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Eliciting antibiotics active against the ESKAPE pathogens in a collection of actinomycetes isolated from mountain soils

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The rapid emergence of multidrug-resistant (MDR) bacterial pathogens poses a major threat for human health. In recent years, genome sequencing has unveiled many poorly expressed antibiotic clusters in actinomycetes. Here, we report a well-defined ecological collection of >800 actinomycetes obtained from sites in the Himalaya and Qinling mountains, and we used these in a concept study to see how efficiently antibiotics can be elicited against MDR pathogens isolated recently from the clinic. Using 40 different growth conditions, 96 actinomycetes were identified – predominantly *Streptomyces* – that produced antibiotics with efficacy against the MDR clinical isolates referred to as ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and/or *Enterobacter cloacae*. Antimicrobial activities that fluctuated strongly with growth conditions were correlated with specific compounds, including borrelidin, resistomycin, carbomethoxy-phenazine, and 6,7,8- and 5,6,8-trimethoxy-3-methylisocoumarin, of which the latter was not described previously. Our work provided insights into the potential of actinomycetes as producers of drugs with efficacy against clinical isolates that have emerged recently and also underlined the importance of targeting a specific pathogen.

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INTRODUCTION

Infections caused by multidrug-resistant (MDR) bacteria continue to be a worldwide problem. Society currently faces rapidly growing resistance among Gram-positive and Gram-negative pathogens that cause infection in the nosocomial environment and the general community (Giske *et al.*, 2008; Rice, 2008; Spellberg *et al.*, 2008). Several MDR pathogens, especially *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. – collectively referred to by the acronym ESKAPE – cause the majority of infections within the nosocomial environment (Rice, 2008). Current antimicrobial therapies have reduced efficiency or are inactive against these isolates, which has led to increased mortality among patients (Rice, 2008), and more people now die of these increasingly antibiotic resistant pathogens than of HIV/AIDS and tuberculosis combined (Boucher & Corey, 2008; Klevens *et al.*, 2006). This underlines the urgent need for new antimicrobials.

The actinomycetes are a diverse family of filamentous bacteria that produce a wealth of secondary metabolites, including two-thirds of the antibiotics in clinical use (Miyadoh, 1993; Okami & Hotta, 1988). Of the actinomycetes, streptomycetes are particularly prolific antibiotic producers, which grow as a branched multicellular network of hyphae, called the mycelium (Claessen *et al.*, 2014); on solid surfaces they reproduce through spores formed by a specialized aerial mycelium, which coincides with antibiotic production (Hopwood, 2007). Many studies have been conducted in the search for novel actinomycetes (Lazzarini *et al.*, 2000; Ng & Amsaveni, 2012; Singh *et al.*, 2006; Takahashi & Omura, 2003; Thakur *et al.*, 2007).

Abbreviations: ESI, electrospray ionization; MDR, multi-drug resistant; MM, minimal media; MRSA, meticillin-resistant *Staphylococcus aureus*; MS, mass spectrometry.

The GenBank/EMBL/DDBJ accession number for the batch of 16S sequences is BankIT 1698416.

Five supplementary figures and two supplementary data files are available with the online version of this paper.

However, the intensive screening programs in the pharmaceutical industry failed to yield significant numbers of novel drugs (Payne *et al.*, 2007). New antibiotics have been estimated to occur at frequencies of less than one per million in randomly chosen actinomycetes grown under routine conditions (Baltz, 2007, 2008). Sequencing of *Streptomyces* genomes established the presence of silent antibiotic biosynthetic gene clusters, suggesting that the potential of these organisms for novel drug production is much larger than anticipated originally (Challis & Hopwood, 2003; Hopwood, 2007). As an example, many novel gene clusters were identified in the extensively studied model streptomycetes *Streptomyces coelicolor* (Bentley *et al.*, 2002), *Streptomyces griseus* (Ohnishi *et al.*, 2008) and *Streptomyces lividans* (Cruz-Morales *et al.*, 2013).

A major challenge is therefore to find ways to elicit the production of yet unknown antibiotics. Such strategies have been in use in industry for many decades, but the discovery of cryptic antibiotics has renewed the interest in identifying specific conditions or agents that elicit the expression of antibiotics (Craney *et al.*, 2012; van Wezel *et al.*, 2009; Zhu *et al.*, 2014). An example is *N*-acetylglucosamine, which in streptomycetes is imported by the phosphoenolpruvate-dependent phosphotransferase system and converted into glucosamine 6-phosphate (Nothaft *et al.*, 2010; Świątek *et al.*, 2012); once imported, it forms a signalling cascade with glucosamine 6-phosphate acting as a ligand for the antibiotic repressor DasR, thereby rendering it inactive and triggering antibiotic production in *Streptomyces coelicolor* (Rigali *et al.*, 2006, 2008). Recently, screening of a chemical library for new elicitor molecules resulted in the identification of chemical probes that activated antibiotic production by interfering with fatty acid biosynthesis (Ahmed *et al.*, 2013; Craney *et al.*, 2012). These approaches show the power of elicitors for rational drug discovery approaches.

Media composition has a major impact on antimicrobial agent production, with glucose and phosphate as well-known suppressors of antibiotic production (reviewed by Sánchez *et al.*, 2010; van Wezel & McDowall, 2011). Screening under different growth conditions is a strategy that has been used by the pharmaceutical industry for many decades, although with less success in recent years (Payne *et al.*, 2007). In this study, we present a novel collection of actinomycetes from soil samples obtained from remote mountains and analysed their antibiotic-producing potential. This provided new insights towards the efficacy of the antibiotics produced against MDR pathogens that currently threaten the health of hospitalized patients. We identified a number of antibiotics whose expression varied greatly with the growth conditions, including a previously unidentified isocoumarin-type antibiotic.

METHODS

Selective isolation of actinomycetes from soil. Soil samples were collected from the Qinling and Himalaya mountains (China). The Qinling samples were obtained from different places in Xi'an, Shaanxi Province, China: Shandi Village at longitude 109° 22′ 39″ and latitude 34° 3′ 28″, height 660 m; Gepai Village at longitude 109° 30′ 8″ and latitude 33° 54′ 54″, height 1088 m; Muzhai Village at longitude 109° 22′ 03″ and latitude 34° 21′ 02″, height 985 m; Huafeng Village at longitude 108° 39′ 4″ and latitude 34° 00′ 58″, height 533 m; Daohe Village at longitude 108° 41′ 10″ and latitude 34° 00′ 58″, height 692 m; Langshan Village at longitude 109° 0′ 53″ and latitude 34° 1′ 5″, height 720 m; and Xinlian Village at longitude 108° 48′ 16″ and latitude 34° 1′ 43″, height 692 m. The Himalayan sample was obtained at 5000 m height, collected near a hot-water spring. Every soil sample was collected at a depth of 10–20 cm, put in sterilized plastic bags and stored at 4 °C before processing.

