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Dark, rare and inspirational microbial matter in the extremobiosphere: 16 000 m of bioprospecting campaigns

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Abstract

The rationale of our bioprospecting campaigns is that the extremobiosphere, particularly the deep sea and hyper-arid deserts, harbours undiscovered biodiversity that is likely to express novel chemistry and biocatalysts thereby providing opportunities for therapeutic drug and industrial process development. We have focused on actinobacteria because of their frequent role as keystone species in soil ecosystems and their unrivalled track record as a source of bioactive compounds. Population numbers and diversity of actinobacteria in the extremobiosphere are traditionally considered to be low, although they often comprise the dominant bacterial biota. Recent metagenomic evaluation of 'the uncultured microbial majority' has now revealed enormous taxonomic diversity among 'dark' and 'rare' actinobacteria in samples as diverse as sediments from the depths of the Mariana Trench and soils from the heights of the Central Andes. The application of innovative culture and screening options that emphasize rigorous dereplication at each stage of the analysis, and strain prioritization to identify 'gifted' organisms, have been deployed to detect and characterize bioactive hit compounds and sought-after catalysts from this hitherto untapped resource. The rewards include first-in-a-class chemical entities with novel modes of action, as well as a growing microbial seed bank that represents a potentially enormous source of biotechnological and therapeutic innovation.

INTRODUCTION

Bioprospecting in its widest sense refers to the discovery of novel products from biological sources and while bioactive therapeutic agents are the most sought after natural products, other economically important targets include biocatalysts, plant growth promoters, new feed, food and cosmetic components and functional chemicals such as chelating agents. The pharmaceutical industry provided the mainstay for natural product research but subsequently abandoned bioprospecting for a variety of technical and commercial considerations including that of the acute rediscovery of products. However, a recent upturn of interest in the search and discovery of new specialized (secondary) metabolites is being driven by innovative research in academe and small biotechnology companies and not least due to our ability to access an enormously expanded biodiversity through the identity and quantification of microbial rare and dark matter. These latter categories of organisms have featured strongly in our bioprospecting campaigns and merit careful definition: rarity refers specifically to very low abundance populations within a microbial community and can be defined with a high degree of accuracy based on the work of Lynch and Neufeld [1], dark microbial matter refers to those organisms that can be identified but as yet are uncultivated [2]. Molecular interrogation of environments and of culture collections using metagenomic and single-cell genomic techniques is enabling dark and rare micro-organisms to be defined in a wide range of situations frequently revealing novel Candidatus taxa at the supra-generic rank and above. Our bioprospecting activities have focused on the phylum *Actinobacteria* (*sensu* Goodfellow [3]), the rationale for which we have rehearsed previously: unparalleled size and diversity of the taxon, extensive global and environmental distribution, continued discovery of new taxonomic radiations, genomes rich in natural product biosynthetic gene clusters (NP-BGC) and notable ecological and economic value. Although members of the genus *Streptomyces* continue to be the richest source of new actinobacterial specialized metabolites, members of non-streptomycete genera as pointed out by Hug *et al.* [4] increasingly are being

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Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; NP-BGC, natural product biosynthetic gene clusters; VRSA, vancomycin resistant *Staphylococcus aureus*.

shown to produce chemically unique natural products, a fact well-illustrated by our work with *Verrucosispora* [5], *Dermacoccus* [6] and *Lentzea* [7].

THE PARADIGM SHIFT

We will start by discussing the questions of where to look and how to look in the context of bioprospecting. Our prioritization of the extremobiosphere for bioprospecting has been based on the likelihood that it contains novel microorganisms that express new chemistry and metabolism, which in turn stimulates innovative biotechnology. The term extremobiosphere defines the environmental limits to life on Earth and encompasses the extreme boundaries of the known physico-chemical world; the extremobiosphere is characterized by various combinations of extreme temperature, pH and aridity, intense radiations, hyperbaric pressure, anoxia, strong oxidizing conditions, high salinity, oligotrophy, xenobiotic pollution and toxic elements (e.g. As, Cu, Pb). Here it is important to distinguish between extremophilic and extremotrophic (also known as extremotolerant) actinobacteria [8]; to date, the majority of isolates from the extremobiosphere appear to express extremotrophic phenotypes.

The decisive change in bioprospecting has resulted from *how* to detect novelty at the organism level, that is the shift from culture-dependent to culture-independent microbiology resulting in the ability to distinguish and identify rare and dark biodiversity. Our own realization of the scale of actinobacterial dark matter first resulted from analyses of deep Atlantic Ocean sediments [9], which revealed that between 86 and 94 % of the clone libraries examined represented putatively novel taxa (estimates based on 16S rRNA sequence identity of 98.7 % [10, 11]; or 38–71 % novelty based on the previously advocated 97 % sequence identity [12]). Other more detailed examples of dark and rare populations are discussed below.

It should be noted also that significant developments have occurred regarding the detection and identity of natural products. Established approaches such as bioassay-guided screening coupled with compound purification and structure determination, and metabolomics (chemical fingerprinting of an organism, i.e. the product of genotype and environment), have been complemented by genome-based techniques such as mining of whole and metagenomes, and crucially by database developments designed to promote chemical dereplication [13].

