Sponge-associated sp. RM66 metabolome induction with N-acetylglucosamine: Antibacterial, antifungal and anti-trypanosomal activities

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Original article

1. Introduction

Members of the order Actinomycetales produce a multitude of antibiotics and other natural products (Vicente et al., 2013; Abdelmohsen et al. 2014a, 2014b; Muller and Wink 2014). Sponge-associated actinomycetes showed diverse pharmacological activities including antiparasitic, antimalarial, antibacterial, anti-fungal, anti-inflammatory, antioxidant, anticancer, and immunomodulatory properties (Mayer and Hamann 2005; Bull and Stach 2007; Pimentel-Elardo et al. 2010; Abdelmohsen et al. 2012; Blunt et al. 2013; Eltamany et al. 2014; Grkovic et al. 2014; Cheng et al. 2015). The actinomycete genomes have showed many biosynthetic genes that encode for natural products that not observed under standard fermentation conditions, this was figured out with the advances in sequencing technologies, (Cimermancic et al. 2014; Marmann et al. 2014; Ziemert et al. 2014; Abdelmohsen et al. 2015). Some of these methods, previously used to induce cryptic metabolites include chemical, biological and molecular elicitation (Brakhage 2013; Letzel et al. 2013; Liu et al. 2013; Luo et al. 2013; Zhu et al. 2014; Abdelmohsen et al. 2015). Altering fermentation conditions (media composition, pH, and temperature) using the (OSMAC) “one strain many compounds” approach and has been used to induce silent and/or poorly expressed metabolic pathways (Bode et al. 2002; Wei 2010; Abdelmohsen et al. 2014a, 2014b). Furthermore, using the technique of microbial strains co-cultivation is widely known to induce significant changes in the microbial metabolome (Pettit 2009;
Dash et al. 2014; Marmann et al. 2014) Chemical elicitors produced from one microorganism in co-culture, could upregulate the metabolite expression in the other microorganisms. N-acetylglucosamine (GlcNAc) is considered one of the chemical elicitor that acts as a signalling molecule and induces significantly increases the production of antibiotics in actinomycetes (Konopka 2012; Naseem et al. 2012). The amino sugar GlcNAc is considered a part of peptidoglycan in the cell walls of bacteria, chitin in fungal cell walls, and the extracellular matrix of animal cells. (Moussinan 2008; Konopka 2012) When the cell wall is remodelled during cell growth, a significant amount of GlcNAc is released into the environment and becomes available for cell signalling. (Konopka 2012; Naseem et al. 2012) Some reports have demonstrated that GlcNAc implementation into the culture medium of fungi (Simonetti et al. 1974; Pérez-Campo and Dominguez 2001) and bacteria (Sohsanpal et al. 2004; Barnhart and Chapman 2006; Rigali et al. 2008) activates signalling pathways in these microorganisms. It was shown that the production of uncyclodigiosin and actinorhodin antibiotics in Streptomyces coelicolor under poor nutritional conditions (Rigali et al. 2008). On the other hand, the addition of GlcNAc to the culture medium of Pseudomonas aeruginosa shows to enhance the phenazine antibiotic type yield, pyocyanin (Korgaonkar and Whiteley 2011). Advances in high resolution mass spectrometry e.g. Orbitrap or Quadrupole Time of Flight (Q-TOF) technology enables provision of accurate mass data, which can be utilised to rapidly dereplicate metabolite-rich extracts (Rainville et al. 2014; Rubbert et al. 2015). These instruments offer high sensitivity (nano- or pico-gram level) and the simultaneous collection of positive and negative mode data in a single experiment. Additionally, MS2 and MS3 data provides structural information, that enables tentative matches with existing natural products databases (Yuliana et al., 2011, Abdelmohsen et al. 2014). Chemical dereplication using bioinformatic tools allows a series of extracts to be compared in terms of chemical novelty, helping to prioritise strains for isolation work (Harvey et al. 2015). Metabolomic tools, for example, multivariate analysis can then be used to uncover trends e.g. qualitative alterations in metabolite production under various culture conditions by probing intensity of m/z values in a series of mass spectra from a collection of extracts (Frank et al. 2008; Hou et al. 2012; Forner et al. 2013; Tawfike et al. 2013; Krug and Muller 2014; Cheng et al. 2015). This technique allows for high-throughput multi-strain comparisons of thousands of complex metabolite extracts, and can reveal considerable metabolite diversity (Yang et al. 2013).

