. Other complementary tools were developed in our laboratory, focusing on detection of Polyketide Synthase (PKS) and Non Ribosomal Peptide Synthetase (NRPS) biosynthetic genes. They were also used to develop rapid strain fingerprints on the basis of biosynthetic genes restriction pattern that might provide additional information on their diversity and uniqueness from the biosynthetic perspective [4, 5]. The systematic 16S rDNA sequence analysis applied on strains producing biological activities was extremely useful to reveal the high rate of strains from a given niche and associated with different taxonomic affiliations that produced the same or related compounds. We have not evaluated whether horizontal transfer events lie beneath these observations, but studies on microbial evolution and convergence of biosynthetic pathways suggest that they may be responsible to a large extent [1, 25, 27, 50]. This is why intensive exploitation of a substrate to identify novel lineages from the taxonomic point of view does not always guarantee a final outcome of novel chemistry. These are some questions that we needed to take into account when we developed the strategy to build the screening extract collection. The growth of this dynamic sample collection was limited in the number of samples by a given combination of strains and production conditions, making always critical the selection criteria applied.

Miniaturized fermentation and scale-up

Whereas the actinomycetes, together with filamentous fungi, are the most prolific producers of bioactive metabolites, and the rediscovery problem has become a hurdle in all screening programs, genome sequence analyses of most important actinomycetes producers have shown that a large fraction of their genomes still remains unexploited, and that switching on silent pathways might reveal production of novel molecules [8, 22, 35, 53, 73]. Other mining studies applied on a large collection of reference strains suggested that almost all actinomycete lineages contain biosynthetic information and should have the potential to produce bioactive compounds [5]. Traditionally, production of most known compounds has been triggered just by empirical changes in cultivation parameters and a limited number of stress conditions (media composition, rheology, aeration). In fact, use of a limited number of three to four conditions at a time was generally accepted as sufficient to exploit production of new secondary metabolites [81]. Today, in spite of the numerous whole-genome studies confirming the presence of this huge biosynthetic potential, in many cases only revealed as cryptic pathways, we still do not know the nutritional requirements and physiology of most of the groups of strains screened and the key elements involved in the regulation of their secondary metabolite production. Furthermore, when turned on, many of these pathways probably only show residual expression, producing therefore novel molecules quite far below our

analytical detection limits. The one strain, many activities (OSMAC) approach aims to exploit in part this microbial biosynthetic potential by manipulating the production conditions of the strains [12]. We wanted to evaluate the possibility of triggering production of new secondary metabolites with empirical testing of multiple nutritional conditions using miniaturized parallel fermentation devices. The use of high-throughput miniaturized formats for cultivation of bacteria has been previously explored by other groups [21, 23, 86], but the high-throughput cultivation of bacteria offered by System Duetz allowed fermentation in volumes as low as 750 µl in 96-well plates that were later easily integrated into highly automated extraction and screening platforms [23, 24]. This technology was applied to study and develop new production conditions for major taxonomic groups of actinomycetes (*Streptomyces*, *Micromonosporaceae*, *Streptosporangiaceae*, *Pseudonocardiaceae*, *Nocardiaceae*, and *Thermomonosporaceae*), according to their terrestrial or marine origin, environmental stresses or nutrients. Whereas the original microplates described in the literature had only been tested with a limited number of taxa [24], we found no major issues in cultivation and optimization of growth conditions for all major taxonomic groups of actinomycetes, in a large variety of complex and synthetic liquid media, even with long incubations extending up to 14 days without observing well evaporation. This new possibility of testing a large number of nutritional conditions in parallel offered the possibility to explore minor groups of isolates and understand their requirements in some cases in as many as 20 different media. These included varied compositions in terms of carbon sources, inorganic or complex nitrogen sources, trace elements, buffers, and phosphate levels, with relatively low investment in time and resources. An average of eight to ten production conditions was shown to be a good balance between the number of bioactivities obtained from the fermentation extracts and the effort invested in their preparation. Without increasing our resources, we managed to increase by fivefold the number of extracts ready to be evaluated for production of antibiotic activities. Activities against the Gram-positive pathogen *Staphylococcus aureus* were scored as zones of inhibition and as a representative measure of production rate. This progressive increase in the number of nutritional conditions was translated to all the taxonomic groups tested in terms of an increase in the number of antibiotic activities produced, reaching a plateau only after addition of the eighth to tenth condition. In the course of the screening we studied the effect of the progressive increase of the number of cultivation conditions investigated on antibiotic activities and confirmed that production of antibacterial activities in the traditional three-media approach could be increased in *Streptomyces* by 25% with the introduction of a combination of eight media. Figure 3 shows the results obtained with two groups of strains of 400 *Streptomyces* and 480 non-*Streptomyces*, respectively, cultivated in arrays of combinations of eight media optimized specifically for each group. The addition of more production media had a more drastic effect on the production of activities by the so-called non-*Streptomyces* strains, which increased by 32%. We confirmed, after a cumulative experience of fermentation weeks using different sets of media and strains, that only good selection of media compositions could reduce the number of strains active in multiple conditions. The use of eight conditions ensured as many as 30% of the *Streptomyces* activities obtained in only one medium, whereas in non-*Streptomyces* this number reached 40% of the activities. These and similar results already reported with microfermentations of filamenting fungi cultivated in multiple conditions [10] provide evidence supporting that proper manipulation of media conditions can further promote the biosynthetic potential of microorganisms [12] and increase the chances of detecting novel molecules from active extracts selected to be pursued in chemical isolation projects.