BIOPROSPECTING BIOMES AND SITES

Our bioprospecting campaigns have targeted major extreme biomes and within them distinct sites displaying various degrees of environmental extremities. Thus, the choice of deep sea and desert biomes provides strong selective pressures, for example in hyperbaric pressure and aridity respectively, that determine the composition of their actinobacterial communities.Fig. 1 shows the global distribution of extreme biome sampling sites that we have researched and which, in addition to the principal focus on deep seas and deserts, also has included polar, thermal and volcanic environments. Specific details of these sites are given in Table 1, where the range of altitude over which our 'bioprospecting campaigns'

Fig. 1. Global distribution of extremobiosphere sites sampled during 1992–2017.

Table 1. Extremobiosphere sites sampled for actinobacteria[a]

Deep sea	Pacific Ocean:	1. East: Sagami Bay (1168 m), Suruga Bay (1151–1948 m), Sea of Japan (289 m), Ryukyu Trench (5110–6600 m), Izu-Bonin Trench (2670–9780 m), Mariana Trench (10897 m)
		2. West: Golfo Corcovaclo (200 m)
	Atlantic Ocean:	1. Mid: Canary Basin (3814)
		2. North: Raune Fjord (187 m), By Fjord (316 m), Norway
	Indian Ocean:	Indus Rise
Polar	Antarctica:	Terra Nova Bay, Edmundson Point
Thermal	Indonesia, Java	Dieng Volcanic Complex (2000 m)
	Chile	El Tatio geothermal field (4320 m)
Volcanic	Indonesia, Java	Tangkuban Perahu, Bandung (2000 m)
		Gunung Merapi, Yogyakarta (1500 m)
Desert	Australia, Northern Territory East of Kings Canyon	
	Namibia	Kalahari Desert, Waterberg
	Chile	1. Atacama Desert: Yungay (915–1015 m), Salar de Atacama (2220–2300 m), Cordillera de Sol (2470–2540 m), Lomas Bayas
		2. Altiplano: Salar de Tara (4366 m)
		3. Central Andes: Cerro Chajnantor (3000–5000 m)

a, Depths and elevations given in metres.

have unfolded is evident; a pictorial representation of this mega range of depth and elevation is featured in Fig. 2.

Before highlighting some of the scientific outcomes from this 16 000 m exploration we direct attention to the salient properties that define the two major biomes that we have surveyed. Deep seas are generally defined as marine environments of greater than ca. 200–500 m depth and which were once regarded as being biologically inert (Forbes' azoic theory [14]), a notion that was dispelled in the late nineteenth century. Abundant life and biodiversity is now well-documented even at abyssal (>2 000 m) and hadal (>6 000 m) depths and actinobacteria have been isolated from sediments taken at all of these extremes [8, 15–18]. Conditions found in these oceans include high pressure, temperature and salinity extremes, low nutrient status and anoxia. Although our ocean bioprospecting has been made over a wide geographic range and included coastal and remote marine locations it cannot claim to be systematic either in terms of depth or ocean conditions, a situation that stems from the major logistical problems associated with sampling these environments [it is sobering to note that the ultra-deep robotic vehicles Kaiko (Japan Agency for Marine-Earth Science and Technology, JAMSTEC) and Nereus (Woods Hole Oceanographic Institution) both were lost during research missions].

In addition to many of the environmental conditions cited above that characterize the extremobiosphere, that of severe aridity is probably the most defining for the existence of microbial life in deserts, indeed the core region of the Atacama Desert in northern Chile has been designated 'the dry limit of microbial life' [19]. Moreover, high mountain desert habitats that include the Chajnantor plateau (see below) are subject to a combination of extreme environmental conditions including the world's highest levels of surface UV radiation [20]. Access to all of the major habitats in the Atacama Desert have enabled our bioprospecting campaigns to adopt a more systematic approach and have encompassed hyper-arid and extreme hyper-arid soils (*sensu* Houston [21]), altitude gradients, and saline and geothermal environments. From each of these habitats we have recovered viable actinobacteria and/or obtained DNA evidence for their presence.

BIOPROSPECTING OUTCOMES

Novelty both of actinobacterial diversity and chemical diversity has been detected and characterized unequivocally over the complete depth-altitude and geographic ranges of our bioprospecting campaigns. Highlighted results are considered first in terms of taxonomic novelty followed by new natural product chemistry produced by novel representatives of deep sea and desert species.

