Investigation on the synthesis of the aminopolycarboxylate metallophores EDHA and [*S*,*S*]-EDDS in actinomycetes

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Unterschrift

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A) Zusammenfassung

Spurenelemente, wie beispielsweise Eisen oder Zink, sind für alle Lebewesen essentiell, wenngleich sie jedoch nur in geringen Mengen benötigt werden. Um die Aufnahme dieser Spurenelemente zu erleichtern, sind Bakterien in der Lage sogenannte Metallophore zu produzieren. Metallophore sind meist niedermolekulare Substanzen, die sekretiert werden, um dann extrazellulär die oft schwer löslichen Metallionen zu binden. Dadurch erhöhen sie als natürliche Chelatoren (Komplexbildner) die Löslichkeit der Metallionen und vermitteln deren verbesserte Aufnahme in den Organismus. Chelatoren werden jedoch nicht nur von Bakterien verwendet, um die Löslich- und Verfügbarkeit von Metallionen zu beeinflussen. So ist beispielsweise der synthetische Komplexbildner Ethylendiamintetraessigsäure (EDTA) einer der am häufigsten kommerziell genutzten Chelatoren. Er findet unter anderem in zahlreichen Waschmitteln, Kosmetikprodukten, Lebensmitteln, Düngern oder Medizinprodukten Anwendung. Der massive industrielle Einsatz von EDTA in den letzten Jahrzehnten, kombiniert mit einer schlechten Abbaubarkeit, führt jedoch dazu, dass dieser synthetische Chelator in Gewässern akkumuliert. Um daraus resultierende, potenzielle Gefahren zu vermeiden, wird seit einigen Jahren versucht, andere Chelatoren mit ähnlich guten Eigenschaften als Komplexbilder, aber mit einer höheren Bioabbaubarkeit, als nachhaltige Alternativen zu etablieren. Einer dieser möglichen Surrogate ist Ethylendiamindibernsteinsäure (EDDS). Bei EDDS handelt es sich um ein Strukturisomer von EDTA, das von verschiedenen Bakterien der Gattung *Amycolatopsis* produziert wird, unter anderem von *Amycolatopsis japonicum*. EDDS hat im Vergleich zu EDTA ähnliche Fähigkeiten als Komplexbildner, das [*S*,*S*]-Isomer von EDDS verfügt jedoch im Gegensatz zu EDTA über eine ausgesprochen gute Bioabbaubarkeit. Daher stellt [*S*,*S*]-EDDS eine sowohl effektive als auch nachhaltige Alternative zu EDTA dar. In *A. japonicum* fungiert das [*S*,*S*]-EDDS vermutlich als Zincophor, um die Zinkaufnahme zu verbessern, da die Biosynthese dieses Komplexbildners durch Zink reguliert wird. Bereits Spuren von Zink, die in jedem Glas- oder Stahlfermenter vorhanden sind, können die Produktion komplett inhibieren. Um eine angestrebte biotechnologische Produktion von [*S*,*S*]-EDDS rentabel zu machen, wurde die Produktion in *A. japonicum* gezielt mittels *metabolic engineering* optimiert. Da die Biosynthesegene in diesem Stamm bereits identifiziert und der Mechanismus der Zinkregulation untersucht worden waren, beruht der Ansatz im ersten Schritt auf dem Austausch des nativen Promotors der

[*S*,*S*]-EDDS-Biosynthesegene durch einen starken, konstitutiven Promotor. Dadurch konnte sowohl die Zinkinhibierung durch den sogenannten Zur-Regulator umgangen, als auch die Produktion gesteigert werden. In weiteren Schritten wurde die Anzahl der Biosynthesegenkopien erhöht und die Produktion der für die [*S*,*S*]-EDDS-Biosynthese benötigten Vorstufe *O*-Phosphoserin verbessert. Durch diese Maßnahmen wurde die Produktion von [*S*,*S*]-EDDS in *A. japonicum* im Labormaßstab bei Verwendung von Minimalmedium von 0,3 g/L auf 3,0 g/L erhöht. Bei ersten Tests des neu entwickelten [*S*,*S*]-EDDS-Produzentenstammes in 10 L Fermentern mit Komplexmedium konnte abschließend ein Titer von 9,8 g/L erreicht werden.