A second challenge to this miniaturization of production conditions is scaling up and reproducing antibiotic activities detected with microfermentations in larger fermentation formats. Different approaches were tested to mimic in large volumes the fermentation conditions of oxygen transfer and shear forces developed in the microwells, including selection of square bottles for cultivation instead of Erlenmeyer flasks. The application of Liquid Chromatography–Mass

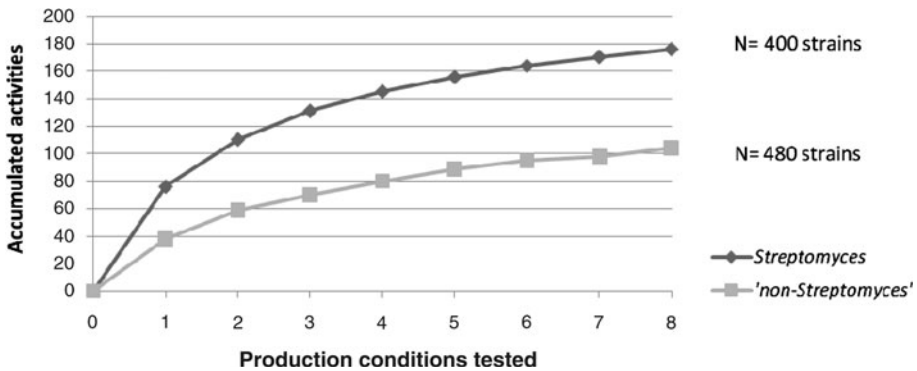

Fig. 3 Influence of nutritional conditions on production of bioactivities using 96-deep-well miniaturized fermentations

Spectrometry (LC–MS) to track total masses of positive ions as a direct measure of production of metabolites has been extremely useful to monitor the performance of strains in these different formats. We observed that, in most cases studied, the total volume and geometry of the container was rarely found to be a limiting factor in the production of antibiotic activities, and very good correlations were obtained between LC–MS profiles from strains grown in microwells and those grown in square bottles or Erlenmeyer flasks.

These microfermentations have been shown to be extremely useful for upfront evaluation of large numbers of strains and nutritional conditions to identify the best production media for a metabolite of interest. One of the limitations of this approach is determined by the small amount of extract (no more than 0.5 ml) that can be generated from microwell fermentation, which can be an issue if larger volumes are required for primary screening and hit detection. In spite of these limitations, the good reproducibility of microfermentations ensures that large-scale regrowth resources are focused purely on hits of interest.

Early LC–MS annotation and dereplication of antibiotic activities

One of the approaches used for discovery of novel antibiotics from massive screening of extracts generated by high-throughput cultivation included upfront empirical evaluation of antimicrobial activities against a wide panel of multiresistant bacterial and fungal pathogens. To be considered active, extracts were required to exhibit potent activity against at least *Staphylococcus aureus* and/or *Candida albicans* (Fig. 4). One of the major expected problems associated with this empirical screening was the continued rediscovery of known molecules and the need for implementation of an analytical approach to identify and eliminate these molecules from the process as early as possible. Different analytical approaches have been developed by other groups to address these limitations [19, 43, 71], and early dereplication of known molecules was ensured in our case through systematic analysis of combined LC–MS and Ultra Violet (UV) profiles of each of the active extracts, and screening of these data against a proprietary LC–MS library of known antibiotic compounds.

In a period of 2 years, we performed intensive low-resolution LC–MS analysis of more than 28,150 bioactive extracts obtained from 8,670 actinomycetes, from which we identified 22% of strains producing molecules matching 190 different entries in our antibiotic library. The matching patterns differed according to the group of producing strains, and 42% of the 5,530 *Streptomyces* strains tested were found to produce known compounds in spite of efforts to challenge these strains with new nutritional conditions. Besides the widely occurring ionophore nocardamine, actinomycins, polyenes, and nigericins are among the compounds repeatedly found in *Streptomyces* extracts, and in some cases in as many as 8% of samples (Fig. 5). In only 2 years of screening, more than 130 different known molecules from major classes of known antibiotics and