The soil was enriched with 6 % yeast extract (Hayakawa & Nonomura, 1989) and dilutions plated onto selective media, which were modified starch casein agar (MSCA) (Küster & Williams, 1964), humic acid agar (HA) (Hayakawa & Nonomura, 1987), glucose casein agar (GCA) (soluble starch 15.0 g, KNO_3 0.5 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 5.0 g, KCl 5.0 g, $FeSO_4.7H_2O$ 0.01 g, vitamins 0.5 mg, agar 18.0 g, add water to 1000 ml, pH 7.2–7.4), soy flour mannitol medium (SFM) or minimal medium (MM) (Kieser *et al.*, 2000). Nystatin (50 μg ml^{-1}) and nalidixic acid (10 μg ml^{-1}) were added for the inhibition of fungi and bacteria, respectively during initial selection. Single actinomycete colonies were streaked onto SFM agar plates until pure cultures were obtained. Imaging of actinomycetes by phase-contrast, stereo microscopy and cryo-scanning electron microscopy was conducted as described previously (Colson *et al.*, 2008).

Indicator micro-organisms, growth conditions and antimicrobial assays. *Bacillus subtilis* 168 (no resistance markers) and *Escherichia coli* ET8 (Amp^R $Apra^R$ Cam^R Kan^R Neo^R Str^R Tet^R) were used as indicator strains. ET8 is a derivative of *E. coli* ET12567 (which contains the *cmr*, *aadA* and *tet* genes that confer resistance to chloramphenicol, streptomycin and tetracycline, respectively; MacNeil *et al.*, 1992) harbouring cosmid supercos 1 (Agilent; containing the *bla* gene for ampicillin resistance and *aph* gene for resistance to kanamycin and neomycin), and in addition the apramycin resistance cassette *aacC4*. MDR clinical isolates of *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter cloacae* were obtained from Erasmus Medical Centre (Rotterdam, Netherlands). Indicator bacteria were cultured in LB media at 37 °C. Antimicrobial assays were conducted using the double-layer agar method. Briefly, MM agar plates, containing supplements as detailed in Table 1, were inoculated with actinomycetes. Plates were incubated for 4 days at 30 °C, or longer if lower temperatures were used, overlaid with LB top agar (0.6 % w/v agar) containing one of the indicator strains pregrown in liquid LB to exponential phase (OD_{500} 0.4–0.6) and then incubated overnight at 37 °C. The following day, antibacterial activity was determined as zones of inhibition (in mm) surrounding the actinomycete colonies. Antimicrobial activity was determined following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2004). Briefly, bacterial cells were cultured overnight on Colombia agar plates with 5 % sheep blood (Becton Dickinson). Colonies were picked and suspended in NaCl 0.9 % to a density of 0.5 MacFarland. This was diluted 1 : 100 in Mueller-Hinton Broth (MHB; Oxoid), and 100 μl of this suspension was added to wells containing antibiotics ranging from a concentration of 0.02 to 37.5 μg ml^{-1} of the different tested antibiotics in MHB. U-bottom 96-well plates (Greiner Bio One) were incubated for 18–24 h at 37 °C and MIC values were determined visually. MIC determinations were performed in triplicate.

16S ribosomal gene sequencing and phylogenetic analysis. The 16S rRNA genes of the actinomycetes were amplified by PCR from liquid-grown mycelia using primers F1 (5′-GCGTGCTTAACA-

Table 1. Media supplements used to elicit antibiotic production (for details, see Methods)

No.	Supplement	No.	Supplement	No.	Supplement
1	25 mM *N*-acetylglucosamine (Fluka)	14	0.1 % Vitamins (vitamins with minerals tablets; Hangzhou, China) (w/v)	27	1 % Cellobiose (w/v) (Sigma)
2	0.3 % $NaNO_2$ (w/v) (Fluka)	15	1 % Xylose (w/v) (Sigma)	28	1 % Galactose (w/v) (Merck)
3	0.7 % KNO_3 (w/v) (Merck)	16	0.6 % Yeast extract (w/v) (Bacto)	29	1 % Lactose (w/v) (Sigma)
4	pH 10 (adjusted using 5 M NaOH)	17	0.5 % Malt extract (w/v) (Bacto)	30	1 % Mannose (w/v) (Janssen)
5	pH 8 (adjusted using 5 M NaOH)	18	0.8 % Peptone (w/v) (Difco)	31	1 % Sucrose (w/v) (BDH)
6	pH 6 (adjusted using 2 M HCl)	19	0.001 % Jasmonic acid (w/v) (Sigma)	32	0.5 % Trehalose (w/v) (Brunschwig)
7	pH 4 (adjusted using 2 M HCl)	20	2.5 % Fungal extract (w/v) (in-house extraction)	33	0.8 % Dextrin (w/v) (Merck)
8	1 % Starch (w/v) (Difco)	21	1.2 % Milk powder (w/v) (Friso)	34	0.5 % Glycine (w/v) (Merck)
9	1 % $MgCl_2$ (w/v) (Merck)	22	2 % Oatmeal (w/v)	35	0.5 % Cellulose (w/v) (Sigma)
10	0.1 % $CaCl_2$ (w/v) (Merck)	23	1 % Maltose (w/v) (Sigma)	36	0.1 % Glucosamine (w/v) (Sigma)
11	0.6 % NaCl (w/v) (Merck)	24	0.8 % Glucose (w/v) (Sigma)	37	1 % Chitin (w/v) (Sigma)
12	1 % Casein (w/v) (Difco)	25	1 % Fructose (w/v) (Sigma)	38	High temperature (45 °C) for overnight
13	25 mM Na/K phosphate buffer	26	1 % Arabinose (w/v) (Sigma)	39	Low temperature (4 °C) for overnight

CATGCAAG-3′) and R1 (5′-CGTATTACCGCGGCTGCTG-3′), corresponding to nt 15–34 and 465–483 of the 16S rRNA locus of *Streptomyces coelicolor* A3(2), respectively. PCRs were performed as described (Kieser *et al.*, 2000) and sequenced using oligonucleotide Seq F1 (5′-TGCTTAACACATGCAAGTCG-3′). Sequencing was done by BaseClear. 16S sequences were deposited in the GenBank database as a batch under the accession number BankIT 1698416. 16S rRNA sequences were used as query for BLASTN analysis. Subsequently, a phylogenetic tree based on these partial 16S rRNA sequences was reconstructed, essentially as described previously (Girard *et al.* 2013). To that aim, sequences of known actinomycetes were retrieved from GenBank. Sequences were aligned with MAFFT (Katoh *et al.*, 2009). After visual inspection, alignments were trimmed for gaps where >5 % of the sequences were missing using the Extractalign tool of the eBioX package (http://www.ebioinformatics.org/ebiox/). The phylogenetic tree was generated using maximum-likelihood algorithms with default parameters as implemented in MEGA version 5 (Tamura *et al.*, 2011). The tree reliability was estimated by bootstrapping with 1000 replicates. As groupings supported by poor bootstrap values were not reliable, internal branches with a bootstrap value of <50 % were collapsed so as to emphasize the reliable branching patterns.