In this study, the effect of GlcNAc on the metabolic profile of the sponge-associated actinomycete, namely Streptomyces sp. RM66, using three different cultural media, both solid and liquid fermentation free and amended with N-acetylglucosamine. The detailed analysis of the chemical profile of the Streptomyces sp. RM66 before and after treatment with GlcNAc using LC-HRMS data via metabolomic tools was performed. Moreover, the antibacterial, antifungal and antityrrapsonal activities of crude extracts were reported.

2. Material and methods

2.1. Sponge collection, strain isolation and fermentation

The sponge Amphimedon sp. was collected about 5 km to the north of Hurgada (Egypt) at latitudes 27° 17’ 01.0” N, and longitudes 33° 46’ 21.0” E. The Sponge biomass was transferred into plastic bag containing seawater then transported to the laboratory. The sponge specimens were rinsed in sterile seawater, then cut into pieces of ca. 1 cm², and then thoroughly homogenized in a sterile mortar with 10 volumes of sterile seawater. The supernatant was diluted in ten-fold series (10⁻¹, 10⁻², 10⁻³) and subse-quentely plated out on agar plates. Using three different media (ISP2, M1 and Marine Agar (MA) for the isolation of actinobacteria. All the media were supplemented with 0.2 µm pore size filtered cycloheximide (100 µg/mL), nystatin (25 µg/mL) and nalidixic acid (25 µg/mL) to help the isolation of slow-growing actinobacteria. Cycloheximide and nystatin were used to inhibit fungal growth, while nalidixic acid used to inhibit many fast-growing Gram-negative bacteria. All media contained Difco Bacto agar (18 g/L) and were prepared in 1 l artificial sea water (NaCl 234.7 g, MgCl₂ 6 H₂O 106.4 g, Na₂SO₄ 39.2 g, CaCl₂ 11.0 g, NaHCO₃ 1.92 g, KCl 6.64 g, KBr 0.96 g, H₂BO₃ 0.26 g, SrCl₂ 0.24 g, NaF 0.03 g and ddH₂O to 10.0 L). The inoculated plates were incubated at 30°C for 6–8 weeks. Distinct colony morphotypes were picked and re-streaked until visually free of contaminants. Kocuria, Dietzia, Micrococcus, Microbacterium and Streptomyces were cultivated on ISP2 medium. The isolates were as maintained on plates for short-term storage and long-term strain collections were set up in medium supplemented with 30% glycerol at ~80°C. Streptomyces sp. RM66 was subjected to solid fermentation as follows, the strain was spread on five ISP2 agar plates (500 µL/plate) and after 2 h, 300 µl of N-acetylglucosamine (50 µM, Sigma-Aldrich, Germany) were spread over. After incubation, colonies and agar were cut into small pieces and macerated overnight with twice volume of ethyl acetate (Roth, Germany) with continuous shaking. Ethyl acetate macerate was filtered and maceration with ethyl acetate was repeated to produce the ethyl acetate extract. Combined filtrates were dried under vacuum stored at 4°C for analysis and bioassays. For liquid fermentation, Erlenmeyer flasks (2 L), each containing 1 L of ISP2 medium in artificial sea water and incubated at 30°C for 7 days with shaking at 150 rpm. For the elicitation experiments, N-acetylglucosamine (50 µM, Sigma-Aldrich, Germany) was added immediately after inoculation. After fermentation, the broth was filtered, and the supernatant was extracted with ethyl acetate (2 > 5 L) to yield the ethyl acetate extract.