Actinobacterial diversity

Initial studies of deep sea actinobacteria were founded on culture-dependent surveys where our collaboration with JAMSTEC enabled sediments from a complete range of ocean depths (289–10 898 m) to be examined. With the exception

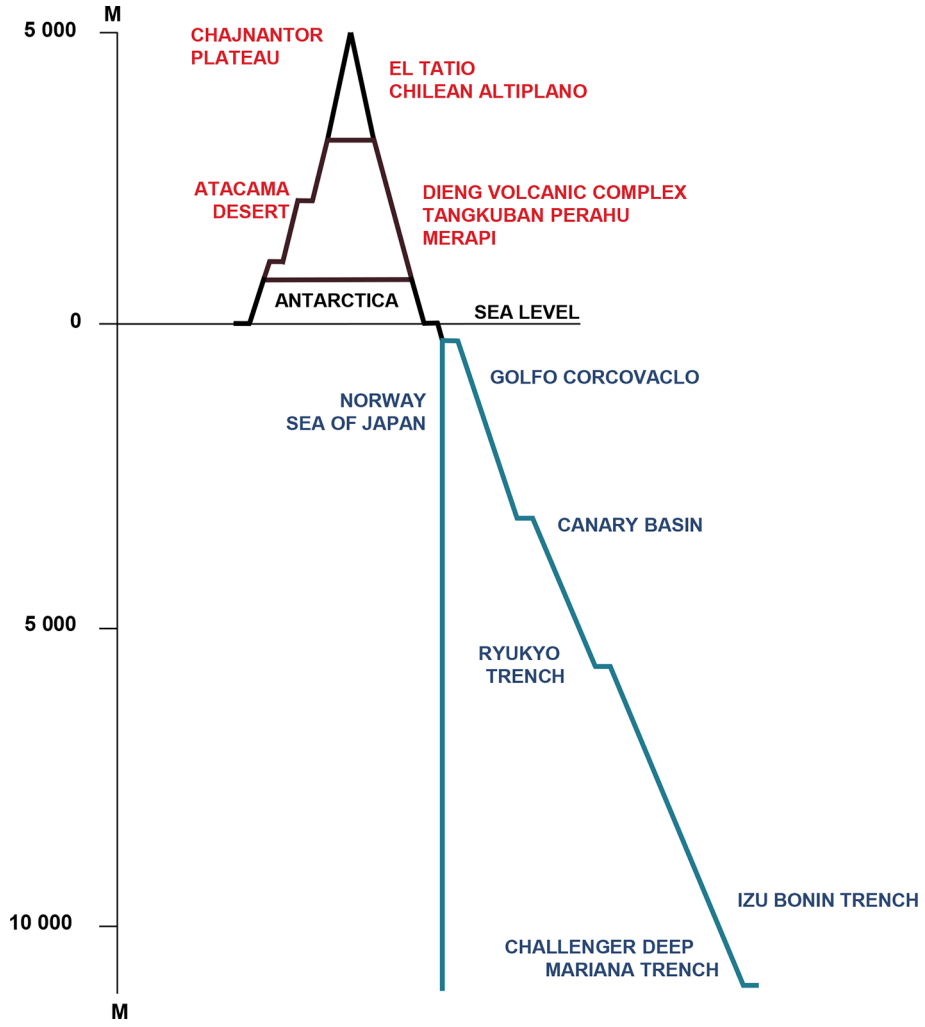

Fig. 2. A 16 000 m extremobiosphere bioprospecting campaign

of Mariana Trench samples, actinobacteria initially were isolated from all depths and evidence obtained to support the hypothesis of indigenous taxa, especially in the very deep Ryukyu, Izo Bonin and Japan Trenches [22]. Among the genera detected were *Corynebacterium, Dietzia, Gordonia, Mycobacterium* and *Rhodococcus* and 16S rRNA phylotyping indicated putative new species within several of these taxa while recent phylogenomic analyses show that rhodococci belong to several lineages that can be equated with genera [11, 23]. Moreover, simulated deep sea conditions confirmed that these actinobacteria grew at the sediment salinities and temperatures from which the isolates were recovered while a number of abyssal and hadal rhodococci were capable of growth at hyperbaric pressures (40–60 MPa). Subsequent efforts resulted in the isolation of three new species of *Dermacoccus* one of which, *D. abyssi*, was piezotolerant [15, 16]. Other examples of novel actinobacterial diversity are treated in relation to natural product discoveries.

Actinobacteria are globally distributed in the world's oceans, particularly in marine sediments [24, 25]. We referred briefly above to the analysis of sediment cores taken in the Atlantic Ocean near to the Canary Basin. New primers designed specifically for actinobacteria were used in the construction of clone libraries from sediment sections at depths up to ca. 1 m below the ocean floor and diversity statistics applied to define actinobacterial library coverage, taxonomic richness and phylogenetic diversity. Significant differences were observed in the taxonomic diversity as a function of sediment depth, but both taxonomic richness and phylogenetic diversity decreased with depth [9]. Statistical diversity estimators such as those used in this study provide a powerful bioprospecting adjunct. Culture-independent analysis of the Canary Basin sediment also yielded clones of the obligate marine genus *Salinispora* [24] pointing to its wider biogeographical distribution in terms of ocean temperature and depth than previously reported.