Des Weiteren wurde im Rahmen dieser Arbeit untersucht, in welchem Maße das Potential EDDS-ähnliche Metallophore zu produzieren in Aktinomyceten vorhanden ist und welche Rolle diese dort spielen. Mittels einer *MultiGene-Blast* Analyse, bei der nach EDDS-ähnlichen Biosyntheseproteinen in Aktinomycetengenomen gesucht wurde, konnte ein neues, in Bakterien unterschiedlicher Gattungen verbreitetes Biosynthesegencluster entdeckt werden. Aufgrund der hohen Ähnlichkeit der im Cluster befindlichen Gene zu den bereits bekannten Genen der EDDS-Biosynthese, sowie einem Gen der Viomycin-Biosynthese, konnte abgeleitet werden, dass das Cluster für die Biosynthese des Metallophors Ethylendiaminbernsteinsäure-hydroxyarginine (EDHA) codiert. Obgleich die Substanz EDHA bereits bekannt war, wurde das entsprechende Gencluster bisher noch nicht beschrieben. In zwei näher untersuchten Trägern des EDHA-Biosyntheseclusters, *Streptomyces scabies* und *Streptomyces* sp. MA5143a, konnte mittels bioinformatischer Analysen das Bindemotiv eines IdeR-Regulators innerhalb des Biosyntheseclusters identifiziert werden. Bei IdeR-Regulatoren handelt es sich um Eisen-abhängige, DNA-bindende Proteine, die die Expression von Siderophore-Biosyntheseclustern regulieren. Anhand von Expressionsanalysen mittels reverser Transkriptase- und Polymerasekettenreaktion (RT-PCR) wurde eine Eisen-reprimierte Transkription der EDHA-Biosynthesegene in beiden Stämmen nachgewiesen. Des Weiteren konnte mittels Hochleistungsflüssigkeitschromatographie-Elektrospray-Massenspektrometrie (HPLC-ESI-MS) eine durch Eisen inhibierte Produktion von EDHA belegt werden. Dies legt nahe, dass das EDDS-ähnliche Metallophor EDHA im Gegensatz zu EDDS in den untersuchten Arten *S. scabies* und *Streptomyces* sp. MA5143a wohl als sogenanntes Siderophor fungiert, um die Eisenaufnahme zu verbessern.

B) Abstract

Although they are only needed in small amounts, trace elements, e.g. zinc or iron, are still essential for all living organisms. To facilitate the uptake of those trace elements, bacteria are able to produce so-called metallophores, which are usually low molecular weight compounds. These naturally produced chelators (ion complexing agents) are secreted to extracellularly bind hardly soluble metal ions. When metal ions are complexed by metallophores, their solubility increases and thus their uptake improves. However, not only bacteria rely on chelators to increase the solubility and bioavailability of metal ions. The synthetic chelator ethylenediaminetetraacetic acid (EDTA) e.g. is one of the most abundantly used commercial complexing agents. Its field of application ranges from an ingredient in various washing agents, cosmetics or fertilizers to a use as additive in foods and medicinal products. Due to a combination of both, the abundant industrial usage as well as a poor biodegradability, the synthetic chelator EDTA has accumulated in rivers and lakes in the last decades. Since this poses a putative environmental threat, there is an ongoing effort to replace EDTA with a sustainable alternative with a higher biodegradability, while having similar chelating capacities. One of the compounds that is considered as a potential substitute is ethylenediaminedisuccinc acid (EDDS). EDDS is a structural isomer of EDTA and is naturally produced by various species of actinomycete bacteria of the genus *Amycolatopsis,* e.g. by *Amycolatopsis japonicum*. Although both compounds share similar chelating capacities, the [*S*,*S*]-isomer of EDDS is extremely-well biodegradable compared to EDTA, making it an efficient as well as a sustainable alternative to EDTA. Since the biosynthesis of [*S*,*S*]-EDDS in *A. japonicum* is zinc regulated, the metallophore is presumably used as zincophore in order to facilitate the uptake of zinc. However, even trace amounts of zinc, which occur ubiquitously in glass or steel fermenters, are able to inhibit the production of [*S*,*S*]-EDDS. Aiming at an economical biotechnological production of this compound, the production of [*S*,*S*]-EDDS in *A. japonicum* was optimized by metabolic engineering. Since both the biosynthesis genes as well as the molecular mechanisms of the zinc regulation had already been elucidated in this strain, the first step of the optimization process focused on the exchange of the native promoter of the [*S*,*S*]-EDDS biosynthesis genes with a strong, constitutive one. Hence, the zinc inhibition by the so called Zur-regulator was abolished and the productivity increased. Furthermore, additional copies of the biosynthesis genes were introduced and the