TLC and HPLC. Cultures were extracted with ethyl acetate, which was then removed under vacuum at 40 °C, after which the residue was dissolved in methanol. Chemical analysis was conducted by thin layer chromatography (TLC) and HPLC. Specifically, TLC silica gel 60 F_{254} (Merck) plates were developed using chloroform and methanol as the solvent system, and visualized under UV light at 254 and 365 nm; HPLC analysis was performed using an Agilent Technologies 1200 series chromatographic system with a photodiode array detector and separated on a Phenomenex Luna C18 column (4.6 mm × 250 mm, 5 μm) with methanol/water as the mobile phase, applying a gradient of 20–100 % methanol over 40 min at a flow rate of 1 ml min^{-1}. The TLC bioautography assay was performed by placing the developed TLC plate onto the bioassay Petri dish overlaid with soft LB agar (Hispanagar) (0.6 %) containing *B. subtilis* as an indicator. Following 2 h incubation, the TLC plate was removed and incubated overnight at 37 °C. A control plate was also processed using the same solvents excluding test material to ensure the TLC and solvents themselves did not affect the growth of the indicator strain. The activity assessment was based on inhibition zones of the indicator strain.

For chromatographic methods, we used normal-phase silica gel (Merck; pore size 60 Å, 230–400 mesh), Sephadex LH-20 (Pharmacia), reversed-phase C18 semi-preparative HPLC and preparative TLC (PLC silica gel 60 F_{254} TLC; Merck).

NMR and mass spectrometry. Structure elucidation of pure active compounds was done by NMR measurement and MS analysis. The spectra of ^{1}H NMR, correlation spectroscopy, *J*-resolved, heteronuclear single quantum coherence, heteronuclear multi-bond correlation spectroscopy and ^{13}C attached proton tests of purified compounds were recorded on a 600 MHz DMX-600 spectrometer (Bruker) operating at a proton NMR frequency of 600.13 MHz (^{1}H) and 150.13 MHz (^{13}C). The specific ^{1}H NMR experiment measurement parameters were set as described (Kim *et al.*, 2010).

Fourier transform MS (Bruker) was used to determine the exact mass of the compounds. The analyses were performed by direct infusion nano-electrospray ionization (ESI)-MS in the positive-ion mode using the automated Advion NanoMate TriVersa system (type 'A' chip) coupled to a LTQ FT Ultra (Thermo Fisher Scientific).

RESULTS

A collection of actinomycetes and growth conditions to elicit antibiotic production

A new collection of actinomycetes was created from soil samples obtained from unspoilt areas in the Qinling and Himalaya mountains (China). In total, 816 different actinomycetes were recovered from the soil samples (see Methods for details). The strains were tested initially for their ability to inhibit the growth of indicator strains *B. subtilis* 168 and *E. coli* ET8, which carries several resistance cassettes. The vast majority of the actinomycetes inhibited growth of *B. subtilis* even under routine growth conditions and production of antibiotics with efficacy against Gram-positive bacteria was therefore only pursued with MDR pathogens (see below). When analysing inhibition of *E. coli* ET8, 108 actinomycetes inhibited its growth under control growth conditions, whilst an additional 207 could also be induced by at least one of the media supplements to inhibit growth of ET8, i.e. a total of 315 out of 816 strains (39 % of

the strain collection) produced inhibitory activity against this multiresistant Gram-negative strain. Of the 40 different growth conditions, pH 10, starch (1 % w/v) and peptone (0.8 % w/v) had the strongest stimulatory effect on antibiotic production. The strongly stimulatory effect of high pH values is particularly noteworthy as it by no means a common condition for screening of antibiotics. All growth conditions also repressed antibiotic production by a number of the species in the collection (Fig. 1). As an example, we compared the producing capacity of eight *Streptomyces* spp. from the collection, i.e. MBT1, MBT7, MBT11, MBT12, MBT22, MBT27, MBT28 and MBT33, which were all grown on MM agar plates or MM with added *N*-acetylglucosamine, starch or peptone, or MM pH 10. Again using *E. coli* ET8 as the indicator strain, the inhibition zones varied strongly by species and by growth conditions (Fig. 2). MBT1 and MBT12 inhibited growth of *E. coli* ET8 most strongly when grown on routine MM agar, whilst production of antibiotics by these species was blocked by growth at pH 10; conversely, MBT7 and MBT11 were induced by growth at pH 10. Antibiotic production by MBT22 and MBT33 was induced by *N*-acetylglucosamine, whilst the same compound repressed antibiotic production by MBT27 and MBT28 (Fig. 2).

To see if the specific changes to the growth media – and in particular growth at pH 10 – affected the susceptibility of the indicator strains against antibiotics, we determined the inhibition zones for reference antibiotics against *E. coli* ET8 and *B. subtilis*. This showed that the zone of inhibition caused by ampicillin, apramycin, chloramphenicol or kanamycin did not increase when the indicator strains were grown at pH 10 (Fig. S1, available in the online Supplementary Material). These data corroborate that the growth conditions did not affect the susceptibility of *B. subtilis* or *E. coli*, but instead elicited antibiotic production in the actinomycetes.