The main efforts of our arid biome prospecting have focused on the Atacama Desert where actinobacteria are a dominant taxon in many habitats including those defined as extreme hyper-arid. Culture-based surveys of recent years have

Table 2. High taxonomic rank dark matter among Atacama actinobacteria

Median altitude ecosystems (915–1540 m)		
No. of families detected	67	
Candidatus[a] Families	14	(21 % total)
High altitude ecosystems (3000–5000 m)		
No. of families detected	55	
Candidatus families	19	(35 % total)
Candidatus classes	2	(increase of 33%)[b]
Candidatus orders	4	(increase of 22%)[b]

a, *Candidatus* defines well-characterized but as yet uncultured organisms.
b, Putative new taxa in addition to those validly published.

reported members of over 23 validly named genera and many new and putatively new species have been highlighted including ones belonging to the poorly studied genera *Actinomadura*, *Cryptosporangium*, *Kribbela, Nonomuraea, Pseudonocardia* and *Saccharothrix* [26, 27]. However, it is only from the results of metagenomic surveys that the spectacular extent of actinobacterial diversity in Atacama habitats has been fully recognised [28, 29]. The data in Table 2 show the massive extent of actinobacterial dark matter existing at supra-generic ranks while at the genus rank 234 and 297 phylotypes were detected at low and high altitude sites, respectively. High proportions (43–54 %) of genuinely rare taxa (*sensu* Lynch and Neufeld [1]) were also detected. Preliminary evidence was advanced for an Atacama Desert core microbiome of actinobacteria (*sensu* Shade and Handelsman [30]) and it will be interesting to discover if this is a characteristic of the Atacama or represents that of global desert biomes.

Bioactive natural products

The impressive array of members of chemical classes found in actinobacteria recovered from the two selected extreme biomes is shown in Table 3, which, with the exception of the abenquines [31], have been discovered by our group. Each of these products represents a new-in-a-class or first-in-a-class chemical. Similarly, a range of bioactivities is presented by these compounds with antibacterial and anticancer activities being the most widely distributed. The occurrence of phosphodiesterase and glycogen synthase kinase inhibitors is notable in providing potential therapeutic targets for anti-inflammatory diseases, and Alzheimer's disease and type II diabetes, respectively. However, in the overall context of drug discovery and development it should be stressed that at this stage these compounds are simply putative drug hits; nevertheless, it is encouraging that within a short time following their discovery several have been synthesized chemically thus opening up the way to enhance efficacy and/or ameliorate cytotoxicity via structural modification.

Table 3. Natural product diversity of actinobacteria cultured from deep sea and hyper-arid desert

Chemical class	Natural product[a]	Source	Bioactivity
Amino furan polyamides	Proximicins[a]	Raune Fjord (187 m)	1, 2
Aminoheptosyl glycosides	Spicamycins A-E	Yungay (1016 m)	2
Aminoquinones	Abenquines A-D	Salar de Tara (4500 m)	1, 6
Ansamycins	Chaxamycins[a]	Laguna de Chaxa (2300 m)	1, 2
Benzoxazoles	Benzoxacystol	Canary Basin (3814 m)	2, 7
	Caboxamycin[a]	Canary Basin (3814 m)	2, 6
Diene glycosides	Lentzeosides	Cerro Chajnantor (5046 m)	5
Beta-diketones	Asenjonamides A-C	Yungay (1016 m)	1*
Macrolactones	Atacamycins	Laguna de Chaxa (2300 m)	2, 6
	Chaxalactins A-C	Laguna de Chaxa (2300 m)	1
Peptides	Chaxapeptin	Salar de Atacama (2219 m)	1, 2
Phenazines	Dermacozines[a]	Mariana Trench (10 897 m)	2, 3, 4
Spirotetronate polyketides	Abyssomicins	Sea of Japan (289 m)	1, 2, 5
	Levantilide C	Golfo Corcovaclo (200 m)	2

(1) Antibacteria, (1*) antibacteria including Gram-negative, (2) anticancer, (3) antioxidant/radical scavenging, (4) antitrypanosome, (5) anti-HIV-1 integrase/HIV reactivator, anti-HIV and HIV replication inducer, (6) phosphodiesterase (PDE-4B2) inhibitor, (7) glycogensynthase kinase 3b inhibitor.
a, Compounds that have been synthesised chemically.

While the strategies used for detecting microbial specialized metabolites are well-established (bioassay-guided, metabolomics, genome mining and sequencing [32–34]) those used to elicit the expression of their biosynthetic gene clusters (BGC) have arisen either by more random or intuitive routes such as the one strain-many compounds scheme involving variations of the fermentation protocol, and co-culture systems. The latter approach appears not to have been tested extensively but two examples arising from our Atacama Desert work have produced interesting and unpredictable results. Co-cultivation of the Chaxa de Laguna isolate *Streptomyces bullii* KBCC15426^T with a strain of *Aspergillus fumigatus* resulted in the synthesis of nine specialized metabolites none of which were produced by axenic cultures of either partner [35]; while co-culture of the fungus with

Streptomyces leeuwenhoekii strain C34^T elicited synthesis of the lasso peptide chaxapeptin not previously detected in extracts of this strain. Moreover, chaxapeptin synthesis by the known producer strain of *S. leeuwenhoekii* (C58) was significantly increased when it was co-cultured with *A. fumigatus* [36]. It can be argued that such discoveries are at best fortuitous and serendipitous although the co-culture of organisms renowned for their abilities to produce specialized metabolites can be viewed as a sensible starting point. Future ecology-guided prospecting along these lines could profit from defining the originating microbial communities per se, and using continuous culture systems for analysing evolutionary trends within such communities [37].