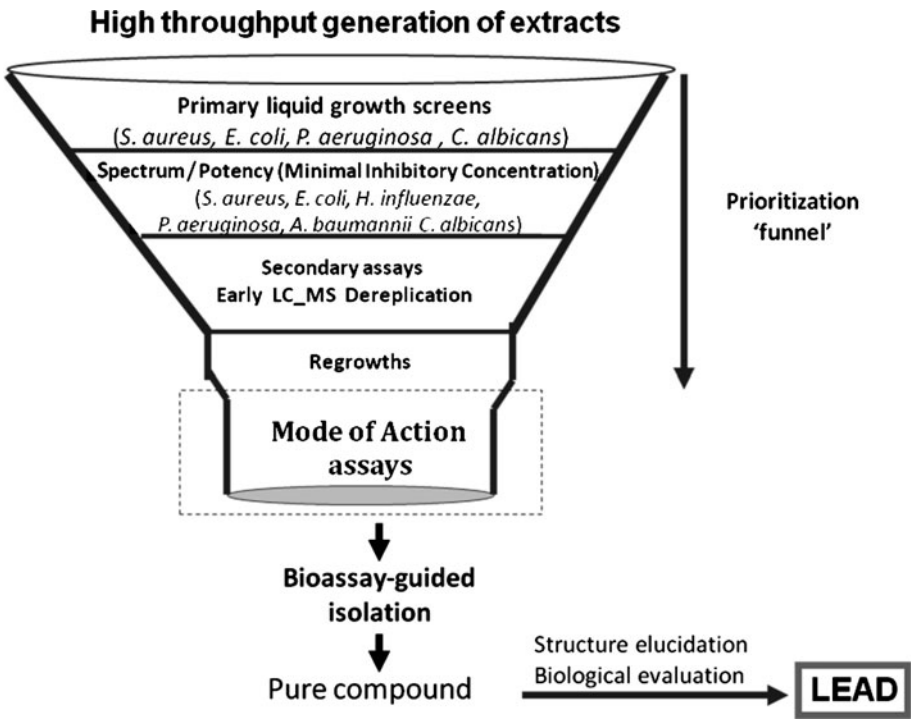

Fig. 4 Empirical high-throughput screening

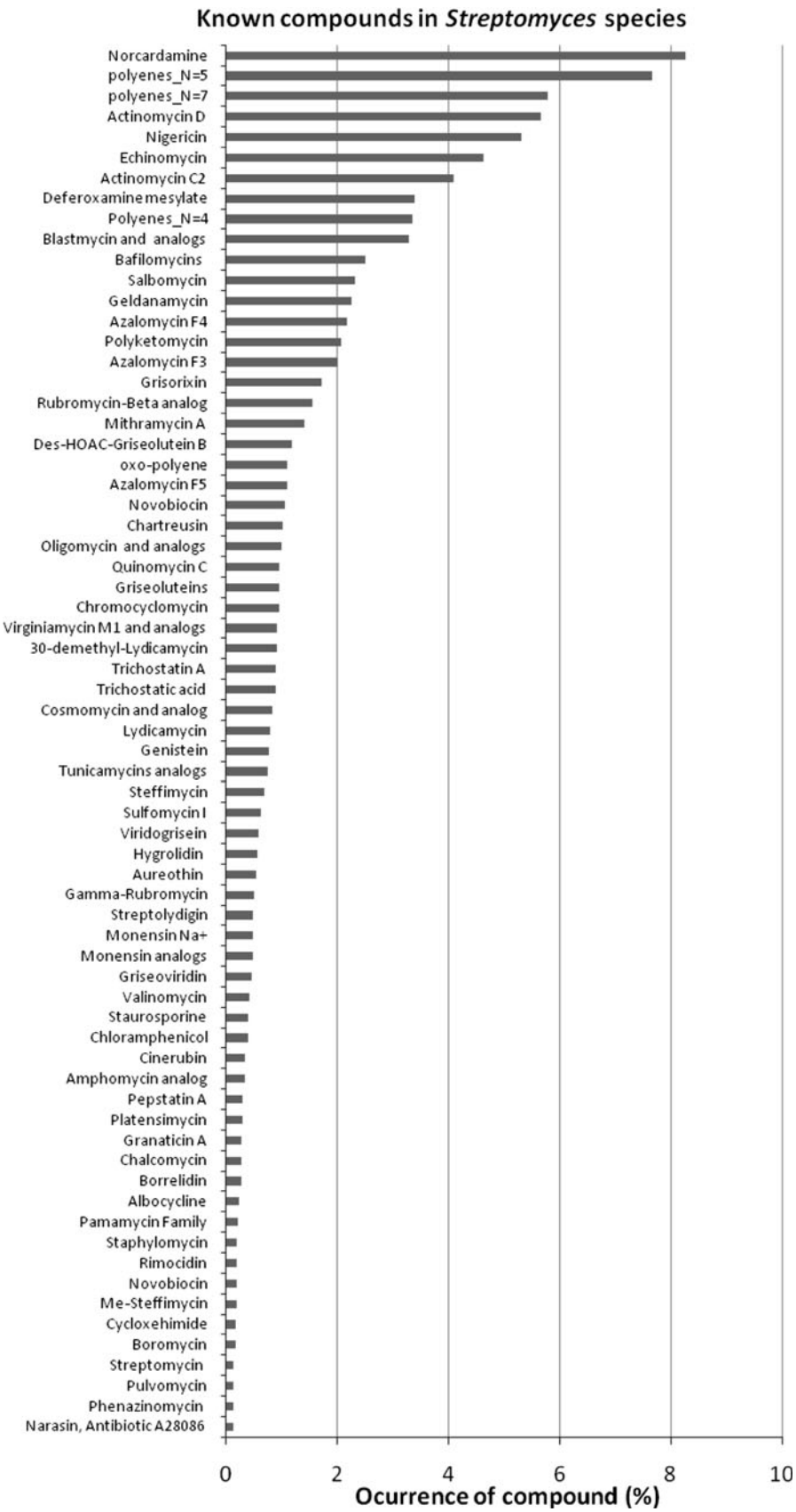

Fig. 5 Known compounds detected in *Streptomyces* extracts. Only those with percentages above 1% are presented

their analogs were identified in *Streptomyces* extracts. In spite of the wide occurrence of known molecules, and the difficulty in identifying these compounds early in the process, *Streptomyces* may produce unrevealed treasures continuously masked by potent or highly produced bioactive molecules. There is no general agreement about the best strategy to tap these novel compounds, but new approaches should take advantage of the continued expanding knowledge of the genome and metabolome of *Streptomyces* [1, 16], technical advances that permit semi-automated generation of enriched semifractionated samples in screening [15, 76], high-resolution analytical LC–MS-NMR techniques to identify the presence of scaffolds in partially purified samples [42, 47], and generation of 3 dimensional (3D) databases that will foster future virtual mining of natural products [56].

On the contrary, many fewer molecules produced by so-called minor genera were found in our database of known compounds, as observed from the accumulated data from 3,140 strains of as many as 44 actinomycetes genera. The pattern of known compounds was completely different, and only a limited number of extracts (19%) matched an entry in the database. The occurrence of known compounds varied depending on the taxon. For the genera including the most active strains studied, the percentage ranged from 5% in *Actinomadura* spp. to 9% in *Nocardia* spp., 13% in *Micromonospora* spp., 21% in *Actinoplanes* spp., to 38% and 47% in *Pseudonocardia* spp. and *Amycolatopsis* spp., respectively. Furthermore, most of the compounds frequently observed in these groups did not correspond to those frequently produced by *Streptomyces*. Our data did not suggest any direct relationship between any given genus and families of compounds. Frequently the same compound could be detected as being produced from unrelated species, supporting the wide distribution of many of these biosynthetic clusters (Table 1).

Natural products and actinomycetes lineages

