Ecology and genomics of Actinobacteria and their specialised metabolism

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Animal stress hormones enhance siderophore production in *Streptomyces*

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Abstract

Actinobacteria are a rich source of bioactive molecules, and genome sequencing has shown that the vast majority of their biosynthetic potential has yet to be explored. However, many of their biosynthetic gene clusters (BGCs) are poorly expressed in the laboratory, which prevents discovery of their cognate natural products. To exploit their full biosynthetic potential, better understanding of the signals that promote the expression of BGCs is needed. Here, we show that the human stress hormone epinephrine (adrenaline) elicits siderophore production by Actinobacteria. Catechol was established as the likely eliciting moiety, since similar responses were seen for catechol and for the catechol-containing molecules dopamine and catechin, but not for molecules with related chemical structures. The response could be linked to the iron-chelating properties of the catechol moiety. Quantitative proteomics revealed that the expression of proteins involved in iron uptake, siderophore production and dithiolopyrrolone biosynthesis is increased in response to catechol. Thus, we show that plant- and animal-associated molecules increase siderophore production in *Streptomyces*.

Introduction

The phylum Actinobacteria represents a highly diverse group of bacteria with extraordinary metabolic potential. Their specialised metabolites include most of the clinically used antibiotics along with numerous cancer chemotherapeutics, immunosuppressants, and pesticides, and are therefore of great importance for application as clinical drugs or in agriculture [9]. This metabolic versatility makes Actinobacteria attractive sources for drug discovery, for which there is an urgent need due to the global rise of drug resistance [53,54]. However, traditional high-throughput screening suffers from low return on investments due to dereplication, in other words, the rediscovery of bioactive compounds that have been identified before [239,240].

Next-generation sequencing technologies revealed a huge repository of previously unseen biosynthetic gene clusters (BGCs) in Actinobacteria, which showed that their potential as producers of bioactive molecules had been grossly underestimated [20,50,241]. However, these BGCs are often not expressed under laboratory conditions, most likely because the environmental cues that activate their expression in their original habitat are missing [2,28]. Indeed, molecules of actinobacterial origin continue to be discovered that have important new structural and/or functional features, for example the antifungal cyphomycin [43], the glycopeptide antibiotic corbomycin [170], and the angucycline-derived polyketide lugdunomycin [60]. To exploit Actinobacteria more efficiently as resource of chemical diversity, we need to first understand the triggers and cues that promote their expression. This knowledge can then be translated to eliciting approaches to activate the expression of BGCs and produce their cognate bioactive compounds at high throughput [42].

One approach to identify the cues that activate BGC expression lies in understanding the ecological context of specialised metabolite production [42]. Actinobacteria inhabit a wide range of terrestrial and aquatic ecosystems [2]. Within these environments, specialised metabolites play an important role in survival through mediation of resource competition [7,8], protection against oxidative stresses [3] and uptake of essential nutrients [5]. This requires careful timing of production and it is therefore likely that environmental signals indicative of specific stresses (such as nutrient levels and the presence of competitors) have been incorporated in the regulation of BGC expression [32-34]. Indeed, nutrient availability and co-culture of Actinobacteria with other microorganisms significantly influence their specialised metabolite production [35,36].

Actinobacteria live in and around a wide variety of other organisms, including higher eukaryotes such as plants, insects, marine organisms, and mammals [42,43]. As part of the microbiomes of these hosts, Actinobacteria are exposed to host-associated signaling molecules, many of which will likely influence their specialised metabolism [42]. Indeed, plant stress hormones, such as salicylic

acid and jasmonic acid, increase the antibiotic activity of endophytic streptomycetes [44]. These hormones are excreted by plants under pathogenic stress and their release might represent a 'cry for help' through which the plant may activate the production of bioactive substances by members of their microbiome in order to counteract a pathogenic attack [44]. Besides plant hormones, also animal stress hormones influence bacteria. For example, the human opioid dynorphin stimulates production of pyocyanin in *Pseudomonas aeruginosa* [242]. Specifically, catecholamines, which include the well-known 'fight or flight' hormone adrenaline (also known as epinephrine), influence bacterial growth [243,244], biofilm formation [245], and horizontal gene transfer [246]. We hypothesise that animal stress hormones may play a role in the control of antibiotic production of Actinobacteria.

In this work, we demonstrate that the animal stress hormone epinephrine can influence specialised metabolism of streptomycetes, specifically siderophore production. The catechol moiety was key to this response and this finding could be translated to other host-specific compounds (human and plant) containing a catechol moiety. Proteomics revealed increased expression of proteins involved in iron uptake, siderophore production and dithiolopyrrolone biosynthesis. Taken together, these results illustrate that catechol, by itself and as part of plant- and animal-associated molecules, can serve as elicitor of specialised metabolism.

Results

Epinephrine alters antibiotic production of Actinobacteria

Actinobacteria live in close association with higher eukaryotes, such as plants and animals. Host stress molecules might play a role in the regulation of actinobacterial specialised metabolism, perhaps reflecting a 'cry for help' from host to bacterium [44]. We therefore investigated if animal stress hormones can affect growth and metabolism of Actinobacteria. For this, we analysed the effect of epinephrine, also known as adrenaline, on a selection of our in-house actinobacterial strain collection that was previously shown to require particular growth conditions for the production of antibiotics [37]. We used the Gram-positive *Bacillus subtilis* 168 and the Gram-negative *Escherichia coli* ASD19 as indicator strains. Minimal Medium agar (MM) and Nutrient Agar (NA) plates with or without epinephrine (bitartrate salt) or tartaric acid (as control for the added bitartrate) were inoculated with spots from spore stocks of different actinobacterial strains. As a concentration we used 100 μM [244]. To see if the addition of epinephrine to the growth media affected the susceptibility of the indicator strains against antibiotics, we added a diffusion disc with ampicillin (6 μg) to each plate and tested whether the presence of epinephrine in the growth medium affected the size of the inhibition zone of ampicillin against *E. coli* and *B. subtilis*. No differences were observed, confirming that addition of epinephrine to the growth medium did not affect the susceptibility of the indicator strains.