supply of the precursor *O*-phosphoserine was optimized. This optimization process resulted in an increase of [*S*,*S*]-EDDS production in *A. japonicum* from 0.3 g/L to 3.0 g/L when minimal medium was used in small scale cultivation. Moreover, when tested in complex medium in bioreactors, the newly generated [*S*,*S*]-EDDS-producer strain reached a production titer of 9.8 g/L.

Another focus of this work lies on investigating the distribution of the potential to produce [*S*,*S*]-EDDS or EDDS-like compounds in actinomycetes and their functional role in the producer strains. A MultiGene-Blast analysis searching for EDDS-like biosynthesis proteins in actinomycetes genomes led to the discovery of a new biosynthesis gene cluster, which is present is various species from different actinomycete genera. Based on the high similarity of the genes in this cluster to the known genes of the EDDS biosynthesis as well as to another known gene of the viomycin-biosynthesis, the metallophore ethylendiaminesuccinic acid hydroxyarginine (EDHA) could be assigned as the putative gene cluster product. Although the compound EDHA had already been discovered, the corresponding gene cluster has not been described so far. Bioinformatic analysis of two EDHA cluster containing strains, *Streptomyces scabies* and *Streptomyces* sp. MA5143a, revealed a putative DNA binding motif of an IdeR-regulator in this gene cluster. These IdeR regulators are known to be iron dependent, DNA binding proteins, which regulate the expression of siderophore biosynthesis clusters in Gram-positive bacteria. Transcription of the EDHA biosynthesis genes in those two strains was analyzed by reverse transcriptase and polymerase chain reaction (RT-PCR) and was shown to be iron repressed. Additionally, in both strains iron inhibited EDHA production could be detected via high performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS). These experiments resulted in the conclusion, that the EDDS-like metallophore EDHA in contrast to EDDS presumably functions as siderophore to facilitate the iron uptake in the strains *S. scabies* und *Streptomyces* sp. MA5143a.

C) List of Publications

Publication 1

Spohn, M., Edenhart, S., Alanjary, M., Ziemert, N., Wibberg, D., Kalinowski, J., Niedermeyer, T., Stegmann, E., Wohlleben, W. (2018). Identification of a novel aminopolycarboxylic acid siderophore gene cluster encoding the biosynthesis of ethylenediaminesuccinic acid hydroxyarginine (EDHA). *Metallomics*, 10, 722-734. https://doi.org/10.1039/C8MT00009C

Publication 2

Edenhart, S., Denneler, M., Spohn, M., Doskocil, E., Kavšček, M., Amon, T., Kosec, G., Smole, J., Bardl, B., Biermann, M., Roth, M., Wohlleben, W., Stegmann, E. (2020). Metabolic engineering of *Amycolatopsis japonicum* for optimized production of [*S*,*S*]-EDDS, a biodegradable chelator. *Metabolic Engineering*. https://doi.org/10.1016/j.ymben.2020.04.003

D) Contributions

Publication 1

In publication 1, I performed the *in silico* DNA-DNA hybridization of *Streptomyces* sp. MA5143a and was involved in the multi-locus sequence analysis. I wrote the corresponding part of the manuscript and prepared the figure of the phylogenetic tree for the supplementary data of the publication. Furthermore, I was also assisting in the editing of the manuscript.