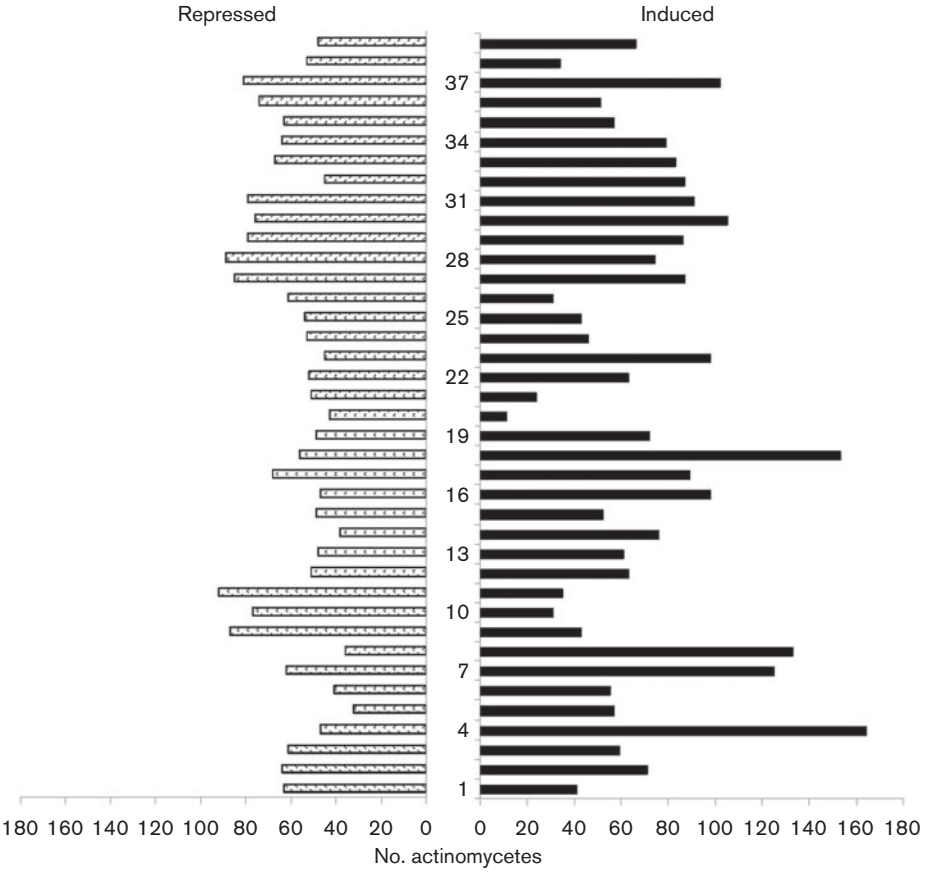

Fig. 1. Quantification of induction or repression of antimicrobial activity in actinomycetes against *E. coli* ET8 depending on the growth conditions. Numbers on the vertical axis represent growth conditions as listed in Table 1 and those on the horizontal axis represent the total number of actinomycetes whose antibiotic production was either induced or repressed under each of the 40 growth conditions. Strains were grown on MM agar plates with 0.5 % mannitol and 1 % glycerol as carbon sources, with or without additives, and then overlaid with top agar containing indicator strain *E. coli* ET8. Inhibition zones were then measured as zones of clearing. Only significant changes (changes of >1 mm and 10 % in diameter) in the size of the zones in all replicates of the triplicate experiment were considered.

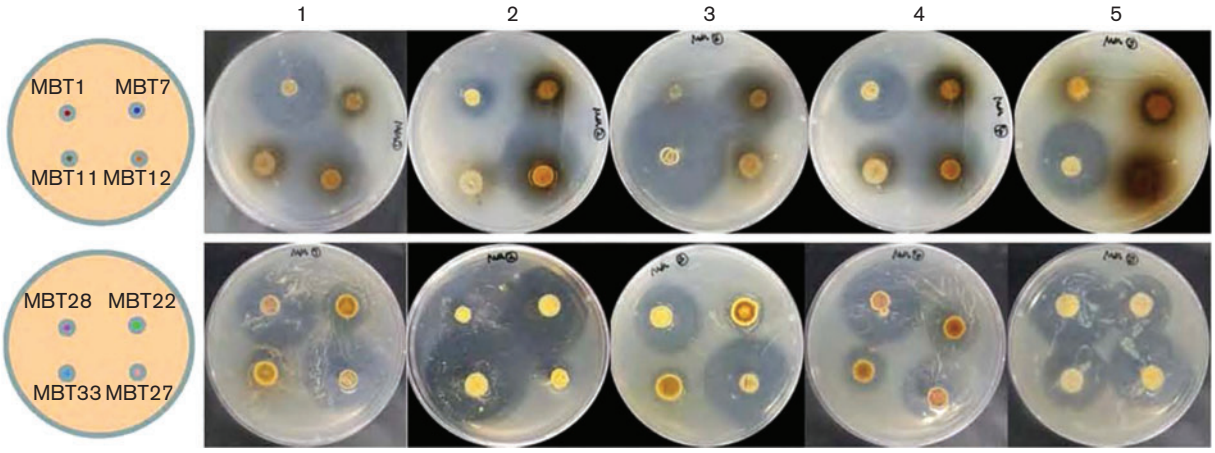

Fig. 2. Antimicrobial activity of eight actinomycetes against *E. coli* ET8 under five different growth conditions. Strains were grown on MM agar plates with 0.5 % mannitol and 1 % glycerol as carbon sources, with or without additives. 1, No additives; 2, 25 mM *N*-acetylglucosamine; 3, pH adjusted to 10; 4, starch (1 % w/v); 5, peptone (0.8 % w/v). The experiment served as a representative example of the inducibility of antibiotic production in actinomycetes, under the chosen conditions. For details on the effect of elicitors on the 96 strains, refer to Tables S1 and S4.

Analysis of 96 promising strains for antibiotic production against MDR clinical isolates

Based on the screening of the strain collection, the 96 most prolific antibiotic-producing actinomycetes were identified, with their antibiotic production typically induced under specific growth conditions. Of these 96, in total 89 strains were identified as *Streptomyces* (Zhu *et al.*, 2014; Table S2, Fig. S2). Other genera were *Kitasatospora* (MBT63, MBT64, MBT66 and MBT69), *Nocardia* (MBT52), *Micromonospora* (MBT87) and *Amycolatopsis* (MBT80). All of the 96 actinomycetes inhibited *B. subtilis* when grown on MM agar plates (not shown). To obtain detailed insight into their producing capacity, antibiotic production was further assessed with the multiresistant *E. coli* ET8 as the indicator strain. For this, the actinomycetes were grown for 4 days on MM agar, MM agar with starch (1 % w/v), MM agar with peptone (0.8 % w/v) or MM agar at pH 10 and inhibition zones determined (Table S1). This revealed that all strains were able to inhibit growth of *E. coli* ET8 under at least one of the growth conditions.

The antibiotic-producing potential of the 96 strains that all showed strong antimicrobial activity against the MDR *E. coli* strain ET8 was then assessed for possible clinical relevance by analysing their ability to inhibit the growth of six MDR clinical isolates. These clinical isolates were all isolated recently at the Erasmus Medical Centre. The resistance profile of these isolates (Table S3) was determined as MIC values using the VITEK II (bioMérieux) system (AST-P586 for *Enterococcus faecium*, AST-P608 for *Staphylococcus aureus*, and AST-N140 for *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter cloacae*).

In accordance with previous reports on antimicrobial susceptibility (Burt, 2004; Nair & Chanda, 2006), Gram-positive pathogens (*Staphylococcus aureus*, *Enterococcus faecium*) were more sensitive to the antibiotics produced by the actinomycetes than Gram-negative pathogens (*A. baumannii*, *Enterobacter cloacae*, *K. pneumoniae*, *P. aeruginosa*) (Table S4). An example of the differential antimicrobial activity produced by 24 of the actinomycetes of the collection against the six pathogens is shown in Fig. S3. Growth of the Gram-positive meticillin-resistant *Staphylococcus aureus* (MRSA) was inhibited by 82 of the actinomycetes grown on routine MM agar plates and by another 10 strains when they were grown under specific conditions. Under the same growth conditions, 53 actinomycetes inhibited growth of the Gram-positive *Enterococcus faecium* and an additional 36 actinomycetes inhibited *Enterococcus faecium* under other growth conditions. Most Gram-negative bacteria were inhibited by ~40 % of the actinomycetes growth on MM agar plates, namely *K. pneumoniae* by 41 strains, *A. baumannii* by 37 strains and *Enterobacter cloacae* by 34 strains. Changing the growth media activated antibiotic production against the latter three pathogens in another 16, 27 and 15 strains, respectively. Notably, the opportunistic pathogen *P. aeruginosa* was extremely resilient, with only seven of the 96 actinomycetes able to exert some degree of growth inhibition to this strain when grown on standard MM agar plates. However, antibiotic production against *P. aeruginosa* could be elicited in 33 of the 89 non-producing actinomycetes by growth on MM with added starch or peptone, or at pH 10 (Fig. 3a).