On MM, only a small number of strains showed a change in bioactivity in response to epinephrine (Fig. S1A). Both promotion and inhibition of bioactivity were observed. For some strains, this decrease in bioactivity could be linked to inhibition of growth in presence of epinephrine. On NA, no significant changes in bioactivity were observed except for the elicitation of a large semi-transparent halo surrounding *Streptomyces* sp. MBT42 by epinephrine, indicative of reduced growth of *B. subtilis* (Fig. S1B).

The catechol moiety is key to the eliciting effect of epinephrine on *Streptomyces* sp. MBT42

To analyse the eliciting effect of epinephrine in more detail, we selected *Streptomyces* sp. MBT42, as this strain reproducibly showed a strong response to the hormone when the strain was grown on NA plates (Fig. 1A). In particular, we were interested to see whether epinephrine itself or any of its chemical constituents would be responsible for the eliciting effect. To do so, the effects of different catecholamines and structurally related compounds were assessed (Fig. 1B). Besides epinephrine, we also included catechin, dopamine, levodopa, and norepinephrine. Interestingly, all of these compounds had an effect similar to or stronger than epinephrine and significantly elicited *Streptomyces* sp. MBT42 compared to control (Fig. 1C). Since all of the tested compounds contain a catechol moiety, we wondered if catechol (1,2-dihydroxybenzene) may be the moiety primarily responsible for the observed eliciting effect. We therefore tested the effect of catechol itself, and as controls we used several structural analogues, namely the *meta* isomer resorcinol (1,3-dihydroxybenzene) and the *para* isomer hydroquinone (1,4-dihydroxybenzene), as well as molecules containing a monohydroxy-substituted benzene ring, such as phenol, phenylephrine, and tyramine. Importantly, of these compounds, catechol itself significantly enhanced antibiotic production by *Streptomyces* sp. MBT42 compared to control, while most other compounds failed to elicit antibiotic production (Fig. 1C). Hydroquinone had a mild eliciting effect, similar to that of norepinephrine. However, this effect was significantly lower compared to the effect of the compounds with a catechol moiety such as epinephrine and catechol. These data strongly suggest that the catechol moiety is key to the response of *Streptomyces* sp. MBT42 to the stress hormone epinephrine.