Detailed information on all of the compounds reported in Table 3 is available in primary publications but here we refer to selected ones in order to highlight some salient aspects of the bioprospecting process.

Abyssomicins

These spirotetronate polyketides, first isolated from the deep sea *Verrucosispora maris* AB-18-032^T, and detected by assay-guided screening possess a unique chemical scaffold and antibacterial mode of action [5]; they are among those dubbed 'inspirational natural products' by Nicolaou [38]. Subsequently members of this chemical class have been found in species of *Streptomyces* and currently 32 natural abyssomicins and many chemically synthesized analogues have been described [39]. Following the defining of anti-Gram-positive bacteria activities [including methicillin- and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA)], particular abyssomicins have been shown to express anti-TB and anticancer properties, to reactivate latent human immunodeficiency virus (HIV), induce HIV replication, and to have anti-HIV action. To our knowledge, none of these diverse abyssomicin attributes has yet led to pharmaceutical development but the recent resurgence of interest coupled with an increasing ability to improve drug efficacy and lower toxicity via medicinal chemistry holds promise for such pro-drug development. The excellent review of Sadaka *et al.* [39] elaborates these and related aspects of the abyssomicin family.

Dermacozines

This new ten-membered family of phenazines was detected by metabolite screening and is notable for its discovery of a novel piezotolerant species of *Dermacoccus* isolated from the deepest oceanic trench on Earth [6, 40]. Several of the dermacozine analogues displayed moderate activity against a human leukemia cell line and one (dermacozine C) has antioxidant titres greater than that of ascorbic acid. The total synthesis of dermacozines A and B was reported recently [41].

Chaxamycins

These new ansamycin antibiotics were detected using a genome mining approach that targeted the gene encoding 3-amino-5-hydroxybenzoic acid synthase [42]. The producing bacterium was characterized as *S. leeuwenhoekii* and assigned as the representative of a deeply rooted 16S rRNA gene clade (C34, see below). In addition to their antibacterial activities (including highly selective anti-MRSA) chaxamycins are promising anticancer agents. To date total chemical synthesis of chaxamycins has not be reported although synthesis of the polypropionate stereocentres has been successful [43]. This latter achievement and the promise of mutasynthesis with this strain [44] recommend routes for producing novel chaxamycin analogues having enhanced and/or expanded bioactivity.

Chaxapeptin

This novel class II lasso peptide inhibits cell invasion activity in a human lung cancer cell line. It was discovered by genome mining of another member of the *S. leeuwenhoekii* clade (C58) [45] and, as noted above, its fermentation titre can be increased effectively by co-culture.

Genome analysis

Genome sequences of three of the deep sea and six of the desert actinobacteria discussed above have been obtained recently (Table 4); most of these are draft sequences and only that of *S. leeuwenhoekii* C34^T is a full, high-quality sequence [46]. The GC contents of these strains were typical for actinobacteria ranging from 70.6 to 74.0 mol %.

Genomes of members of the family *Geodermatophilaceae* isolated from extreme hyper-arid soils were relatively small (3.89–5.51 Mb) but contained a high proportion of genes related to environmental stress response including heat and cold shock responses, osmotic stress, carbon starvation and stress factors that give rise to reactive oxygen species [28, 29, 49]. Perhaps surprising is the high capacity of these bacteria to synthesis specialized metabolites, a feature also noted in novel species of *Micromonospora* isolated from extreme hyper-arid Lomas Bayas soil [52], the assumption being that competitive success in extreme, sparsely populated ecosystems might be more likely determined by stress resistance rather than by antibiosis. The genomes of all the Atacama actinobacteria included in Table 4 contained large numbers [35–46, 49, 52, 56–71] of biosynthetic gene clusters (BGCs), a fact that promises well for bioprospecting action. However, the findings from draft sequences should be interpreted with some caution given problems associated with sequence coverage and mis-assemblies of contigs using some sequencing platforms and failure to distinguish between BCGs encoding for natural products and primary metabolites [46, 56]. Such caveats notwithstanding, draft sequences have proved effective in genome mining prospecting as exemplified by chaxamycin and chaxapeptin discovery.

Regarding genome characteristics, are we justified in describing our extremobiosphere isolates as 'gifted', a concept introduced by Richard Baltz to define micro-organisms having large genomes, preferably greater than 8 MB, and encoding 20–50 BGCs [56], or 'biosynthetically talented', as other authors describing large actinobacterial BGCs have advanced [54]? While only *Amycolatopsis vasitatis* and *S. leeuwenhoekii* strain C34^T meet this requirement in the