2.2. Molecular identification and phylogenetic analysis

The systematic position of the 16S rDNA sequences were analysed with the SINA web aligner and the Search and classify option (Pruesse et al. 2012). Closest relatives and type strains were obtained from GenBank using nucleotide Blast against nt and ref-seq_rna databases, respectively (Altschul 1990).

2.3. Metabolomics analysis

Ethyl acetate extracts from samples were prepared at 1 mg/mL to subject to mass spectrometry analysis. The recovered ethyl acetate extract was subjected to metabolic analysis using LC-HR-ESI-MS according to Abdelmohsen et al.; 2014 (Abdelmohsen et al. 2014a, 2014b)[23][23]. An Acquity Ultra Performance Liquid Chromatography system connected to a Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer (Waters, Milford, USA) was used. Positive and negative ESI ionization modes were utilized to carry out the high-resolution mass spectrometry coupled with a spray voltage at 4.5 kV, capillary temperature at 320°C, and mass range from m/z 150–1500. The MS dataset was processed and data were extracted using MZmine 2.20 based on the established parameters (Tawfike et al. 2019). Mass ion peaks were detected and accompanied by chromatogram builder and chromatogram deconvolution. The local minimum search algorithm was addressed and isotopes were also distinguished via the isotopic peaks grouper. Missing peaks were displayed by means of the gap-filling peak finder. An adduct search along with complex search were done. The processed data set was next subjected to molecular formula prediction and peak identification. The positive and negative ionization mode data sets from the respective extract.
were dereplicated against the DNP (Dictionary of Natural Products).

2.4. Antibacterial activity

The antibacterial activity was tested against *Staphylococcus aureus* NCTC 8325 (Culture Collections Public Health England, Porton Down, UK). After 24 h incubation at 37 °C, broth cultures were diluted in Müller-Hinton broth (1:100) and cultivated again until the cells reached the exponential growth phase. 10⁵ cells/ml were incubated in the presence of various concentrations of the tested extracts in DMSO to a final volume of 200 μl in a 96-well plate at 37 °C. The final concentration of DMSO was 0.8% in each well. After 18 h of incubation, the optical density of the cultures was determined at 550 nm using an ELISA microplate reader (Dynatech Engineering Ltd., Willenhall, UK) with respect to the control without bacteria. The lowest concentration of the compound that inhibits bacterial growth was defined as the minimal inhibitory concentration (MIC), where chloramphenicol was used as positive control (0.3 μg/ml).

2.5. Antifungal activity

Antifungal activity testing was done by re-suspending a colony of *Candida albicans* 5314 (ATCC 90028) (Culture Collections Public Health England, Porton Down, UK) (Lum et al. 2015), in 2 ml of 0.9% NaCl. Four microliters of this suspension was transferred to 2 ml of HR medium. Various concentrations of the test extracts were diluted in 100 μl of medium in a 96-well microplate with final DMSO concentration of 0.4%. One hundred microliters of the Candida suspension was added to each well then incubated at 37 °C for 48 h. Optical density was measured at 530 nm with respect to a control well without Candida cells, and the MIC was detected. Amphotericin B was used as a positive control (MIC 0.4 μg/ml).

2.6. Anti-Trypanosomal activity

Anti-trypanosomal activity was tested following the protocol of Huber and Koella 104 trypanosomes per ml of *Trypanosoma brucei brucei* strain TC 221 were cultivated in Complete Baltz Medium. Trypanosomes were tested in 96-well plate chambers against dif-

Fig. 1. Heat map generated from LC-HRMS data of 12 crude extracts displaying distinct metabolic profiles amongst the extracts. Samples are grouped according to experimental conditions as described in Table S1.
different genera representing belong to genera Kocuria, Dietzia, Micrococcus, Microbacterium and Streptomyces. The peptone implemented to M1 medium resulted in a higher number of actinomycetes and representing more genera. The genus *Streptomyces* as cultivated preferentially on ISP2. Bacteria of the genus *Streptomyces* are well known for their natural products diversity encompassing different metabolite classes, therefore, *Streptomyces* sp. RM66 was selected for further chemical analysis.