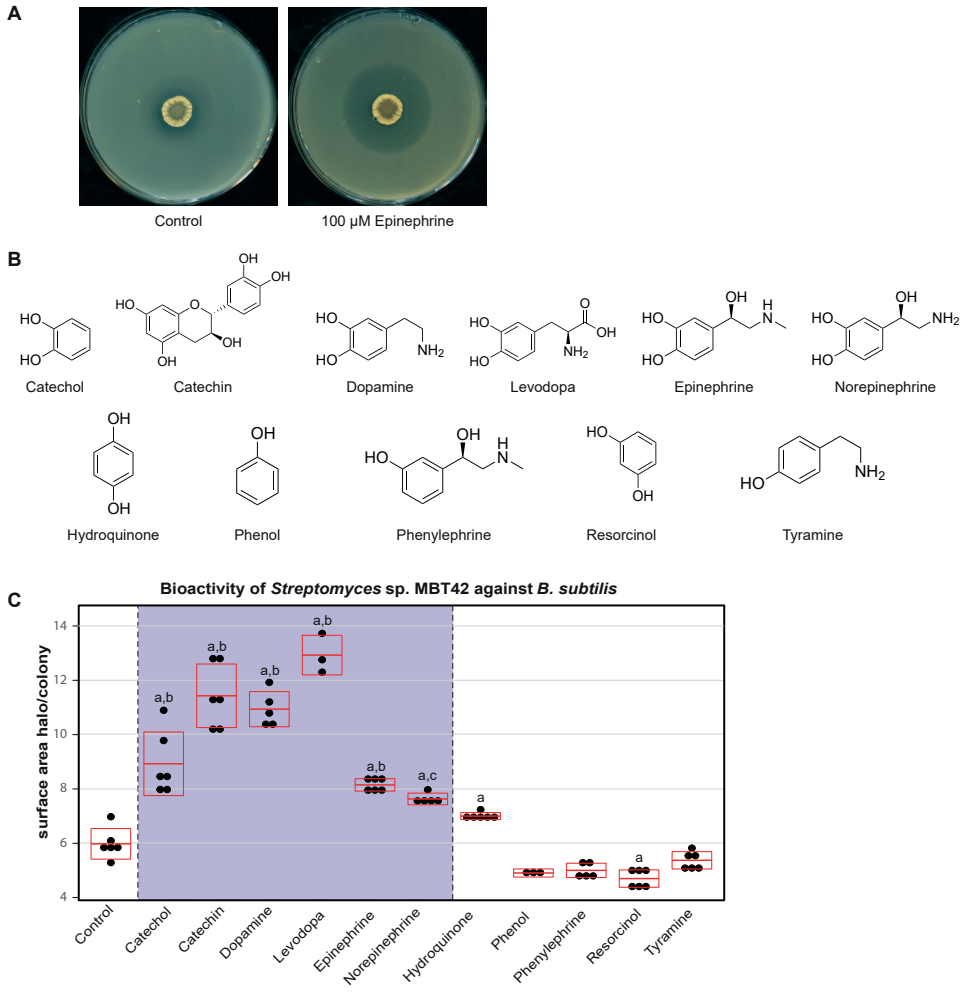

Figure 1. The catechol-moiety is key to the eliciting effect of epinephrine on *Streptomyces* sp. MBT42. A) *Streptomyces* sp. MBT42 was grown on NA supplemented with and without 100 μM epinephrine ($n = 3$). After 4 days of growth, plates were overlaid with *B. subtilis* to test for antimicrobial activity. Note the increased semi-transparent halo surrounding *Streptomyces* sp. MBT42 grown in presence of epinephrine. **B)** An overview of the structurally-related compounds tested to assess the specificity of the response **C)** Effect of different compounds on the bioactivity of *Streptomyces* sp. MBT42 against *B. subtilis*. Bioactivity was quantified by measuring the ratio between the surface area of the inhibition zone and the colony (data represent two independent experiments, the number of biologically independent replicates (= *n*) for each group is indicated in the figure, mean and standard deviation are indicated in red, the grey box highlights the response to compounds with a catechol moiety). One-way ANOVA, followed by a *post hoc* Tukey's honest significant difference (HSD) test, was performed to compare the difference in bioactivity between the growth conditions. The symbols indicate a significant ($p < 0.05$) increase in bioactivity compared to (**a**) the control, (**b**) to compounds lacking a catechol moiety, and (**c**) to compounds lacking a catechol moiety except hydroquinone.

Catechol enhances siderophore production in *Streptomyces* sp. MBT42

The catechol moiety has iron-chelating properties, and catechin is a strong siderophore [247]. We therefore hypothesised that the addition of catechol-containing molecules might result in lower iron availability, which may trigger siderophore production and further iron depletion, thus inhibiting growth of *B. subtilis*. To test this hypothesis, MBT42 spores were spotted onto NA with or without 100 μM catechol, and the plates overlaid with CAS agar solution after four days of growth. An orange halo was formed that is indicative of siderophore production, which was strongly increased in the presence of catechol (Fig. 2). The siderophore-induced halos matched the semi-transparent halos that were observed when *Streptomyces* sp. MBT42 was overlaid with *B. subtilis.*

Siderophore production is suppressed by iron. We therefore tested whether the addition of iron to the growth medium could compensate for the effect of catechol on *Streptomyces* sp. MBT42. Indeed, when 5 μM $FeCl_3$ was added to the medium, siderophore production was strongly reduced and this coincided with disappearance of the semi-transparent inhibition zones (Fig. 2). The same result was obtained when $FeSO_4$ was added (data not shown). Other metal ions did not have this effect (Fig. 2). Additionally, we tested whether the reduced growth of *B. subtilis* was related to iron depletion by adding a disc containing either 10 mM $FeCl_3$ or dH_2O on top of the *B. subtilis* overlay close to the spot of *Streptomyces* sp. MBT42. Within the semi-transparent halo, growth of *B. subtilis* was restored when $FeCl_3$ was present, but not in the control (Fig. S2). This strongly suggests that catechol reduces iron availability, which then enhances siderophore production by *Streptomyces* sp. MBT42 and leads to reduced growth of *B. subtilis.*

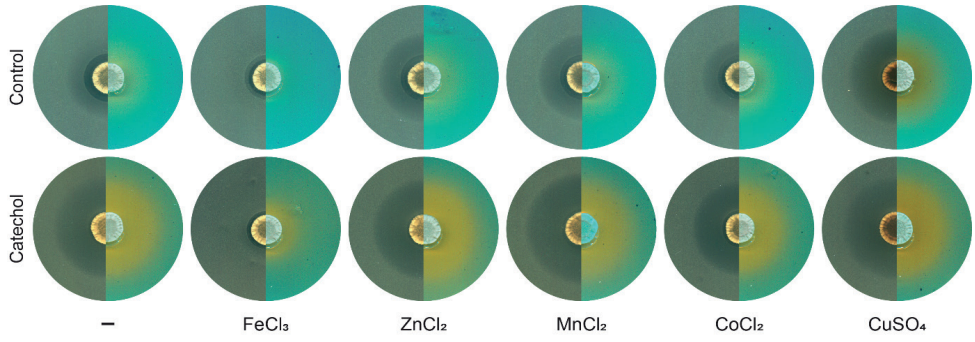

Figure 2. Catechol elicits siderophore production by *Streptomyces* sp. MBT42. MBT42 was grown on NA with and without 100 μM catechol, supplemented with various metal salts (5 μM) (n = 3). After 4 days of growth, plates were overlaid with *B. subtilis* (left) to test for antimicrobial activity or with CAS agar to detect the extracellular production of iron-chelating molecules (orange halos; right). Note that catechol inhibits growth of *B. subtilis* and induces siderophore production, and that these zones are highly comparable. When iron was added to the medium, siderophore production was almost completely inhibited and the semi-transparent halo was no longer visible.