Table 4. Genome characteristics of deep sea and Atacama Desert actinobacteria

Species	Genome size (MB)	tRNAs	Contigs	DNA GC content (%)	BGCs
Verrucosispora maris[a]	6.7	51			20
Streptomyces sp. strain NKT397[b]	7.44	81		71.9	37
Blastococcus atacamensis[c]	3.89	53	163	73.1	
Modestobacter caceresii[d]	4.96	50	139	73.6	48
Geodermatophilus chilensis[e]	5.51	52	374	74	56
Micromonospora arida[f]	7.15	56	345	71	54
Micromonospora inaquosa[g]	7.75	58	408	70.6	64
Streptomyces leeuwenhoekii strain C34[h]	8.29	65	1	72.6	35
Amycolatopsis visitatis[i]	10.67			70.8	38

a, Roh *et al*. [47]; *b*, Olano *et al*. [48]; *c*, Castro *et al*. [49]; *d*, Busarakam *et al*. [50]; *e*, Castro *et al*. [51]; *f, g,* Carro *et al.* [52]; *h*, Busarakam *et al*. [53], Gomez-Escribano *et al*. [54]; *i*, Adamek *et al*. [55], Idris *et al*. [28].

strictest sense (Table 4; [46]), it is remarkable that some of the small genome actinobacteria isolated from the extreme hyper-arid core of the Atacama Desert displayed even greater capacities for specialized metabolite production. Clearly chemical screening of these latter members of the family *Geodermatophilaceae* deserve prior attention. Nevertheless, bioprospecting campaigns should remain focused on the detection of cultivable actinobacteria with large genomes given their propensity to produce novel drug-like compounds [33, 56]. Metagenomic analyses have shown that such gifted actinobacteria are a feature of natural habitats [57, 58]. Creative cultivation techniques are available for the isolation of rare and novel actinobacteria with large genomes [27, 59], though there is a need to marry innovative selective isolation procedures with the ecology of target organisms [60–63], as demonstrated by adaptations shown by *Salinispora* and *Streptomyces* strains [64, 65]. There is also a need to obtain complete genome sequences from especially gifted actinobacteria [33, 34] for systematic genome mining for new molecules.

WHITHER BIOPROSPECTING?

By way of concluding this review we pose a number of questions that should influence decisions on whether or not to engage in bioprospecting. We preface these questions by a brief aside on the necessity of being granted official permission to access prospecting sites (often located within countries deemed to be less-developed and/or reservations of indigenous peoples and that have minimal relevant expertise); of effective collaboration with in-country personnel; of joint reporting and benefit sharing; and of being cognizant of the Convention on Biological Diversity and the Nagoya Protocol.

Why bother with natural products?

The fact that natural products occupy a much greater area and variety of chemical space and chemical scaffolds than combinatorial synthetic compounds provides a compelling *a priori* reason for prioritizing them in the search for novel drugs. The former problem of rediscovery of products is now greatly minimized or prevented by sophisticated methods for organism and chemical dereplication [66]. It is becoming apparent that expanded chemical novelty is to be found by screening rare or poorly researched microbial taxa particularly among actinobacteria (e.g. the discovery of abyssomicins, proximicins and lentzeosides in novel species of *Verrucosispora* and *Lentzea,* respectively). Genomic-guided identification of new drug targets such as protein–protein interactions also will stimulate natural product bioprospecting given the extent of such interactions in nature. Moreover, as Hug *et al*. [4] point out if production scale cultivation of target organisms compromises the exploitation of a promising natural product, culture-independent strategies that include metagenomics and heterologous expression of BGCs provide exciting approaches for the discovery of novel natural products among biosynthetic dark matter, as does the prospect of coupling genome sequencing with synthetic biology [33, 34].

Is focus on the extremobiosphere justified?

We note first that the extent of the known extremobiosphere has increased dramatically during recent times, most notably with the exploration of the marine deep biosphere [67], the deepest sediment analysed at 1626 m [68]; deep terrestrial boreholes [69]; and high elevation barren tephra (Volcan Llullaillaco, Atacama region 6330 m [70, 71]). Prokaryotic life has been detected in each of these environments using culture-dependent and culture-independent methods and, interestingly, actinobacteria have been reported to be among the dominant bacteria. Members of a large number

of actinobacterial genera have been reported in subseafloor sediments over 1 km in depth [72]. While recognizing that more mundane environments can contain novelty for the bioprospector (e.g. forest soil [73], street sediments [74]) we opine that the case for prioritizing extreme biomes is sound as it moves bioprospecting action away from well-probed terrestrial streptomycetes towards microbial communities that have evolved under harsh selective conditions during the course of which novel biochemistry has been generated [75]. As Rateb *et al*. report [66], a total of 46 natural products representing diverse chemical classes have been isolated from Atacama Desert actinobacteria during the past 7 years. Symbiotic associations frequently have evolved in harsh selective environments and there is increasing recognition of them producing a wealth of new chemical space in insect [76] and marine invertebrate [77] mutualisms, for example. The discovery of novel antimicrobial pentacyclic polyketides produced by *Streptomyces formicae* [78] isolated from a fungus-growing plant-ant illustrates very effectively the benefits of exploring under-investigated ecosystems for the discovery of micro-organisms having the capacity to synthesize novel natural products [79].

Is biogeography a roadmap to drug discovery?