In addition to the LC–MS annotation of the active extracts, we generated in parallel 16S rDNA sequences of all the active strains to confirm their taxonomic affiliation and to evaluate the potential relatedness of the strains. The collection and analysis of more than 3,500 16S rDNA sequences from active strains provided an extremely useful way to assess their taxonomic identification, although it rapidly became evident that the 16S rDNA sequence was insufficient to resolve the relatedness of many of the strains of the same species, especially in the inner branches, and with regard to the compounds produced. This information was used to map the known metabolites produced in all these taxa, trying to correlate the phylogenetic data with the compounds produced. Besides the partial information from public culture collections, few studies that monitor the distribution of known molecules across large numbers of different actinomycetes species known to produce bioactive molecules have been reported. To assess whether species- or genus-specific molecules detected in our screening could be associated with any taxon, we evaluated the phylogenetic relationship and known compounds being produced by the strains. In addition to the small numbers of molecules identified by LC–MS, families of compounds could not be specifically associated with any given lineage (Table 1). In the case of *Micromonosporaceae*, no general pattern was observed, although rifamycin was detected repeatedly in different and unrelated *Micromonospora* species [34], and as previously described, in isolates of *Salinispora* spp. (Fig. 6) [39]. Rifamycin is also produced by different *Amycolatopsis* spp. (Fig. 6) as observed in the analysis of the *Pseudonocardiaceae* spp., where a reduced number of strains produce known compounds. Contrary to what has been observed in filamentous fungi [10], where small molecules could be associated to some extent with specific families of fungi, the production of frequently occurring compounds studied in several actinomycetes families is widespread across the different lineages and cannot be related to any specific taxon, probably reflecting horizontal gene transfer events that have been reported as occurring among actinomycetes [25, 45, 69] in the environment.