Catechol enhances expression of proteins involved in dithiolopyrrolone and siderophore production

We next wondered which siderophore may be produced by *Streptomyces* sp. MBT42. For this, the full genome of *Streptomyces* sp. MBT42 was sequenced (GenBank accession number: JAJNOJ000000000) (Table S1) and the natural product BGCs predicted using antiSMASH 6.0 [217] (Table S2). This revealed four candidate BGCs for siderophores; one was identified as the desferrioxamine BGC, one with high similarity to the BGC for salinichelin and albachelin, and two predicted clusters that are not known to be associated with siderophore biosynthesis. To find out which siderophore BGC was induced by catechol compounds, MS-based quantitative proteomic analysis was applied, which is an efficient way to establish changes in expression of BGCs in response to eliciting signals [248,249]. The proteome samples of *Streptomyces* sp. MBT42 grown on NA plates with and without 100 μM dopamine, catechin, or phenylephrine were compared. Dopamine and catechin were chosen, because these compounds induced a similarly strong response despite their structural differences, while PE was added as a control, since this compound lacks a catechol moiety.

After five days of growth, biomass was harvested and snap-frozen in liquid nitrogen. Subsequent quantitative proteomic analysis was performed on four replicate samples per growth condition, yielding 1534 quantifiable proteins present in at least one treatment group. Of the 41 putative BGCs annotated by antiSMASH, proteins of 13 BGCs were expressed under these growth conditions (Table S2). To identify proteins involved in the response to catechin and dopamine, $\log_2$ fold changes in protein level were calculated compared to control cultures (no addition to growth medium). Proteins were considered significantly differentially expressed when a significant change was found in both dopamine- and catechin-cultures ($p \leq 0.05$) but not in phenylephrine-cultures ($p > 0.1$). According to these criteria, 13 proteins were significantly differentially expressed, three of which were predicted to be involved in NP biosynthesis; MBT42_4625 belongs to BGC2.5 that bears significant similarity to the salinichelin BGC, and MBT42_9695 and MBT42_9705 belong to BGC2.14A, which shows similarity to the BGCs for the dithiolopyrrolones holomycin and thiolutin (Fig. 3A). Expression of these proteins was significantly increased in dopamine- and catechin-grown cultures. Comparison of the total $\log_2$ fold changes of the proteins within both BGCs showed increased expression of siderophore BGC2.5 in both catechin and dopamine-grown cultures compared to phenylephrine-grown cultures ($p \leq 0.05$) (Fig. 3B+C, BGC border predictions are based on literature [109,250]). For the dithiolopyrrolone BGC2.14A, only part of the cluster showed increased expression in response to catechol-containing compounds.

Besides BGC-associated proteins, also the expression of an EfeM/EfeO family lipoprotein (MBT42_26995) involved in iron uptake was significantly increased in response to catechin and dopamine (Table 1). Taken together, this shows that catechol compounds act as an elicitor of siderophores, which is most likely explained by the sequestering of iron.