Publication 2

In publication 2, M. Denneler carried out the construction of the promoter probe plasmids, the strains containing these promoter probe plasmids and the glucuronidase activity assay under my supervision. E. Doskocil worked under my supervision on the generation of an *A. japonicum ΔserC* mutant. M. Spohn performed cloning of the plasmids pSET-*aesA-D* and pMS_*aesA-D* and started to transfer them to *A. japonicum*. I subsequently proceeded this work. I constructed *A. japonicum* OP1 and OP2 and performed the analysis of the HPLC data for the small scale production, the [*S*,*S*]-EDDS titer quantification as well as the respective statistics. The fermentation and its data analysis was carried out by Acies Bio (T. Amon, G. Smole, M. Kavšček, G. Kosec). The fermentation of *A. japonicum Δzur* in fed-batch fermentation was carried out by B. Bardl, M. Biermann and M. Roth who also provided the corresponding data for the supplementary information of the manuscript. I wrote most of the manuscript except for the parts of the section concerning the scale up of the [*S*,*S*]-EDDS production. E. Stegmann and W. Wohlleben supervised the project, participated in designing the experiments and revised the manuscript.

E) Abbreviations

act	actinorhodin
APCA	aminopolycarboxylic acid
bp	base pair
DMSA	dimercaptosuccinic acid
DNA	deoxyribonucleic acid
EDDS	ethylendiaminedisuccinic acid
EDHA	ethylenediaminesuccinic acid hydroxyarginine
EDTA	ethylenediaminetetraacetic acid
Fur	ferric uptake regulator
HPLC	high performance liquid chromatography
IDS	iminodisuccinic acid
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
MLSA	multi-locus sequence analysis
Mur	manganese uptake regulator
Nur	nickel uptake regulator
NIS	NRPS-independent synthetase
NRPS	non-ribosomal peptide-synthetase
NTA	nitrilotriacetic acid
OP	over producer
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
WT	wild type
ZRP	zinc regulated producer
Zur	zinc uptake regulator

1 Introduction

1.1 Crucial trace elements in bacteria

In biology, trace elements like iron, copper, cobalt, zinc, molybdenum and manganese are ions that are only needed in small amounts, but are essential for the health and proper growth of all organisms. In humans for example a daily uptake of less than 100 mg of trace elements are sufficient and their concentration is less than 50 mg/kg bodyweight (Matissek and Baltes, 2016). The inalienability of these metals is based on their vital role in metabolic activities in cells such as catalysis of enzymatic reactions, as protein co-factors or in electron transfer in cell respiration. In bacteria, magnesium is by far the most common metal associated with enzymes, although magnesium-enzyme interactions are usually of a more transitory nature. Besides magnesium, zinc plays the most important role in the group of the redox-inactive metal ions (Andreini et al., 2008). These two redox inert metals and to a lower extend also calcium are used to stabilize negative charges in enzymes or to activate substrates as Lewis acid. Metals with unfilled d-orbitals like iron can additionally be used as redox centres. The most common metal with an unfilled d-orbital used as co-factor in enzymes is indeed iron, followed by manganese, cobalt, molybdenum, copper and nickel (Andreini et al., 2008). In order to satisfy their demand for these essential trace elements, bacteria have developed three main mechanisms for metal acquisition: elemental import systems, piracy from metal containing compounds originating from other organisms, and metallophore-mediated capturing of metal ions.

1.2 Metallophores, naturally produced chelators

Metallophores are low-molecular organic ligands produced by various groups of organisms like bacteria, plants or fungi that have a high affinity to bind metal ions. They are secreted by organisms in order to extracellularly bind the otherwise often inaccessible metal ions and thus facilitate the uptake of these essential trace elements. Although metallophores are mostly able to bind a variety of metal ions *in vitro*, the regulation of their biosynthesis in bacteria is often connected to the presence or absence of one specific metal ion. This connection led to the classification of metallophores according to their metal responsive regulation, e.g. as

siderophore for ferric iron, as zincophore for zinc or as chalkophores for copper. However, metallophores cannot only be classified by their respective metal regulation, but also by their chemical setting and functional groups that coordinate the metal binding. Most metallophores have hydroxamate, α-hydroxycarboxylate or chatecholate functionalities. Some of the strongest siderophores, e.g. enterobactin, which is produced by several *Enterobacteriacae* including *Escherichia coli* and *Salmonella typhimurium* (O'Brien and Gibson, 1970; Pollack and Neilands, 1970), belong to the chatecholate group. Hydroxamate metallophores are also very common, especially in streptomycetes, of whom for example *Streptomyces olivaceus* TÜ 2718 (Meiweis et al., 1990) or *S. coelicolor* produce desferrioxamines in response to iron depletion (Tunca et al., 2007). However, besides the former, metallophores with α-hydroxyimidazole functionalities, α-aminocarboxylates or mixed groups have also been reported. In bacteria, metallophore biosynthesis can occur in different ways. Many of the chatecholate metallophores are synthesized by non-ribosomal peptide-synthetases (NRPS). NRPS-independent synthesis (NIS) pathways or hybrids of NRPS and polyketide synthases (PKS) are also reported for metallophores as in the case of yersiniabactin (Pelludat et al., 1998; Challis, 2005).