The platensimycin case

The discovery of the antibiotic platensimycin at Merck is one of the best examples of success resulting from the confluence of key elements in drug discovery: the right strain, the right production conditions, and the right target-based screening. Platensimycin, a novel molecule with a completely new mode of action, was discovered from an antisense differential sensitivity screening strategy focused on selective inhibitors of synthesis of fatty acids [59]. Platensimycin selectively inhibits the elongation condensing enzyme *Fab*F of the bacterial fatty-acid synthesis pathway, whereas the related compound platencin is an inhibitor of both the initiation condensing enzyme (*Fab*H) and elongation enzymes (*Fab*F) [77, 78]. Platensimycin was originally identified from a strain of the genus *Streptomyces* isolated from a South African soil. A second producer was rapidly identified from another *Streptomyces*, this time isolated from a soil collected in the Balearic Islands. Both strains exhibited similar morphologies and, after 16S rDNA sequencing, both isolates were confirmed as *Streptomyces platensis* in spite of the lack of morphological similarities with the type strain. In the process of screening of the 125,000 extracts that were tested from

Table 1 Taxonomic diversity of species producing known natural products as detected by low-resolution mass spectrometry in crude extracts from minor genera of actinomycetes

NP match	Producing taxon
Rudolfomycin	*Pseudonocardia* sp.
30-Demethyl-lydicamycin	Non-*Streptomyces*
Actinomycin C2 and D	*Amycolatopsis* sp., *Micromonospora* sp.
Amphomycin	*Actinoplanes* sp.
Azalomycin F3, F4, and F5	*Amycolatopsis* sp.
Bafilomycin B1, C, and E	*Micromonospora* sp.
Blastomycin and analogs	*Micromonospora* sp.
Bottromycin A2 and B2	*Amycolatopsis* sp., *Micromonospora* sp.
Chloramphenicol	*Micromonospora* sp.
Chromocyclomycin	*Amycolatopsis* sp.
Cinerubin	*Pseudonocardia* sp.
Cosmomycin D and analog	*Actiinomadura* sp.
Deferoxamine mesylate	*Micromonospora* sp., *Nocardia* sp.
Des-HOAC-Griseolutein B	*Amycolatopsis* sp., *Saccharopolyspora* sp., *Micromonospora* sp., *Nocardia* sp.
Echinomycin	*Pseudonocardia* sp.
Erythromycin	*Saccharopolyspora* sp., *Micromonospora* sp., *Rhodococcus* sp.
Flavofungin II or analog	*Pseudonocardia* sp.
Fluvirucin analog	*Micromonospora* sp.
Gamma-rubromycin	*Actinomadura* sp.
GE2270 A	*Nonomuraea* sp.
Geldanamycin	*Amycolatopsis* sp., *Pseudonocardia* sp.
Genistein	*Actinomadura* sp., *Actinoplanes* sp., *Couchioplanes* sp., *Dietzia* sp., *Kribella* sp., *Micromonospora* sp., *Nocardia* sp., *Nonomuraea* sp.
Heliquinomycin aglycone	*Actinoplanes* sp.
Histidomycin A and B	*Micromonospora* sp.
Hygrolidin	Non-*Streptomyces*
Isoquinocycline B	*Micromonospora* sp.
Lobophorin A and B	*Dietzia* sp., *Rhodococcus* sp.
Lysolipin I	Non-*Streptomyces*
Medermycin methyl ester	*Micromonospora* sp.
Mithramycin A	*Amycolatopsis* sp., *Saccharothrix* sp.
Monensin and analog	*Pseudonocardia* sp.
Mycosubtilin analog	*Nocardia* sp.
Narasin and analogs	*Micromonospora echinospora*
Nargenicin B1	*Nocardia brasilensis*
Nigericin	*Amycolatopsis sacchari*, *Nocardia cyriacigeorgica*, *Saccharothrix flava*
Nocathiacin I, II and analog	*Amycolatopsis fastidiosa*
Norcardamine	*Amycolatopsis orientalis*, *Micromonospora* sp., *Nocardia* sp., *Pseudonocardia* sp.,
Novobiocin	*Micromonospora brunnea*, *Nocardia* sp, *Pseudonocardia* sp.
Oxanthromicin	*Nocardia carnea*, *Pseudonocardia* sp.
Pamamycin family	*Amycolatopsis kentuckyensis*
Pepstatin A	*Pseudonocardia* sp.
Polyene_N = 4	*Actinokineospora diospyrosa*, *Amycolatopsis kentuckyensis*, *Micromonospora* sp., *Saccharothrix australiensis*
Polyene_N = 5	*Actinomadura* sp., *Actinoplanes digitatis*, *Amycolatopsis lexingtonensis*, *Micromonospora* sp., *Nocardia cyriageorgica*, *Saccharothrix cryophilis*
Polyene_N = 7	*Actinoplanes brasiliensis*, *Amycolatopsis orientalis*, *Lechevalieria xinjiangensis*, *Micromonospora* sp., *Nocardia cyriageorgica*
Polyketomycin	*Couchioplanes caeruleus*, *Pseudonocardia* sp.
	Pseudonocardia sp.
Puromycin	*Amycolatopsis* sp.