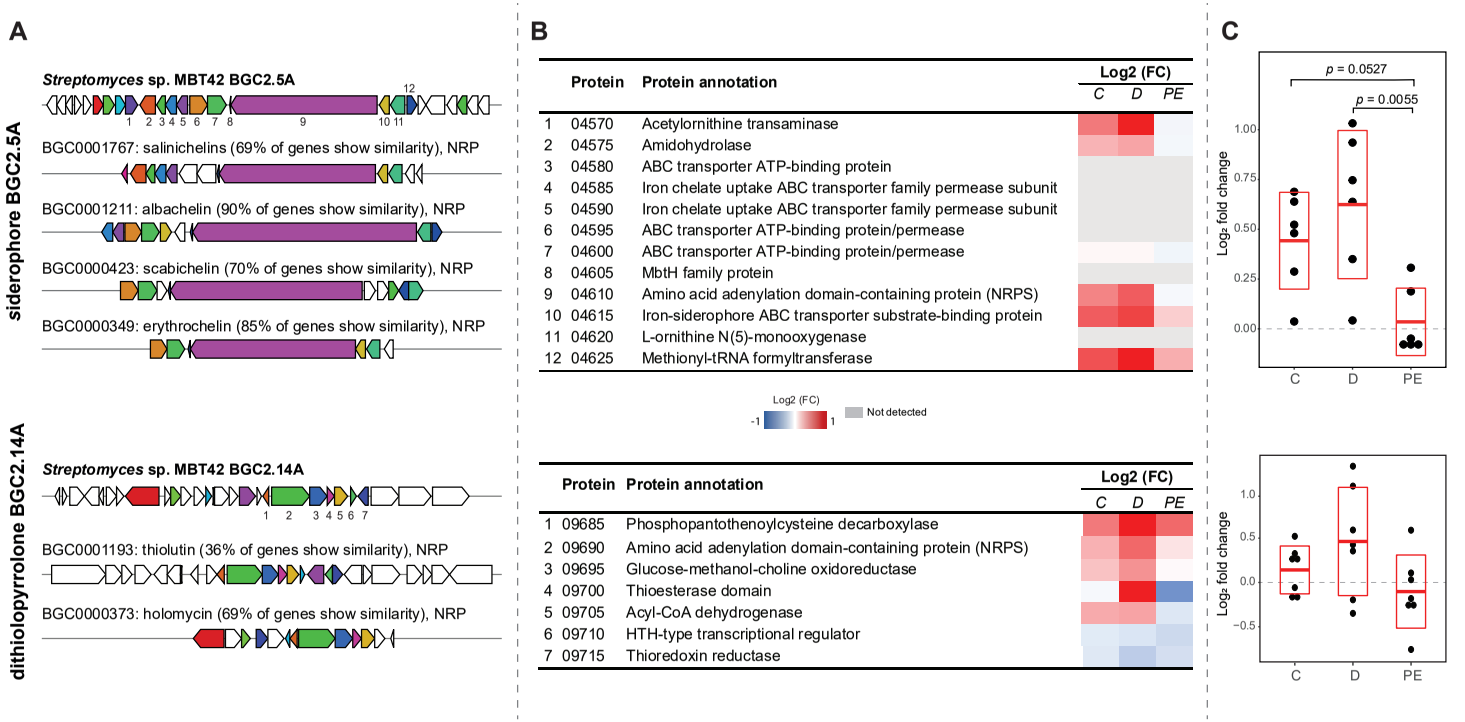

Figure 3. Eliciting effect of catechol compounds on BGCs related to siderophore (top) and dithiolopyrrolones (bottom) production. A) KnownClusterBlast output from antiSMASH which displays the similarity of BGC2.5A and BGC2.14A to clusters from the MIBiG database. Genes marked with the same colour are functionally related; white genes have no relationship. **B)** Changes in protein expression in response to catechin (C), dopamine (D), and phenylephrine (PE). Heatmaps showing $\log_2$ fold changes in protein level compared to control ($n = 4$). Note the similarity between catechin and dopamine. C) Scatter bar plots showing the average $\log_2$ fold change of each protein. The mean $\log_2$ fold change in protein expression of the entire BGC and standard deviation are indicated in red ($n = 4$). The average $\log_2$ fold changes (Log2 (FC)) in protein level were compared between the different groups by one-way ANOVA, followed by a *post hoc* Tukey's HSD test. Top figures represent siderophore BGC2.5A, figures below represent dithiolopyrrolone BGC2.14A.

Table 1. Proteins showing significantly altered expression in response to dopamine and catechin.

Protein ID	Protein annotation	Log2 (FC) *			BGC
		C	*D*	*PE*	
MBT42_04625	methionyl-tRNA formyltransferase	0.69	1.03	0.31	2.5
MBT42_06775	ATP-binding protein	-3.43	-3.43	0.03	-
MBT42_06860	ATP synthase subunit b	-0.53	-0.53	-0.35	-
MBT42_07290	sulfite oxidase	-0.35	-1.00	-0.25	-
MBT42_09695	GMC family oxidoreductase N-terminal	0.23	0.43	0.03	2.14A
MBT42_09705	Acyl-CoA dehydrogenase	0.33	0.36	-0.18	2.14A
MBT42_10280	Vitamin B12-dependent ribonucleotide reductase	-0.32	-0.32	-0.24	-
MBT42_16950	Phosphoserine transaminase	0.31	0.46	0.28	-
MBT42_21175	small secreted protein	-0.29	-0.35	-0.07	-
MBT42_22810	ABC-family ATP-binding cassette	-0.27	-0.36	-0.16	-
MBT42_26995	EfeM/EfeO family lipoprotein	0.25	0.36	-0.01	-
MBT42_28135	Anthranilate synthase component 1	-0.39	-4.27	-0.64	-
MBT42_33795	acyl-CoA/acyl-ACP dehydrogenase	-0.09	-0.12	-0.09	-

* For statistical analysis, Log_2 fold changes compared to control were calculated ($n = 4$). Average protein levels between control and each growth condition (catechin = C, dopamine = D, phenylephrine = PE) were compared using a two-sample t-test. Proteins that showed a significant change in protein level in catechin- and dopamine-cultures ($p < 0.05$) but not in PE-cultures ($p > 0.1$) are shown.

Discussion

Actinobacteria live in close association with a wide variety of eukaryotic hosts, including plants and animals. Plant stress hormones affect the bioactivity of *Streptomyces*, indicating that host-associated molecules can play a role in the regulation of BGC expression [2,44]. Here we show that the animal stress hormones adrenaline, dopamine, and noradrenaline and the plant-associated metabolite catechin increase siderophore production in *Streptomyces*, resulting in reduced growth of *B. subtilis*. We could link this response to the iron-chelating properties of the catechol moiety of these molecules.

Iron is an essential nutrient for bacterial growth and the cause of fierce battle within bacterial communities [5]. In cocultivation experiments, many of the observed changes in bioactivity and metabolome production have been attributed to changes in iron availability and siderophore production [35,83,95,251,252]. Here we present evidence of plant- and animal-associated hormones as important players in the battle for iron. Quantitative proteomics experiments revealed that the expression of proteins associated with iron uptake, siderophore production and dithiolopyrrolone biosynthesis is significantly increased in response to dopamine and catechin. To the best of our knowledge, elicitation of siderophore production by animal- and plant-associated molecules has not been reported in *Streptomyces* before.

Catecholamines increase the growth and virulence of bacteria, mainly by liberating iron from human transferrin, making it more accessible to bacteria [253-255]. They increase the expression of iron uptake systems in *Pseudomonas aeruginosa* and repress the expression of siderophore biosynthetic genes [256]. However, the data presented in our work show that catecholamines can also decrease iron availability, thereby resulting in increased siderophore production, with concomitant inhibition of the growth of *B. subtilis*. The same response was found in the presence of the plant metabolite catechin. Indeed, plant growth-promotion by bacteria has previously been attributed to their ability to produce siderophores thereby depriving other bacteria of iron [257]. This provides a novel perspective for catechol compounds as mediators of host-microbe interactions, and this interesting concept awaits further investigation in an *in vivo* situation.