It is important to note that actinomycetes that inhibited one Gram-negative pathogen often did not inhibit any of the other Gram-negative pathogens, which suggests strongly that different antibiotics were produced (Table S4). As an example, strains MBT32, MBT44, MBT94 and MBT96 only inhibited growth of *A. baumannii*, whilst they

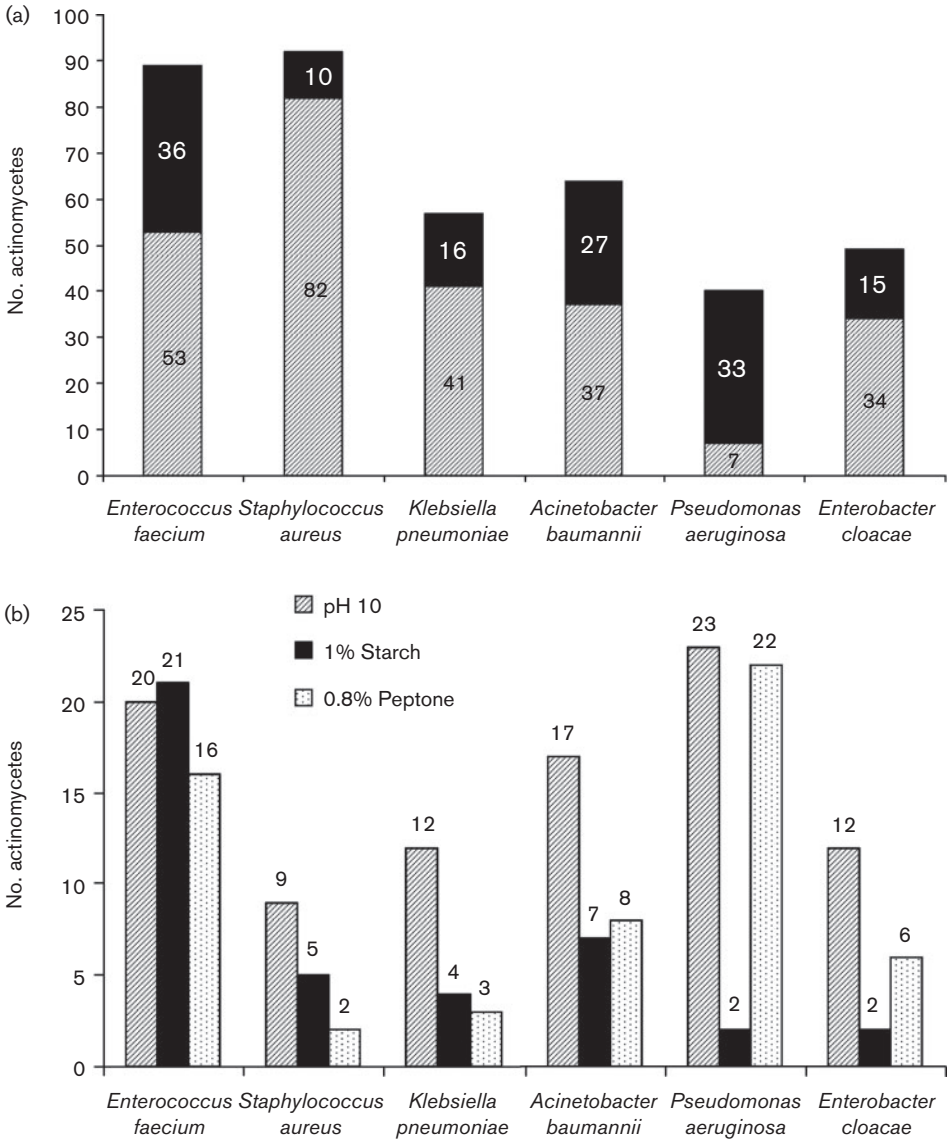

Fig. 3. Inducibility of antimicrobial activity of 96 selected actinomycetes against MDR pathogens and effect of media conditions. (a) Bar diagram of total number of actinomycetes that could inhibit the indicated MDR pathogens when grown under non-induced (light grey) or induced (black) conditions. (b) Bar diagram of the number of actinomycetes that were induced to produce antibiotics with efficacy against the MDR strains by growth on MM at pH 10 (hashed), MM with starch (black) or MM with peptone (small dots). Antimicrobial assays were conducted using the double-layer agar method (see Methods). The tested pathogens were *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. cloacae*.

had no effect on the growth of the other MDR Gram-negative clinical isolates of *K. pneumoniae*, *Enterobacter cloacae* and *P. aeruginosa*; conversely, MBT82 only inhibited the growth of *Enterobacter cloacae*. Intriguingly, MBT10 (when grown on MM with pH adjusted to 10) inhibited exclusively the growth of MDR *P. aeruginosa* and none of the other Gram-negative pathogens, even though *P. aeruginosa* was by far the most resilient of all pathogens in our analyses.

Expectedly, antimicrobial activity varied significantly with the growth conditions (Fig. 3b). When grown on MM with starch, antibiotics were produced with activity against the Gram-positive *Enterococcus faecium* and *Staphylococcus aureus* in 21 and five actinomycetes, respectively; for MM at pH 10, this was the case for 20 and nine actinomycetes, and for MM with peptone for 16 and two actinomycetes, respectively. For the induction of antibiotics active against *K. pneumoniae* and *A. baumannii*, increasing the pH to 10 was most effective, activating the production of antibiotics by 17 actinomycetes against *A. baumannii* and by 12 actinomycetes against *K. pneumoniae*. When grown on MM

with starch or peptone, seven and eight strains inhibited growth of *A. baumannii*, respectively, and for *K. pneumoniae* these numbers were four and three, respectively.

Venn diagrams (Fig. 4) were used to visualize the specificity of the antibiotics produced by the 96 actinomycetes. Despite the multiple resistance cassettes present in *E. coli* ET8 for dereplication purposes, all of the 96 strains inhibited growth of this indicator strain under at least one of the growth conditions. Furthermore, 30 out of 96 actinomycetes could be activated by one or more growth conditions to produce antibiotics against all of the tested MDR pathogens (Table S4). Considering the broad resistance spectra of these pathogens, this underlines the potential of these actinomycetes in terms of antibiotic production. Of these, 34 actinomycetes had the potential to produce antibiotics active against *P. aeruginosa* and *K. pneumonia*, six specifically inhibited growth of *P. aeruginosa* and not of *K. pneumonia*, whilst the reverse was true for 23 actinomycetes. This suggests strongly that many of the actinomycetes produced different antibiotics. In a similar comparison, but with the Gram-negative *Enterobacter cloacae* and *A. baumannii*, a total of 44 actinomycetes were active against both MDR pathogens, with 20 only against *A. baumannii* and five only against *Enterobacter cloacae*. Comparing the Gram-positive MDR pathogens *Enterococcus faecium* and *Staphylococcus aureus*, 86 out of 96 tested actinomycetes were active against both indicator strains, six of which were specific against *Staphylococcus aureus* and three specific against *Enterococcus faecium* (Fig. 4).