Table 1 continued

NP match	Producing taxon
Pyrroxamycin	Non-*Streptomyces*
Quinocycline A	*Micromonospora* sp.
Quinomycin C	*Pseudonocardia* sp.
Rifamycin S	*Actinomadura* sp., *Amycolatopsis vancoresmycetica*, *Micromonospora* sp., *Salinispora* sp.
Rimocidin	*Micromonospora* sp., *Pseudonocardia* sp.
Rubromycin beta	*Actinoplanes penicillatus*, *Pseudonocardia* sp.
Salbomycin	*Pseudonocardia* sp.
Setomimycin	Non-*Streptomyces*
Soframycin	Non-*Streptomyces*
Spiramycin sulfate and analog	Non-*Streptomyces*
Staphylomycin analog	Non-*Streptomyces*
Staurosporine	*Actinomadura* sp., *Rhodococcus* sp., *Salinispora* sp.,
Steffimycin	*Micromonospora melanospora*
Streptothricin F	*Actinoplanes* sp.
Tetracycline analog	*Amycolatopsis* sp.
Thiolactomycin	*Nocardiopsis* sp., *Verrucosispora* sp.
Valinomycin	*Micromonospora* sp., *Nocardia* sp.
Viridogrisein	Non-*Streptomyces*

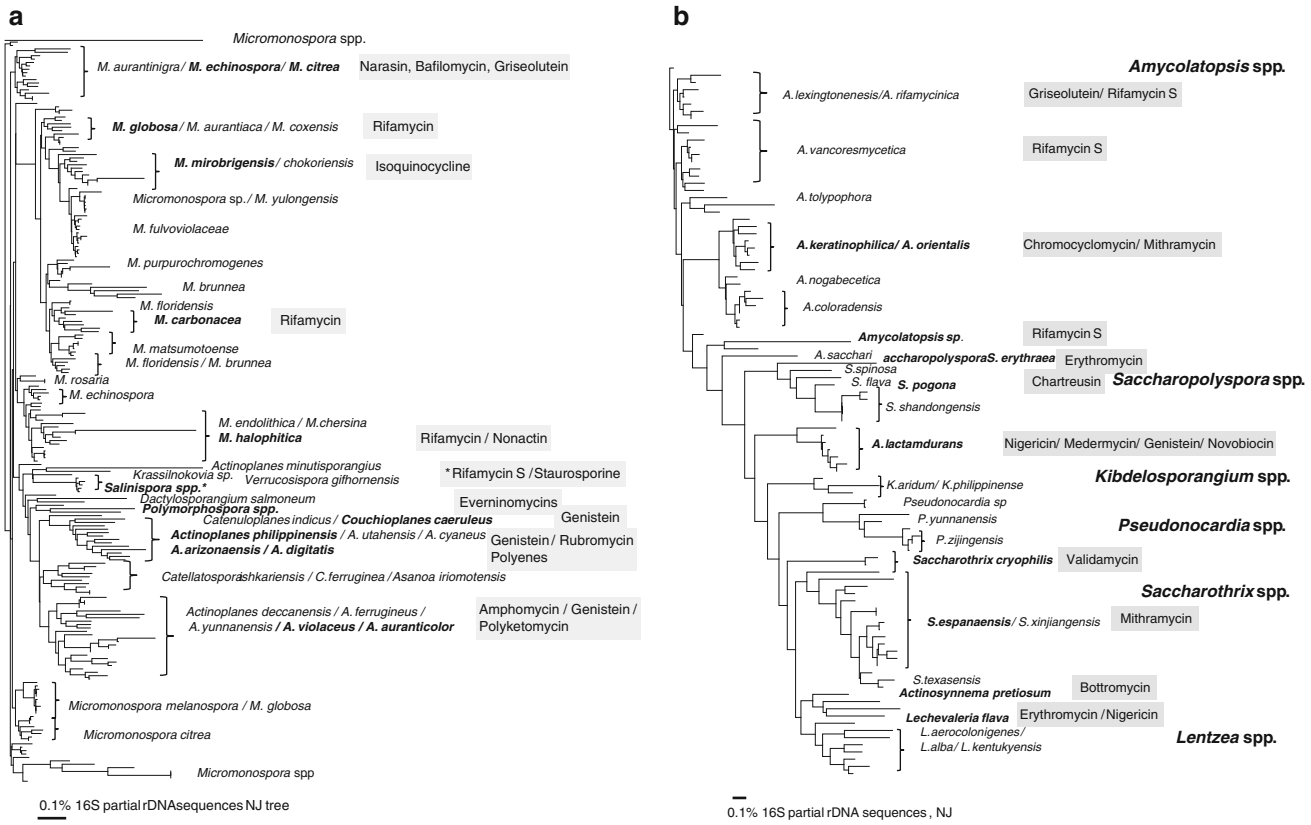

Fig. 6 Detection of known metabolites across the different species of active strains of *Micromonosporaceae* (**a**) and *Pseudonocardineaceae* (**b**) producing antibacterial or antifungal compounds. In both cases the phylogenetic tree was based on a neighbor-joining clustering analysis of partial 16S rDNA sequences of 218 *Micromonosporaceae* and 100 *Pseudocardineaceae*