To coordinate production of specialised metabolites, Actinobacteria have evolved a vast array of complex, multi-level regulatory pathways of which many remain to be elucidated [32]. We propose that the presence of catechol-containing molecules (catechols) results in lower iron availability, which triggers siderophore production. Our data indicate that catechols additionally increase the expression of proteins involved in dithiolopyrrolone biosynthesis, visualizing a possible regulatory connection between siderophore and dithiolopyrrolone biosynthesis. The concurrent increased expression of proteins involved in siderophore and dithiolopyrrolone biosynthesis in response to catechols may also point towards a shared ecological function of these natural products. While a lot of attention is directed towards the discovery of new bioactive molecules for therapeutic use, still relatively little is known about the ecological roles of specialised metabolites within the natural environment of Actinobacteria. Although the ecological purpose of siderophores is well known, little is known about the role of dithiolopyrrolones. Dithiolopyrrolones have antibacterial and anticancer activity, which has been correlated to their ability to inhibit RNA polymerase and to disrupt intracellular metal homeostasis [258,259]. In *E. coli*, mutants defective in iron and zinc were more sensitive to dithiolopyrrolones [258]. Hence, siderophore and dithiolopyrrolones production may be used by *Streptomyces* sp. MBT42 as a strategy to increase the sensitivity of competitors to dithiolopyrrolones. Further studies will be needed to address this hypothesis and to further elucidate the regulatory mechanisms involved.

Methods

Bacterial strains, growth conditions and antimicrobial activity assay

All media and routine *Streptomyces* techniques have been described previously [227]. The actinobacterial strain collection used in this study was obtained from the Leiden MBT strain collection [138]. *Bacillus subtilis* 168 and *Escherichia coli* ASD19 [237] were used as indicator strains for antimicrobial activity and were cultured in LB media at 37 °C.

Antimicrobial activity assays were conducted using the double-layer agar method. Strains were spotted on minimal medium agar plates (MM) supplemented with 0.5% mannitol and 1% glycerol (w/v) as non-repressing carbon sources, and nutrient agar (NA) (Difco) plates, using a pin replicator. For individual testing of strains, 2 μL spore stock was manually spotted. Growth media were supplemented with 25 mM TES buffer and 100 μM of either (+)-catechin hydrate (Sigma-Aldrich, CAS# 225937-10-0) , catechol (Sigma-Aldrich, CAS# 120-80-9), dopamine hydrochloride (Sigma-Aldrich, CAS# 62-31-7), (−)-epinephrine (+)-bitartrate salt (Sigma-Aldrich, CAS# 51-42-3), hydroquinone (Sigma-Aldrich, CAS# 123-31-9), levodopa (Sigma-Aldrich, CAS# 59-92-7), norepinephrine bitartrate monohydrate (MCE, CAS# 108341-18-0), phenol (VWR, CAS# 108-95-2), (R)-(−)-phenylephrine hydrochloride (Sigma-Aldrich, CAS# 61-76-7), tartaric acid (Sigma-Aldrich, CAS# 87-69-4), or tyramine hydrochloride (Sigma-Aldrich, CAS# 60-19-5).

After four days of incubation at 30 °C, plates were overlaid with soft LB agar (1.8% w/v agar) containing one of the indicator strains (2% v/v) pre-grown in liquid LB to exponential phase (OD600 = 0.4 – 0.6) and incubated overnight at 37 °C (± 18 hours). The following day, antibacterial activity was quantified by measuring the ratio between the surface area of the inhibition zone and the spot. All statistical analyses were performed in RStudio v1.4.1717.

Chrome azurol S (CAS) assay for siderophore detection

The medium for 1 L CAS was prepared according to the method of Schwyn and Neilands (1987) [260] without the addition of nutrients: 60.5 mg CAS, 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA-Br), 30.24 g piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and 10 mL of 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl. Agarose (0.9% w/v) was used as gelling agent. The CAS agar solution was overlaid onto the spots of *Streptomyces* sp. MBT42. Following incubation overnight at 30 °C (± 18 hours*)* the plates were examined visually for halos.

Genome sequencing, assembly, and annotation

Streptomyces sp. MBT42 was grown in 25 mL of YEME supplemented with 0.5% glycine and 5 mM $MgCl_2$ and cultivated at 30 °C with 200 rpm shaking speed. Genomic DNA was isolated by phenol-chloroform extraction as described previously 227. PacBio sequencing and assembly of Streptomyces sp. MBT42 was performed by Novogene (Novogene Europe, Cambridge, UK) using PacBio Sequel platform in continuous long reads mode. Raw sequences were demultiplexed with Lima v1.10.0, assembled using Flye v2.8.1 261, and polished using Arrow v2.3.3. The genome has been deposited at GenBank under accession numbers JAJNOJ000000000*.* Biosynthetic gene clusters were identified using the genome mining tool antiSMASH 6.0 [217].