In the context of bioprospecting a secondary question arises: is everything everywhere? While avoiding another discussion of this long-proposed hypothesis (see [80]) we believe that increased efforts to investigate phylogeographic distribution of micro-organisms is a relevant objective. A landmark study of fluorescent *Pseudomonas* genotypes showed that these soil bacteria were not mixed globally [81] and more recent studies of *Streptomyces* populations [82, 83] support the notion of geographic distinction. *Streptomyces* diversity was found to vary as a function of geographic distance and, moreover, phenotypes showed regional endemism with respect to antibiotic production and resistance, features that are likely to encourage global bioprospecting campaigns. At a global level, Charlop-Powers *et al*. [57] probed a large collection of environmental samples for selected biosynthetic genes and found that geographic location and biome type were the most important factors related to their sequence diversity. Moreover, the diversity and endemicity of actinobacteria in fragile desert habitats such as the extremely oligotrophic Cuatro Cienegas Basin in the Chihuahuan desert presents an invaluable source of biological material for ecological and bioprospecting studies [84].

How rational can we make bioprospecting?

In this context, logic can be applied to the selection of biomes and geographic locations, and the prioritization of organisms with regard to novel chemistries. We have touched on the former issues and here consider arguments for prioritizing organisms in bioprospecting projects, the overall case for actinobacteria having been made repeatedly [26, 27]. Previously we have emphasized the need for taxonomic rigour in order to define novelty, establish strain dereplication, predict metabolic potential and guide selective isolation procedures [85–87]. And underlying such an admonition is the knowledge that prokaryotic systematics currently exists in such a dynamic state that genome-based classification is likely to become the norm [11]. *Actinobacteria* provide a case in point where analyses of genome-scale data have prompted significant revision of taxonomic and evolutionary relationships within this phylum [88], including the reclassification of *Verrucosispora* species in the genus *Micromonospora*.

The advent of genomics now enables natural product potentials of target bioprospecting taxa to be determined and hence prioritize screening options. Recent comparative genomics of the genus *Amycolatopsis*, a large, widely distributed genus well-known as a source of bioactive metabolites, illustrates the power of this strategy. The pan-genome of members of this genus comprises a small core genome largely involved with translation and ribosome biogenesis and a 'huge' accessory genome that includes numerous genes encoding biosynthesis of specialized metabolites [55]. Phylogenetic analysis of the genus revealed four distinct lineages that correlated perfectly in their capacities to produce specialized metabolites; moreover, the extensive diversity of BGCs revealed that the majority were unique to *Amycolatopsis*. Such discontinuous distribution of natural product bioclusters within a target taxon underlines the importance of rigorous classification in bioprospecting. Within the strain library used in this study was the high BGC containing, newly described *A. vastitatis* isolated from Cerro Chajnantor soil at 4000 m [89]. Similar conclusions can be drawn from phylogenomic studies on representatives of the genus *Micromonospora* [90]. Amongst actinobacteria, micromonosporae are second only to streptomycetes in their ability to synthesize natural products, notably antibiotics, including ones that are not only in clinical use [32] but considered essential to global health [91]. Interestingly, the application of innovative selective isolation procedures has shown that micromonosporae are a feature of diverse Atacama Desert soils [92].

Without question, the awareness of silent NP-BGCs in microbial genomes, aided by bioinformatics software for their analysis, is beginning to have a major impact on the identity and expression of new chemical entities. The challenge now, as pointedly stated by Zhang and Moore [93] is how to translate sequence information into chemical reality, and 'to access not just small variations in old chemistry, but completely new chemical scaffolds'. In a valuable discussion of the means of activating such BCGs Baral *et al*. [94], while acknowledging that to date no single superior methodology is available, highlight three approaches for harnessing new natural products: pleiotropic methods aimed at modifying the whole genome; BCG-specific methods, including heterologous expression; and, targeted genome-wide methods that focus on previously identified BCGs of interest.

Is it appropriate to continue with culture-dependent searches?

The impressive developments in culture-independent methods of detecting and characterizing dark and rare

components of microbiomes notwithstanding, we affirm that cultivation-based approaches have an integral role in bioprospecting. For example, the availability of organisms per se enables one strain-many compounds and co-culture screening options and may provide for rapid pre-screening of large communities of actinobacteria via colour-group analysis [27]. Indeed, Baltz [33, 56] makes a strong claim in this context asserting that major resurgence in natural product discovery will be facilitated by focusing heavily on culturable, gifted organisms while expressing reservations on the success of mining microbes from the unculturable majority for natural products. The imperative therefore is to develop innovative procedures for bringing members of the dark and rare actinobacterial populations into culture and here functional metagenome mining of extremobiosphere will provide information on ecophysiology and hence vital clues for designing isolation media and incubation conditions.

Can any advantage be gained from screening subspecies of organisms?