1.3 Zinc, an essential trace element in bacteria

Originally, the production of metallophores was considered only as a mechanism for iron acquisition, leading to the discovery of many siderophores in the narrow sense. Besides iron, zinc is the second most important metal bound to enzymes. Zinc binding proteins constitute to about 6 % of the bacterial proteome (Andreini et al., 2009). Therefore, it is not surprising that some bacteria have adapted to zinc-depleted environments by producing zinc chelating metallophores to cover their zinc demand. *Pseudomonas aeruginosa,* for example, synthesizes the zincophore pseudopaline (Lhospice et al., 2017), *Yersinia pestis* yersiniabactin (Bobrov et al., 2014), *Streptomyces coelicolor* coelibactin (Kallifidas et al., 2010) and in *Mycobacterium avium* there is also a zinc-regulated *sidA* operon-encoded metallophore gene cluster (Eckelt et al., 2014). Especially studying pathogenic bacteria revealed how essential zinc is for prokaryotes. In general, the pathogens' hosts are nutrient rich resources. However, the host's immune system often tries to inhibit the metal acquisition of bacteria in order to inhibit bacterial growth. While the

host may try to deprive certain areas of micronutrients like zinc and iron in a process called nutritional immunity, the bacterial pathogens try to parasite and scavenge from the stocks of the host. A severe inflammatory response in mammals can for example lead to increased zinc uptake and sequestration by metallothionein, a cytosolic protein with chelating capacities, into liver cells to decrease the zinc content in the blood serum (Haase and Rink, 2014). Besides metallothionein, there are also other members of the so-called chelating S100 protein family like calprotectin, an abundant neutrophil protein that can chelate Zn^{2+} and Mn^{2+}, which can suppress bacterial growth via chelating essential metals. Calprotectin efficiently competes zinc away from *Staphylococcus aureus*, *Listeria monocytogenes*, *Acetinobacter baumannii*, *Klebsiella pneumoniae*, and *Heliobacter pylori* during infection (Corbin et al., 2008; Zaia et al., 2009; Achouiti et al., 2012; Hood et al., 2012; Gaddy et al., 2014). However, especially for calprotectin several studies showed that it is also subjected to zinc piracy, which means stealing from nutritional immunity proteins. *Neisseria meningitidis* for example binds calprotectin via an outer membrane receptor (CbpA) and then extracts zinc, using a TonB-like receptor (Stork et al., 2013).

In contrast to the zinc depletion following upon calprotectin release, the innate immune system is also capable of using zinc intoxication to reduce the intracellular survival of pathogens. Human macrophages as well as neutrophils are both able to increase free cytosolic zinc levels in order to fend off *M. tuberculosis* and *Staphylococcus pyogenes* upon infection (Botella et al., 2011; Ong et al., 2014).

1.4 Zinc homeostasis regulatory proteins in bacteria

This intense warfare about zinc clearly shows the importance of zinc for both, eukaryotes as well as prokaryotes. Both, zinc deficiency or an excess of zinc in a particular environment can be harmful to a bacterial cell. Since zinc is redox inert, there is no risk of creating dangerous oxidative species, which can cause major cell damage. Hence, its toxicity is presumably caused by mismetallation of metalloenzymes when the intracellular zinc concentration exceeds a certain level. This mismetallation could lead to the loss of enzyme functionality.