Proteomics sample preparation

Streptomyces sp. MBT42 spores were spotted on NA plates supplemented with and without 100 μM catechin, dopamine, or phenylephrine (n = 4) and incubated for five days at 30 °C. Biomass was scraped off and snap-frozen in liquid nitrogen, lysed in a precooled TissueLyser adaptor (Qiagen, The Netherlands) and proteins extracted using lysis buffer [4% SDS, 100 mM tris-HCl (pH 7.6), 50 mM EDTA]. Total protein was precipitated using the chloroform-methanol method[262] and dissolved in 0.1% RapiGest SF surfactant (Waters, USA) at 95°C. The protein concentration was measured using the BCA method. Protein samples were reduced by adding 5 mM DTT and incubated at 60°C for 30 min, followed by thiol group protection with 21.6 mM iodoacetamide incubation at room temperature in the dark for 30 min. Then 0.1 µg trypsin (recombinant, proteomics grade, Roche) per 10 µg protein was added, and samples were digested at 37°C overnight. After digestion, trifluoroacetic acid was added to 0.5% and samples were incubated at 37°C for 30 min followed by centrifugation to degrade and remove RapiGest SF. Peptide solution containing 6 µg peptide was then cleaned and desalted using STAGE-Tips [263]. Briefly, 6 µg of peptide was loaded on a conditioned StageTip with 2 pieces of 1 mm diameter C18 disk (Empore, product number 2215), washed twice with 0.5% formic acid solution, and eluted with elution solution (80% acetonitrile, 0.5% formic acid). Acetonitrile was then evaporated in a SpeedVac. Final peptide concentration was adjusted to 40 ng·µL^{-1} using sample solution (3% acetonitrile, 0.5% formic acid) for analysis.

Proteomics measurement and data analysis

The desalted peptides solution was separated on an UltiMate 3000 RSLCnano system (Thermo Scientific) set in a trap-elute configuration, coupled to QExactive HF (Thermo Scientific) mass spectrometer. The LC system used a Waters nanoEase M/Z Symmetry C_{18} trap column (5 μm, 100 Å, 180 μm × 20 mm) for peptide loading/retention, and Waters nanoEase M/Z HSS T3 C_{18} analytical column (1.8 μm, 100 Å, 75 μm × 250 mm) for peptide separation. Mobile phase A was 0.1% formic acid (FA) in ULC-MS grade H_2O (Biosolve), while mobile phase B was 0.1% FA, 10% H_2O in ULC-MS grade ACN (Biosolve). The flow rate used was 0.3 μL/min. The gradient was programmed with linear increment from 1% to 5% B from 0 to 2 min, 5% to 13% from 2 to 63 min, 13% to 22% from 63 to 85 min, 22% to 40% from 85 to 104 min, 90% at 105 min and kept at 90% to 113 min.

The MS was operated in positive mode with data dependent acquisition. The survey scan for the tuning was set to scan range of 350–1,400 *m/z* at 60,000 resolution, 1 microscan, automatic gain control (AGC) of 1 × 10^6, max injection time (IT) of 50 ms, no sheath, aux or sweep gas, spray voltage ranging from 1.7 to 3.0 kV, capillary temp of 250 °C and an S-lens value of 80. The MS method settings were: the survey scan was taken with a scan range of 350–1,400 *m/z* at 120,000 resolution, AGC target of 3 × 10^6, and maximum IT time of 100 ms. For individual

peaks, a threshold of 1.00×10^3 for the minimum AGC target yielding an intensity of 2.0×10^4 was set to trigger an MS/MS event. No apex trigger was used, unassigned, 1 and charges >8 were excluded with peptide match mode preferred, and dynamic isotope exclusion was set to 20 s. For MS/MS events, the loop count was set to 10 and the settings were: resolution at 15,000, AGC target 1×10^5, max IT time 50 ms, isolation window of 1.6 *m/z*, fixed first mass of 120 *m/z* and normalised collision energy (NCE) at 28.

Raw LC-MS/MS files were analysed using MaxQuant software (v1.6.17.0) [264] with label free quantification (LFQ) method applied. Proteins that were not detected in three out of four replicates in at least one treatment group were removed prior to analysis, resulting in the removal of 132 protein quantification results. Log2 fold changes were calculated compared to control growth conditions (growth without supplementation). Proteins were considered significantly differentially expressed when a significant change was found in both dopamine- and catechin-cultures ($p \leq 0.05$) but not in PE-cultures ($p > 0.1$). The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE [265] partner repository with the dataset identifier PXD029669.

Supplementary information for Chapter 4

Table S1. Features of the genome of *Streptomyces* sp. MBT42

	***Streptomyces* sp. MBT42**
Number of contigs	2
Largest contig	8,925,615
Total length	8,967,886
N50	8,925,615
CDS	7,748
rRNAs	21
tRNAs	85

Table S2. Biosynthetic gene clusters identified by antiSMASH 6.0 [217]

Proto-cluster	Type	Most similar known cluster	Core biosynthetic protein(s)
2.1	lanthipeptide class IV	venezuelin (100%)	01555
2.2A	NRPS		02925, 02930, 02940
2.2B	NAPAA	stenothricin (13%)	03080, 03085
2.3A	T2PKS	spore pigment (83%)	03725, 03730
2.3B	NRPS		03765 - 03780
2.3C *	T1PKS	lobosamide A-C (17%)	03850 - 03865, 03875 - **03890**
2.4A	thiopeptide/LAP		04320 – 04340, 04360
2.4B *	terpene	2-methylisoborneol (100%)	**04395**
2.5A *	NRPS	salinichelin (69%)	**04610**
2.5B	NRPS	paenibactin (83%)	04685, 04690
2.6	melanin	melanin (28%)	05445
2.7 *	lantipeptide class III	SapB (100%)	**06250**
2.8 *	RiPP-like		**06385**
2.9 *	terpene	hopene (69%)	**06695**, 06710, 06715
2.10	RRE-containing/T2PKS	alnumycin A/B/C/P (75%)	07460, 07560, 07565
2.11 *	T1PKS/NRPS	formicamycins A-M	**07975**, 7980
2.12	butyrolactone	coelimycin P1 (16%)	08580
2.13	RiPP-like		09110
2.14A *	NRPS	thiolutin (36%)	**09690**
2.14B *	NRPS		**09770**
2.15	NRPS	friulimicin A-D (24%)	10020, 10025
2.16*	siderophore	ficellomycin (3%)	**10300**, 10310
2.17	siderophore	murayaquinone (6%)	10590, 10595
2.18	thiopeptide, LAP	BD-12 (14%)	11945-11965, 11990
2.19A	other	-	12130
2.19B	butyrolactone	A-factor(100%)	12175
2.2	melanin	istamycin (8%)	14320
2.21	linaridin	cypemycin (88%)	14495, 14505
2.22	NRPS-like	lankamycin (16%)	17300
2.23	siderophore	desferrioxamin B (100%)	24525
2.24	CDPS	malacidin A/B (5%)	27340
2.25 *	NAPAA	-	**33510**, 33525
2.26A	terpene	-	33630
2.26B	lantipeptide class II	-	33670
2.27 *	terpene	geosmin (100%)	**35570**
2.28	ectoine	ectoine (100%)	35825
2.29	NRPS/T1PKS	Herboxidiene (2%)	36730
2.30	CDPS	-	37735
2.31	terpene	-	38015
2.32 *	T1PKS/NRPS	elloramycin (8%)	**38455**, 38480
2.33	terpene	isorenieratene (100%)	38700, 38715