Identification of antibiotics

The activity-guided assay demonstrated that all actinomycetes produced varying levels of growth-inhibiting bioactivities. However, a question that needed to be answered was to what extent the growth inhibition was indeed caused by antibiotics with possible application as clinical drugs, rather than representing general disruptive activities. Examples of the latter include surfactants, acids or lytic agents and agents with a mode of action that could also have a detrimental effect on eukaryotic cells, including agents that target DNA, induce cell lysis or damage the cell membrane. Therefore, bioactivities that fluctuated strongly depending on the growth condition were selected for further characterization. For this validation study, *Streptomyces* spp. MBT28, MBT70, MBT73 and MBT76 (all part of the subcollection of 96 actinomycetes) were selected as they showed strongly inducible activity and therefore served as reference strains for this study. Importantly, as discussed below, all of the antimicrobial bioactivities that were characterized were indeed identified as antibiotics. However, it should be noted that all of the strains produced multiple antibiotics (not shown) and the entire antibiotic-producing capacity of the strains is currently under investigation. A summary of the data is presented below.

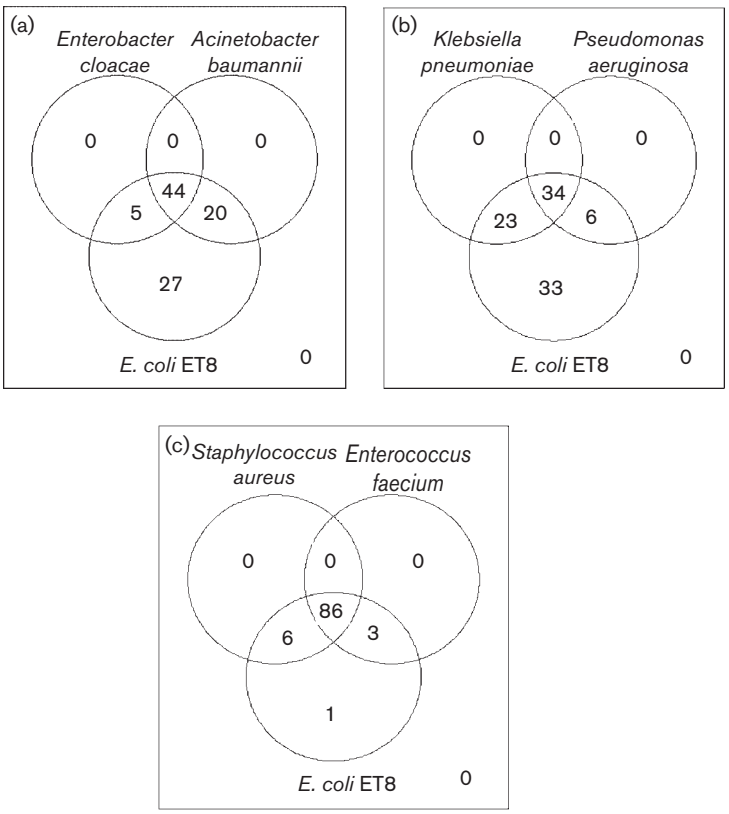

Fig. 4. Venn diagrams of antimicrobial activities of 96 selected actinomycetes against different groups of pathogens. Numbers refer to the number of actinomycetes that produced antibiotics that inhibited the indicated MDR pathogens, under any of the four conditions listed in Fig. 3. The groups of pathogens were (a) *Enterobacter cloacae*, *A. baumannii* and *E. coli* ET8, (b) *K. pneumoniae*, *P. aeruginosa* and *E. coli* ET8, and (c) *Staphylococcus aureus*, *Enterococcus faecium* and *E. coli* ET8.

For details on the compound isolation procedure, the reader is referred to Supplementary Data File 1; for NMR data, the reader is referred to Supplementary Data File 2 and Figs S4 and S5.

A bioactive agent that was produced specifically by *Streptomyces* sp. MBT73 on MM with starch (see HPLC chromatograms in Fig. 5a) was isolated as 4.6 mg of a pure orange compound with a molecular formula of $C_{22}H_{16}O_6$ based on an ESI-MS $[M+Na]^+$ accurate mass of *m/z* 375.08926. This compound was identified by NMR analysis on the isolated compound as the antimicrobial and anticancer compound resistomycin, 3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2H-benzo[*cd*]pyrene-2,6(1H)-dione, which was described previously, and produced by *Streptomyces resistomycificus*, *Streptomyces griseoincarnatus* and *Streptomyces aurantiacus* (Sajid *et al.*, 2011; Vijayabharathi *et al.*, 2011). The proton NMR spectrum is presented in Fig. S4. Further confirmation was provided by genome sequencing, which revealed a gene cluster with high similarity (95 % nucleotide level; not shown) to that of the previously published resistomycin gene clusters from *Streptomyces griseoincarnatus* and *Streptomyces aurantiacus*.

An antibiotic induced during growth at high pH from *Streptomyces* sp. MBT28 (Fig. 5b) was isolated as 8 mg of a pure and colourless compound with a molecular formula of $C_{28}H_{43}NO_6$ based on an ESI-MS $[M+Na]^+$ accurate mass of *m/z* 488.30438. The compound was identified by NMR analysis as the antimicrobial, antiviral and antimalarial agent borrelidin, 2-(7-cyano-8,16-dihydroxy-9,11,13,15-tetramethyl-18-oxooxacyclooctadeca-4,6-dien-2-yl)-cyclopentane-carboxylic acid (Fig. S4). Borrelidin is a natural product first identified in *Streptomyces rochei* (Wakabayashi *et al.*, 1997). The gene cluster was identified by genome sequencing in MBT28 (not shown), and shared 89, 97 and 93 % nucleotide identity with the borrelidin biosynthetic gene clusters of *Streptomyces rochei*, *Streptomyces parvulus* and *Streptomyces griseus*, respectively.

Growth on MM with peptone elicited the production of at least three distinct antibacterial agents in *Streptomyces* sp. MBT70, as shown by TLC (Fig. 5c). For one of these, 1.9 mg of a pure and brown compound was obtained. The compound was readily characterized by NMR on the purified compound (Fig. S4) as 1-carbomethoxy-phenazine, with a molecular formula of $C_{14}H_{10}N_2O_2$ (Römer, 1982). Phenazines are produced by pseudomonads and actinomycetes, and the biosynthetic gene clusters from *Streptomyces* MBT70 and of *Streptomyces cinnamonensis* (Karnetová *et al.*, 1983) share 95 % nucleotide identity (not shown).

Growth of *Streptomyces* sp. MBT76 on MM with peptone resulted in the isolation of two compounds (see HPLC chromatograms in Fig. 5d). For the first compound, 2.3 mg of a colourless compound was obtained with a molecular formula of $C_{13}H_{15}O_7$ based on the ESI-MS $[M+Na]^+$ accurate mass of *m/z* 273.0905. For the second compound, 2.8 mg of a yellowish solid was obtained with the same predicted molecular formula of $C_{13}H_{15}O_7$ (ESI-MS $[M+Na]^+$ accurate mass of *m/z* 273.0932). NMR analysis showed that the two compounds were similar but distinct isocoumarin-type compounds. The first isocoumarin was identified as the previously described 6,7,8-trimethoxy-3-methylisocoumarin (Hegde *et al.*, 1989), whilst the second isocoumarin was identified as 5,6,8-trimethoxy-3-methylisocoumarin, which has not yet been described in the literature. For details on the ^1H and ^{13}C NMR data of the isocoumarins, see Fig. S5, Table S5 and Supplementary Data File 1. These data show that the observed antimicrobial activities were caused by pharmaceutically relevant natural products.