Probably as a consequence of effort involved and intuition, information on this point is limited but our experience with the gifted species *S. leeuwenhoekii* bears comment. A large well-delineated clade of *Streptomyces* was isolated from soils of the Salar de Atacama [95], a representative member of which was subsequently published as the new species *S. leeuwenhoekii* [53]. Four members of a subclade of this species sharing 99.5–99.9 % 16S rRNA sequence identity have been shown to have differential capacities to produce bioactive specialized metabolites that include chaxalactins (strain C34^T), atacamycins (C38), chaxapeptin (C58) and novel compounds (C79) [46]. The recent report from Park and Andam [96] on strain-level variation in the distribution and abundance of BGCs in *Streptomyces rimosus* emphasizes the importance of screening multiple strains of the same species in natural product discovery campaigns. Individual *S. rimosus* genomes were shown to have a unique repertoire of BGCs that ranged from 35 to 71 clusters per genome. In future, such studies can be undertaken with increased confidence now that a digital DNA homology cut off of 79 % can be used to circumscribe subspecies [97] as illustrated by Nouioui *et al.* [88]. This should help to avoid the delineation of ill-defined subspecies; detailed taxonomic analyses of the type strains of subspecies of *Streptomyces hygroscopicus* and *Streptomyces lavendulae,* for instance, showed that the former merited recognition as distinct species within the genus *Streptomyces* [98] whereas the latter formed a genomic species together with the type strain of *S. lavendulae* [99]. Further, the circumscription of ecovars of gifted actinobacterial species such as *Streptomyces griseus* may allow the development of predictive models for explaining the distribution of novel NP-BGCs in defined habitats [60, 100].

What is the feasibility of managing bioprospecting information overload?

Given the rapid accumulation of genomic and metagenomic sequences, and the ever-increasing size of chemical libraries and microbial culture collections, bioprospecting is not immune from information overload, hence the need for effective search and analytical tools. While this is not the place for a detailed discussion of the problem, two recent examples illustrate the type of approaches being made to manage large data sets.

Extracts from a collection of a 1000 largely taxonomically uncharacterized marine bacteria were screened by LC–MS/MS-based metabolomics and the data subjected to molecular networking [101]. Coupled with high throughput culturing such a procedure enabled rapid dereplication and the detection of novel metabolites. The incorporation of multiple culture extraction systems significantly extended the chemical space covered in this project but manual curation of the resulting data sets, having the magnitude of hundreds of thousands of different MS features, would not have been feasible. The authors conclude that the strength of their protocol lies in its untargeted nature: 'Without reliance on genetic, geo-graphic or other data, large numbers of randomly sampled organisms can be prioritized…and guide investigators towards novel molecules'. Our second example refers to the prioritization of bacterial collections for the production of the potent bioactive enediyenes. Preliminary screening of a bacterial collection by our group [102] using library-on-a-slide technology resulted in a hit rate of 15 % from which PCR and sequencing of positives enabled the identification of novel enediyene PKS genes. More recently, Yan *et al.* [103] have surveyed a much larger bacterial collection (3400 strains) also based on enediyene PKS cassettes and showed that the positive strains fells into 28 distinct phylogenetic clades each of which contained a distinct enediyene BGC. Subsequent development of a genome neighbourhood network enabled new enediyene chemistry to be predicted again demonstrating the feasibility of rapid discovery of new natural products from a large strain collections.

CONCLUSION

We conclude by reasserting our optimism in microbial natural products as a source of new therapeutic agents and that the Earth's extremobiosphere provides propitious opportunities for successful bioprospecting. Decisive technical advances in microbiology, molecular biology, bioinformatics and chemical screening give credible support to this optimism while natural product hits that initially appear to fail the tests of druggability on the basis of toxicity or other properties can be further explored by the intervention of medicinal chemistry. Although this review has focused on the search for putative new drugs, the extremobiosphere is a rich source of other natural products not least research enzymes and industrial biocatalysts [104], and compounds with the potential to promote the growth of actinorhizal plants in polluted and nutritionally poor saline soils [105].

The primary requirement, and concern, for successful bioprospecting is the availability of microbial gene pools, an objective that can be met by access to diverse habitats, culture collections and genome data banks. However,

worldwide habitat destruction, large-scale crop monoculture, mining and power generation should be of increasing concern to microbiologists, as should the effect of climate breakdown, as witnessed by the destruction of microbial communities in the hyper-arid core of the Atacama Desert following highly unusual rain events [106]. Although such problems and suggested ways of safeguarding microbial diversity have been voiced from time to time, 'formulating a consistent environmental ethic for the conservation of microorganisms is notoriously difficult' (Cockell and Jones [107]). In a very thoughtful analysis, the latter authors have set out priorities for microbial conservation and proffer practical means for its advancement ranging from a strengthening of ecosystem research to adapting legislation to provide special protection for ecosystems that are acknowledged to contain critically important microbial communities. Progress towards achieving such objectives has not been impressive and we conclude by strongly commending the entreaty made by Cockell and Jones for institutions such as conservation organizations, learned societies and university centres to take more active roles in broadcasting the case for and promoting microbial conservation.

Dedication

This paper is dedicated to the memory of the late Martin Alexander (1930–2017) of Cornell University, outstanding microbiologist, committed internationalist and humanitarian, and meticulous but compassionate mentor, and all aspects of his philosophy that he passed onto the scientific community.

Postscript

This review marks the collaboration of the authors in bioprospecting over more than a quarter century; it reflects their philosophy regarding search and discovery, highlights some of the concepts and practices that have inspired their research, and, by provoking a number of questions relating to bioprospecting, attempts to encourage further work in this field.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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