* BGCs were considered expressed if 1 ≥ core biosynthetic proteins were detected (expressed proteins in **bold**)

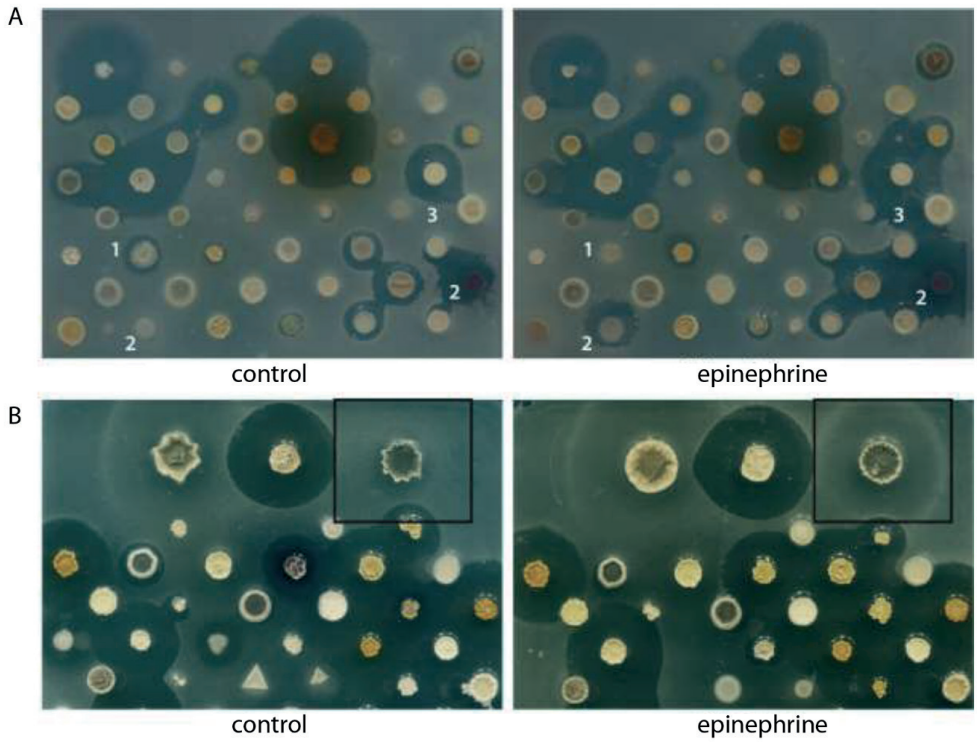

Figure S1. Epinephrine alters the bioactivity of different *Streptomyces*. A) On MM, both inhibition (1) and promotion (2) of antibiotic production in the presence of epinephrine is observed. Additionally, epinephrine can influence interactions between different strains illustrated by the change in halo shape (3) ($n = 3$). B) On NA, epinephrine elicits a semi-transparent halo surrounding *Streptomyces* sp. MBT42 (black square) ($n = 3$).

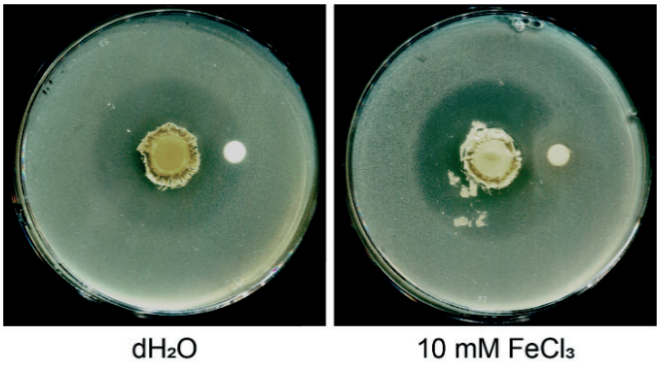

Figure S2. Iron restores growth of *B. subtilis*. *Streptomyces* sp. MBT42 was grown on NA for 4 days, followed by a soft agar overlay containing *B. subtilis*. On top of the overlay, a diffusion disc was added containing either 10 mM $FeCl_3$ or dH_2O followed by overnight incubation. Within the semi-transparent halo, growth of *B. subtilis* was restored when $FeCl_3$ was present, but not in the control.