Therefore, many bacteria evolved complex regulatory systems to maintain the intracellular metal homeostasis. They can get rid of a surplus of intracellular metal

ions like zinc by exporting or by storing them, or by downregulating uptake mechanisms in the first place. On the transcriptional level, this task is assigned to special metal responsive regulatory proteins. Upon binding their respective metal ions, these regulators undergo a conformational change, which affects their binding to the cognate DNA and thus the repression or activation of the transcription of genes involved in metal homeostasis. The Ferric Uptake Regulator (Fur) for example belongs to this group of metalloregulators. Although the eponym of this group, the *E. coli* Fur (Hantke, 1981; Bagg and Neilands, 1987), is, as its name suggests, iron-responsive, there is nowadays a wide variety of regulators known that belong to this group and respond to metals other than iron. Besides iron, regulators of the Fur family for example can also bind zinc (Zur), manganese (Mur) and nickel (Nur) (Patzer and Hantke, 1998; Diaz-Mireles et al., 2004; Ahn et al., 2006). The Zinc Uptake Regulator (Zur) of the actinomycete *S. coelicolor* as well as of *Escherichia coli* and *Mycobacterium tuberculosis* acts as a homodimer (Lucarelli et al., 2007; Shin et al., 2011; Gilston et al., 2014). Each of the monomers can bind more than one zinc ion, though. Upon zinc binding, the structure of the homodimer shifts towards a closed, high-affinity conformation, which is the basis for the allosteric sensing of the metal. In the zinc-bound form, Zur acts mostly as transcriptional repressor by a palindromic motif in the promoter region, usually in the -35 area, of the corresponding genes, and thus presumably prevents the assembly of the RNA polymerase transcription initiation complex (Ma et al., 2011; Shin et al, 2011). Mainly, Zur acts as repressor for genes of zinc uptake systems like *znuABC*, which is rather common in bacteria, *zupT* (*Francisella tularensis*, *F. novicida*, *Cupriavidus metallidurans*) or *tonB-dr* (*Anabaena* sp. PCC 7120, *C. metallidurans*, *Neisseria meningitides*, *Caulobacter crescentus*, *Xanthomonas campestris*, etc.) (Huang et al., 2008; Huang et al., 2009; Napolitano et al., 2012; Pawlik et al., 2012; Mazzon et al., 2014; Schmidt et al., 2014; Sein-Echaluce et al., 2015; Bütof et al., 2017; Moreau et al, 2018). Additionally, the Zur regulator is known to inhibit the expression of ribosomal genes and enzymes involved in zincophore synthesis, e.g. of pseudopaline in *P. aeruginosa*, yersiniabactin in *Y. pestis* and coelibactin in *S. coelicolor* (Kallifidas et al., 2010; Bobrov et al., 2014; Lhospice et al., 2017). In some cases, Zur also acts as transcriptional activator when a certain threshold of intracellular zinc is reached, e.g. in the case of the *zitB* encoded zinc efflux pump of e.g. *S. coelicolor*, *Xanthomonas*

campestris and *Corynebacterium glutamicum* (Huang et al., 2008; Schröder et al., 2010; Teramoto et al., 2012; Choi et al., 2017).

1.5 [*S*,*S*]-EDDS production in *Amycolatopsis japonicum*

In 1984, the production of the aminopolycarboxylic acid (APCA) ethylendiamine disuccinic acid (EDDS) was discovered in the actinomycete *Amycolatopsis japonicum* (Nishikiori et al., 1984). The compound had attracted attention during a screening for phospholipase C-inhibitors, since the zinc dependant enzyme lost activity upon contact with this substance. Besides, EDDS had already been chemically synthesized in the early 1960s. It forms hexadentate complexes with bivalent metal ions (Chen et al., 2009) and its chelating capacities have been well documented (Majer et al., 1968; Neal and Rose, 1968). Due to its two chiral centres there are three stereoisomers of EDDS: [*S*,*S*]-, [*R*,*S*]- and [*R*,*R*]-EDDS.