All compounds were tested for antimicrobial activity against the ESKAPE pathogens using MIC assays as described in Methods. The compounds 1-carbomethoxy-phenazine, borrelidin, resistomycin and the previously undescribed 5,6,8-trimethoxy-3-methylisocoumarin showed activity against the Gram-positive *Enterococcus faecium* (Table 2). However, the known isocoumarin 6,7,8-trimethoxy-3-methylisocoumarin did not display noticeable antimicrobial activity. Borrelidin and resistomycin inhibited growth of the Gram-negative strains *A. baumannii* and *K. pneumoniae*, with resistomycin showing very strong activity (MIC <0.3 μg ml^{-1}) against both of the Gram-negative MDR pathogens, whilst borrelidin showed similarly strong activity against *K. pneumoniae*, but only mild activity against *A. baumannii*. No bioactivity was found for any of the compounds against MRSA nor against the Gram-negative pathogens *Enterobacter cloacae* and *P. aeruginosa*.

DISCUSSION

In this work, we have isolated over 800 actinomycetes from remote mountain areas in the Qinling and Himalaya mountains (China), and tested their capacity to produce antimicrobial agents with efficacy against Gram-positive and Gram-negative bacteria, and in particular antibiotics that inhibit growth of one or more of the six MDR 'ESKAPE' pathogens isolated recently from a nosocomial environment in the Netherlands. The observed bioactivity profiles of the actinomycetes underlined the importance of screening for antibiotics against specific pathogens, in addition to the search for a broadly applicable antibiotic. Gram-negative pathogens are a major problem in the nosocomial environment (Rice, 2008) and therefore many screening efforts focus on finding novel antibiotics that target these pathogens. In our study, 82 of the 96 isolates that were identified as the best antibiotic producers of the strain collection inhibited the Gram-positive MRSA even when grown on control MM agar plates and 53 inhibited *Enterococcus faecium*, whilst a significantly lower number of strains inhibited growth of Gram-negative MDR pathogens when grown on these standard media. Many of the actinomycetes that produced antibiotics against the Gram-negative MDR *K. pneumoniae* or *E. coli* under a certain growth condition did not inhibit *P. aeruginosa* and vice versa. In fact, *P. aeruginosa*, which is the major

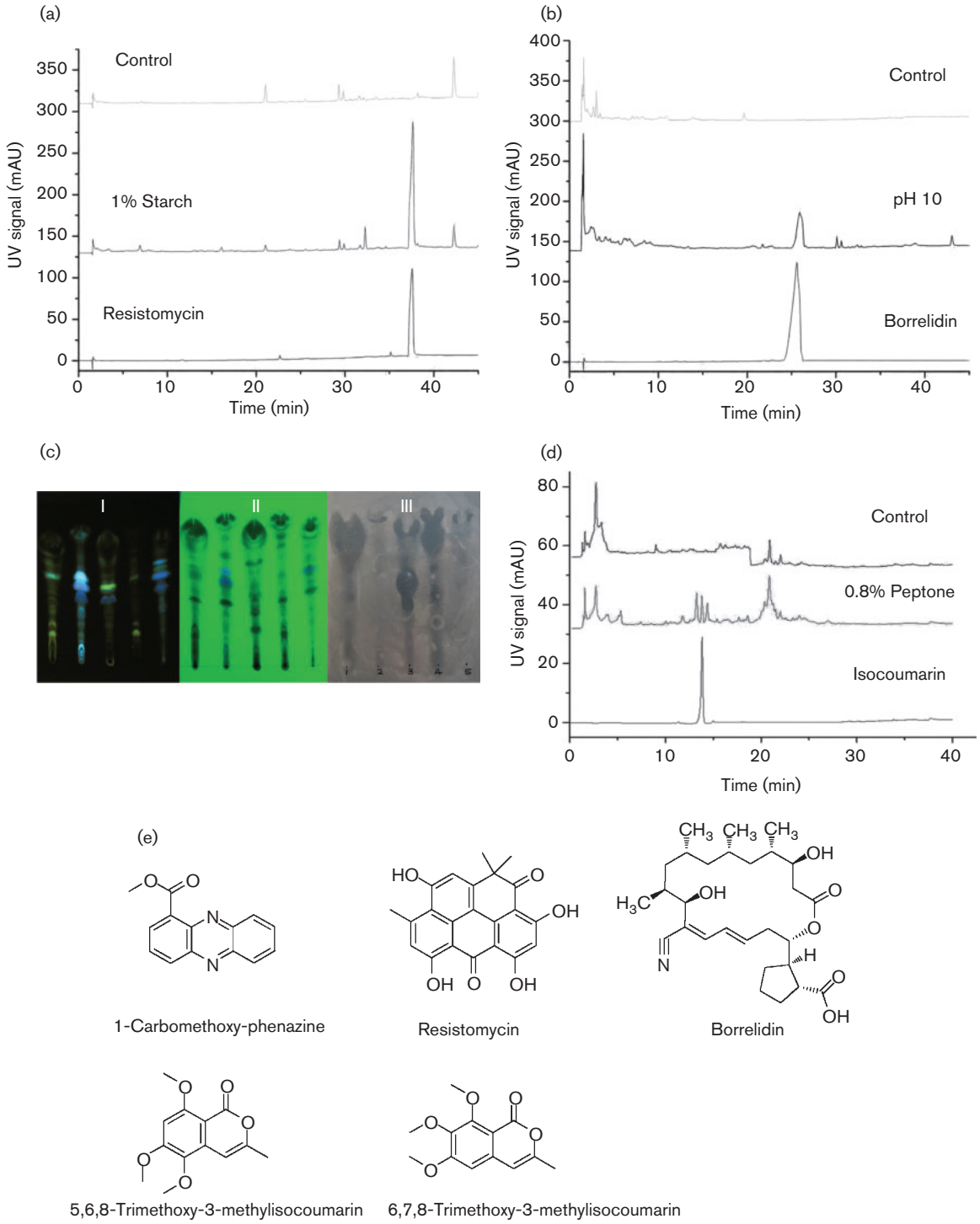

Fig. 5. Induction of antibiotic production. Resistomycin, borrelidin, 1-carbomethoxy-phenazine, and the isocoumarins 5,6,8-trimethoxy-3-methylisocoumarin and 6,7,8-trimethoxy-3-methylisocoumarin were produced by *Streptomyces* spp. MBT73, MBT28, MBT70 and MBT76, respectively, under specific growth conditions. (a) HPLC chromatograms (366 nm) of ethyl acetate extracts of MBT73 grown under control conditions (MM; top) and starch-supplemented cultures (middle); purified resistomycin (bottom) is presented as control. (b) HPLC chromatograms (245 nm) of ethyl acetate extracts of MBT28 grown under control conditions (MM; top) and at pH 10 (middle); purified borrelidin (bottom) is presented as control. (c) TLC of extracts of MBT70 grown in (from left to right): 1, MM; 2, MM with 25 mM *N*-acetylglucosamine; 3, MM with peptone; 4, MM pH 10; 5, MM with starch. TLC solvent was a 10 : 1 mixture of chloroform and methanol. The TLC plates show (I) separated compounds under UV 366 nm, (II) separated compounds under UV 254 nm and (III) antimicrobial activity against *B. subtilis* via

an agar overlay assay. (d) HPLC chromatograms (366 nm) of ethyl acetate extracts of MBT76 grown under control conditions (MM; top) and peptone-supplemented cultures (middle); purified 5,6,8-trimethoxy-3-methylisocoumarin (bottom) is presented as control. (e) Chemical structures of the identified natural products.