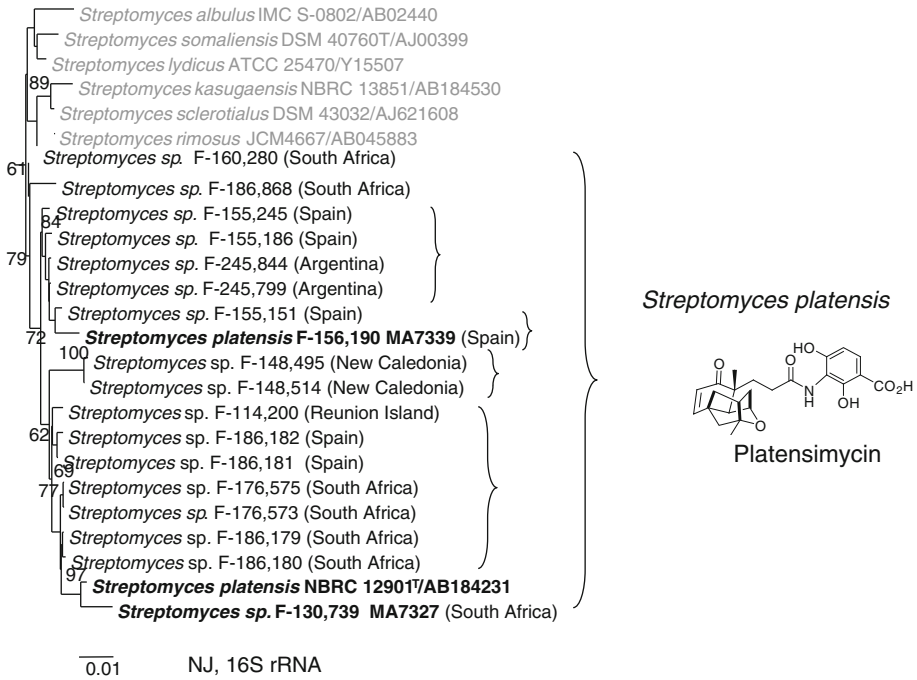

Fig. 7 Diversity of *Streptomyces platensis* strains producing platensimycin. The phylogenetic relationship was established upon neighbor-joining analysis of aligned complete 16S rDNA sequences and compared with the type strain *S. platensis* NBRC 12901^{T}

natural products libraries [82], it rapidly became evident that the molecule could be identified repeatedly in our collection as produced by multiple isolates, although these corresponded exclusively to members of the genus *Streptomyces*. A mass-spectrometry analytical method was put in place to detect early in the process the presence of this compound in extracts with activity against *Staphylococcus aureus*. Following this approach we identified in the early stages of the screening campaign at least 18 actinomycete strains from five different geographical origins initially recognized as producing platensimycin as confirmed by low-resolution mass spectrometry. Independently of their origin, all the strains exhibited similar morphological characteristics and could be identified as *Streptomyces* spp. 16S rDNA sequence analysis of all these isolates confirmed the taxonomic affiliation, being identified as members of the species *S. platensis* (Fig. 7). We tested the production rates of all these strains in several nutritional conditions using microfermentations. In spite of the taxonomic relatedness of the strains, the production conditions were strain dependent, with titers of the compound ranging from 2 to 9 μg/ml being obtained in only one or two liquid media, far from the 323 μg/ml obtained with unregulated overproducing mutant strains [63] (Fig. 8). Further studies with these wild-type strains have shown that intraspecific diversity not revealed by ribosomal sequencing approaches does exist among the platensimycin producers, and that this diversity translates into differential expression of metabolites observed by electrospray injection mass spectrometry (data not shown). Whereas the molecule could not initially be identified in crude extracts without prior knowledge of its presence, platensimycin was detected with rather high frequency later in the course of the screening, being present in as many as 0.3% of tested *Streptomyces* extracts, and it had surprisingly remained untapped for decades until the proper assay was used to bring it to light.