3.2. Metabolomic profiling of *Streptomyces* sp. RM66 crude extracts under different fermentation conditions

Metabolite profiles from crude extracts of *Streptomyces* sp. RM66 using three actinomycetes media (M1, ISP2 and MA) after in solid and liquid fermentation free and amended with N-acetylglucosamine were analysed. LC-HRESIMS analysis of the crude extracts demonstrated a huge diversity of secondary metabolites with a total of 1755 peaks were detected in the twelve crude extracts. Since the metabolites profile in each extract is varying in their physical nature and ionization potential, both the positive and negative ionization modes were used so that detection of the maximum possible metabolites was accomplished.

A heat map of all twelve extracts was used to compare the metabolite production and to visualise the richness in the metabolomes from crude extracts of *Streptomyces* sp. RM66 under different fermentation conditions (Fig. 1). The richest metabolomes (in terms of number of metabolites produced) were observed when the strain *Streptomyces* sp. RM66 was cultivated in solid approach implanted with N-acetylglucosamine (Fig. 1).

Principal component analysis (PCA) analysis was used to investigate the effect of co-cultivation with GlcNAc on metabolite production by *Streptomyces* sp. RM66 (Fig. 2). Separation of crude extracts on the scoring plot indicates that various fermentation conditions are chemically interesting with distinct metabolite profiles. The extract St1 that was observed as outlier from PCA analysis (Fig. 2) is the solid fermentation after addition of GlcNAc. In general, the treatment of *Streptomyces* sp. RM66 GlcNAc observed as more extreme outliers than untreated monocultures. This suggests that their chemistry is more diverse and/or that certain metabolites have higher peak intensities due to their higher expression after GlcNAc addition. The PCA loading plot illustrates the features (m/z ratios), which are responsible for the separation shown in the PCA score plot, indicating the production of additional metabolites or higher peak intensities of metabolites in elicited cultures.

3.3. Metabolic profile of *Streptomyces* sp. RM66

When analyzing *Streptomyces* sp. RM66 metabolic profile, outlying masses were dereplicated and several hits were proposed (Fig. 3). The molecular ion mass peak at m/z 285.2070 [M–H]⁻ gave hits of ethyl plakortide Z (9) and ethyl didehydro-seco-plakortide Z (10) (Harrison and Crews 1998) that were previously isolated from marine sponge *Plakortis* *litata*, or actinopolysporin B that was discovered from a halophilic actinomycete *Actinopolyspora erythreae* YIM 90,600 (Zhao et al. 2011). Peroxidesethyl plakortide Z (9) was reported to be active in vitro against solid tumour and L-1210 leukaemia cell lines (Harrison and Crews 1998). The mass ion peak at m/z 357.2290 [M–H]⁻ fit an antityparanosomal compound manadopederoxide H (12) that was previously isolated from marine sponge *Plakortis cfr.* *Lita* with IC₅₀ value at 0.375 µg/mL against *Trypanosoma brucei rhodesiense* (Chianese et al. 2012). The ion mass peak at m/z 547.2700 [M–H]⁻ gave hits of acanthosterol sulfate F (13) and G (14) which were isolated from marine sponge *Acanthodendrila* sp. but no biological activity was reported from these two compounds (Tsukamoto et al. 1998). Two major ion peaks shown in Fig. 3 were detected in both positive and negative mode

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**Fig. 2.** Principal component analysis of twelve metabolite crude extracts produced in triplicate clustered according to features (m/z ratios) from mass spectral data.
The major ion peak at $m/z$ 446.2226 [M$-\text{H}^-$] was observed in the culture supplemented with GlcNAc (Fig. 3). This parent ion did not match any hits in the MarinLit database, making this molecule a promising target for further investigation. Another major ion peak at $m/z$ 1020.6600 [M$-\text{H}^-$] was observed in a co-culture with *Gordonia* sp. and *Nocardia* sp. and was proposed as one of the surfactin derivatives by searching in the MarinLit database. Surfactins are very well known lipopeptide antibiotics. Several surfactins were reported from members of the genus *Bacillus* (Gong et al. 2009). They have been reported to display a wide range of bioactivities including antimicrobial (Katz and Demain 1977), antiviral (Kracht et al. 1999), antitumor (Kameda et al. 1974), blood anticoagulant (Kim et al. 2006) and immunosuppressive (Zou et al. 2010).