pathogen found in cystic fibrosis lungs (Davies, 2002), was by far the most resilient of the six clinical isolates tested, with only seven of the 96 best antibiotic-producing strains of our collection inhibiting growth of this bacterium under routine growth conditions. For comparison, 34 of the 96 strains inhibited growth of *K. pneumoniae*, which is also a very dangerous MDR pathogen recently associated with major outbreaks in hospitals. It is therefore particularly interesting to note that *Streptomyces* strain MBT10 inhibited exclusively the growth of MDR *P. aeruginosa* and none of the other Gram-negative pathogens, and we are therefore currently investigating the possible application of this strain for the development of drugs against this dangerous opportunistic pathogen. These experiments underline that specific antimicrobial agents that act against a certain pathogen may have advantages over traditional broad-spectrum agents, whereby resistance development and side effects could potentially be reduced (Fischbach & Walsh, 2009). An example of the success of antibiotics acting against a specific clinical pathogen is the recent development of the lipopeptide antibiotic surotomycin for treatment of diseases caused by *Clostridium difficile* – an anaerobic spore-forming Gram-positive bacterium of the lower gastrointestinal tract (Tran *et al.*, 2013). With the rapidly increasing occurrence of MDR pathogens in the clinic, the development of drugs with efficacy against specific infectious diseases appears to be a logical strategy.

Screening under different growth conditions is a strategy that has been used by the pharmaceutical industry for many decades, but the low success rates in more recent years has led to a steep decline in drug discovery efforts (Payne *et al.*, 2007). We analysed the efficacy of the strains of our collection against MDR pathogens that currently threaten the health of patients in the nosocomial environment, whereby the strains were grown under 40 different growth conditions. These included more traditional growth conditions as well as novel conditions, such as the addition of *N*-acetylglucosamine, which inhibits the activity of the global antibiotic repressor DasR and was shown previously to activate antibiotic production, including the cryptic polyketide Cpk in *Streptomyces coelicolor* (Rigali *et al.*, 2008). However, of the conditions tested in this study, high pH values (pH 10) in particular appeared to be a promising new condition for screening, as these efficiently elicited antibiotic production. Previous studies on productivity by the fungus *Sclerotinia sclerotiorum* using a pH range between 5 and 9 showed optimal production at pH 7 (Chater *et al.*, 2010). Here, we show that the effect of pH varies from strain to strain and that in fact there is strong potential for using high pH values for screening purposes, whilst low pH (acidic conditions) was less effective in our assays. Perhaps counter-intuitively, growth at high pH did not affect the susceptibility of the indicator strains *B. subtilis* or *E. coli*, nor did it noticeably influence growth and development of the actinomycetes. As an example of the application of pH 10 during screening, strain MBT68 inhibited growth of *Enterococcus faecium*, *P. aeruginosa*, *K. pneumoniae*, *Enterobacter cloacae* and *A. baumannii* only when grown on MM at pH 10. With the observed strong effects on antibiotic production in many of the strains in our collection, very high pH appears to be a very promising growth condition for antimicrobial screening efforts.

Detailed analysis of the antimicrobial compounds produced by a number of the isolates identified both new and previously identified compounds. This is exemplified by the metabolic profiles of four *Streptomyces* spp. grown under various conditions, which revealed inducible borrelidin production at high pH by MBT28, peptone-inducible production of 1-carbomethoxy-phenazine by MBT70 and of both 5,6,8- and 6,7,8-trimethoxy-3-methylisocoumarin by MBT76, and starch-inducible resistomycin production by MBT73. It should be noted that – as is often the case

Table 2. MIC values (μg ml^{-1}) for the isolated compounds against the ESKAPE pathogens

	Enterococcus faecium	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Enterobacter cloacae*
1-Carbomethoxy-phenazine	37.5	>37.5	>37.5	>37.5	>37.5	>37.5
Borrelidin	9.375	>37.5	0.29	18.75	>37.5	>37.5
Resistomycin	0.59	>37.5	0.29	0.15	>37.5	>37.5
5,6,8-Trimethoxy-3-methylisocoumarin	18.75	>37.5	>37.5	18.75	>37.5	>37.5
6,7,8-Trimethoxy-3-methylisocoumarin	>37.5	>37.5	>37.5	>37.5	>37.5	>37.5

for streptomycetes – all of the four strains can produce multiple antibiotics, so that not all antimicrobial activity can be explained by the identified compounds (not shown). Borrelidin and resistomycin are commercially highly relevant natural products. Borrelidin is a nitrile-containing macrolide that acts as a potent inhibitor of both bacterial and eukaryotic threonyl-tRNA synthetases (Habibi *et al.*, 2012; Nass & Hasenbank, 1970), whilst resistomycin is a pentacyclic polyketide metabolite that inhibits RNA polymerase (Haupt *et al.*, 1975), HIV-1 protease (Roggo *et al.*, 1994) and apoptosis (Shiono *et al.*, 2002), and was first applied as drug for the treatment of tuberculosis. 1-Carbomethoxy-phenazine is known to have weaker antimicrobial activity. Of the isocoumarins, 6,7,8-trimethoxy-3-methylisocoumarin acts on calmodulin-sensitive cAMP- and cGMP-phospho-diesterases (Hegde *et al.*, 1989). Applicability of isocoumarins as an antibiotic, and in particular also that of the novel antibiotic 5,6,8-trimethoxy-3-methylisocoumarin discovered in this work, should be analysed in more detail.

In conclusion, thorough analysis of a novel collection of actinomycetes from soil samples obtained from remote mountains shows their antibiotic-producing potential when grown under a range of different growth conditions, of which high pH proved to be particularly efficient. The study provided new insights into the efficacy of antibiotics produced against recently isolated MDR pathogens and identified a number of antibiotics whose expression varied greatly with the growth conditions, including a previously unidentified isocoumarin-type antibiotic. In particular, the highly differential sensitivity of the various MDR 'ESKAPE' pathogens to the antibiotics produced by the actinomycetes underlines that an important strategy may be to search for drugs that target a specific pathogen.

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