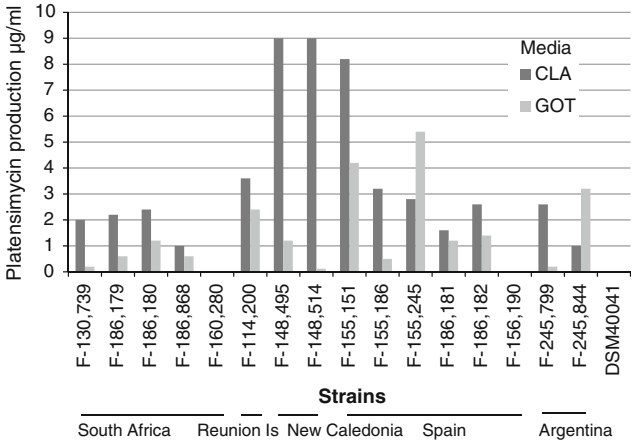

Fig. 8 Platensimycin production in liquid media using miniaturized deep-well fermentations. LC–MS detection: concentration ratios were established with reference to the purified platensimycin level produced by MA7327 (F-130,739): 2 μg/ml. (Production was followed for 7 days in two complex media, CLA and GOT; production was not detected in these media in the strains F-180.280 and F-156.190)

The different approaches mentioned above, including the dereplication tools applied in the early detection of other known compounds across the different groups of actinomycetes tested, have also favored the discovery of

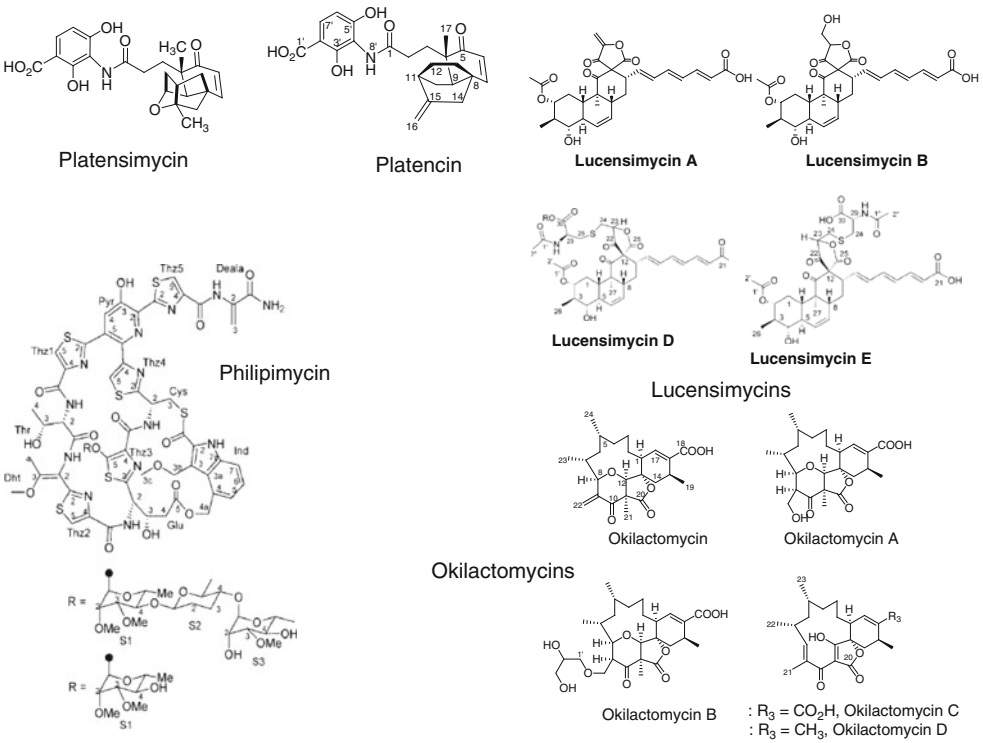

Fig. 9 Novel structures detected from actinomycetes using these screening approaches

other novel compounds. Among these novel molecules can be mentioned the new protein synthesis inhibitors lucensimycins and okilactomycins from *Streptomyces* spp. that were identified using an antisense strategy [61, 85], the new thiazolyl peptides thiazomycin and philipimycin produced, respectively, by *Amycolatopsis fastidiosa* and *Actinoplanes phillipinensis*, or the anthelmintic macrolactam fluvirucins from *Nonomuraea turkmeniaca* [3, 38, 84] (Fig. 9).

Conclusions

The discovery of platensimycin has shown that, in addition to the chemical novelty of the molecules obtained from minor actinomycetes genera, *Streptomyces* continues to be one of the best factories among actinomycetes and can deliver novel scaffolds if appropriate tools are put in place to reveal them in a cost-effective manner. The technological advances that are becoming available are bringing together all disciplines involved in natural products research and shaping new ways in which microbial natural products can be mined and exploited. Without doubt, there are strong indications of renewed interest from many sectors in exploring natural product libraries for novel scaffolds. The challenge today is to be able to translate current developments into industrial-scale processes, and this remains the major hurdle that will have to be overcome if we want to revitalize natural products discovery.

Acknowledgments Authors worked at CIBE, Madrid, Spain until Natural Products at Merck came to an end in September 2008. Most of them are presently members of the scientific staff of the new Fundación MEDINA, Granada, Spain. The authors would like to acknowledge the contribution of all CIBE employees during the years of operation of the research center, and the support of many others at MSD de España who enabled the establishment of this nonprofit research organization that has ensured continuity of research into microbial natural products for more than 54 years.

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