### 3.4. Increasing yield of poorly expressed new metabolites in *Streptomyces* sp. RM66 by GlcNAc

The end points of parent ion masses in the scores plot correspond to the extracts of *Streptomyces* sp. RM66 and suggested they may be attributed to multiple phenazine analogues, for example, phenazine (1), pyocyanine (2), mycomethoxin B (3), phencomycin methyl ester (4), 2-methoxy-1-phenazinecarboxylic acid (5), 1-hydroxy-3-carboxyphenazine (6), phencomycin (7), and tubermycin B (8), indicating an induction of these metabolites by the addition of GlcNAc (Fig. 4, Table S3). Phencomycin was previously isolated from terrestrial microorganisms such as *Streptomyces* sp. HIL Y-9031725 (Chatterjee et al. 1995) and *Burkholderia glumae* strain 411gr-6 (Han et al. 2014), as well as marine *Streptomyces* sp. B 8251 and exhibited antimicrobial properties against *Escherichia coli* and *Bacillus subtilis* (Pusecker et al. 1997). Tubermycin B has been isolated from microorganisms such as *Streptomyces* sp. (Abdelfattah et al. 2011; Sarmin et al. 2013), *Pseudomonas* spp. (Thomashow et al. 1990; Raaijmakers et al. 1997), and a marine sediment derived bacterium *Dermacoccus abyssi* sp. nov. (Abdel-Mageed et al. 2010). It was considered as an environmental compatible antimicrobial agent against various phytopathogens (Zhou et al. 2016), such as *Rhizotonia solani*, *Fusarium graminearum*, *Colletotrichum orbiculare*, *Phytophthora capsici*, *Pythium ultimum*, and *Xanthomonas* sp. (Lee et al. 2003; Xu et al. 2001, 2002).
Furthermore, phenazine compounds are also known to inhibit the growth of *Trypanosoma brucei* (Otoguro et al. 2010) and tumour cell lines (Kennedy et al. 2015), modify cellular redox states (Xu et al. 2015), and regulate patterns of gene expression (Du et al. 2015).

### 3.5. Antibacterial, antifungal and antitrypanosomal activities

All the twelve extracts were tested for their Antibacterial, antifungal and antitrypanosomal activities against *Staphylococcus aureus*, *Candida albicans* 5314, and *Trypanosoma brucei* TC 221, respectively, as shown in Table 1. Extract St1, which was resulted from cultivation of *Streptomyces* sp. RM66 on ISP2, solid media with GlcNAc, was the most active one as antibacterial and antifungal effect. On the other hand it showed the only activity as antitrypanosomal one. Extract St2 and St11 showed moderate antibacterial and antifungal effect but no antitrypanosomal activities. These results may be correlated to the presence of previously identified antitrypanosomal compound manadoperoxide H (12), several surfactins (15) with wide range of bioactivities including antimicrobial, on other hand the induction of phencomycin (7), and tubermycin B (8) in large quantities that previously exhibited antimicrobial properties.

### 4. Conclusions

The rapidly growing number of actinomycete genome sequences highlighted their potential for biosynthesizing a plethora of natural products that is much higher than anticipated during classical laboratory conditions. Chemical elicitation of actinomycetes is an effective strategy to provoke the expression of unexpressed or poorly expressed secondary metabolites and further increasing their chemical diversity. This study highlighted the effect N-acetylglucosamine in induction of producing of many metabolites although they are known or previously isolated, but it was firstly highlighted by this species. On other hand, some peaks showed no hits during dereplication which suggest they might be new metabolites and need further investigations in scale up fermentation. Moreover, the induction of these metabolites qualita-
tively and/or quantitatively may be the difference in biological activities attributed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.04.082.

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