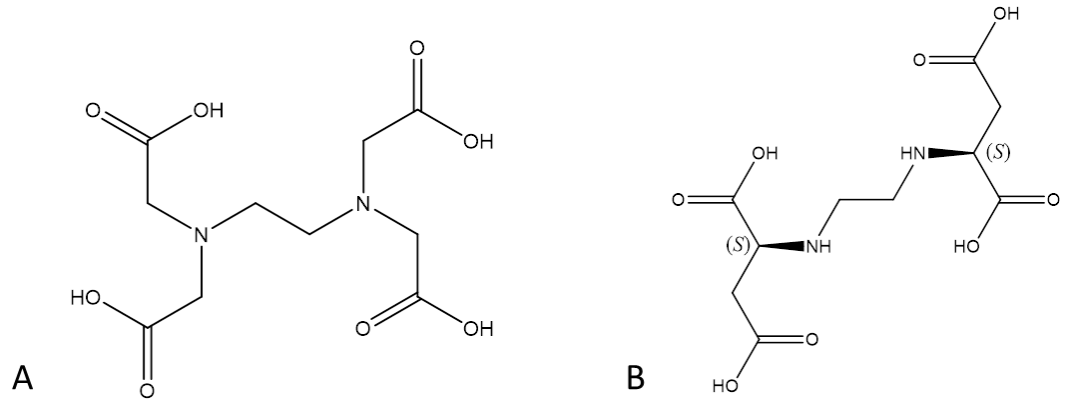

Fig. 1 (publication 2): Structure of the synthetic chelator EDTA (A) and zincophore [*S*,*S*]-EDDS produced by *Amycolatopsis japonicum* (B).

However, the actinomycete *A. japonicum* exclusively synthesizes the [*S*,*S*]-isomer of EDDS (Fig. 1). Interestingly, production of [*S*,*S*]-EDDS by *A. japonicum* only occurs when the strain is grown under zinc depleted conditions (Zwicker et al, 1997; Spohn et al., 2016). This led to the assumption that in *A. japonicum* [*S*,*S*]-EDDS functions as metallophore to facilitate zinc uptake, hence it is a so-called zincophore. For several decades it was not possible to discover the respective biosynthesis genes in *A. japonicum*. Even sequencing the genome of the producer and using bioinformatics

tools with cluster finding algorithms like antiSMASH (Weber et al., 2015) did not lead to the discovery of the [*S*,*S*]-EDDS biosynthesis cluster in *A. japonicum*. In 2016, a new approach to identify the biosynthesis genes by exploiting the knowledge of the zinc-regulation of the [*S*,*S*]-EDDS production was developed (Spohn et al., 2016). Since in several other bacteria the global zinc uptake regulator Zur controls zinc-uptake functions as well as the zincophore biosynthesis gene, Spohn et al. searched for a global zinc uptake regulator in the genome of *A. japonicum* suggesting that this regulator causes the zinc inhibition of the [*S*,*S*]-EDDS production. They discovered the regulator Zur_{Aja}, a homologue of Zur regulators known from e.g. *S. coelicolor*, *C. glutamicum* and *M. tuberculosis*. Following this, they screened the genome of *A. japonicum* for a DNA binding motif of Zur_{Aja} **,** which had been deduced from *S. coelicolor***,** *C. glutamicum* and *M. tuberculosis* binding motives**.** Among others, one Zur_{Aja} binding motif could be determined between the gene *aesE* (AJAP_RS08335) and the operon structure of *aesA-D* (AJAP_RS08340-55). The deletion of the gene region *aesA-D* downstream of the Zur binding motif resulted in the mutant *A. japonicum ΔaesA-C*. This mutant was incapable of producing [*S*,*S*]-EDDS and thus confirmed that the deleted genes are involved in the biosynthesis of [*S*,*S*]-EDDS (Spohn et al, 2016). Complementation of the deletion mutant *A. japonicum ΔaesA-C* with the genes *aesA-D,* led to the duplication of the non-deleted gene *aesD*. Assessing the [*S*,*S*]-EDDS production of this strain with the duplication of *aesD* revealed increased yields of [*S*,*S*]-EDDS compared to the wild type (Spohn et al., 2016). However, this strain still produced [*S*,*S*]-EDDS only under zinc limited conditions (<2 µmol/L). This made a large-scale biotechnological production in steel fermenters, where concentration levels of zinc are ubiquitously above 2 µmol/L, impossible. To solve this problem Spohn et al. (2016) constructed the *zur* deletion mutant *A. japonicum Δzur*. This mutant was indeed able to produce [*S*,*S*]-EDDS in zinc containing media, confirming the transcriptional regulation of the [*S*,*S*]-EDDS biosynthesis genes by Zur and proving the first producer strain for the biotechnological production of [*S*,*S*]-EDDS.

1.6 Industrial application of chelators

Chelating agents are not only used by plants, fungi and bacteria, these compounds are also highly valued for human use. Although their use in everyday life usually

remains unnoticed, chelators are almost omnipresent and thus economically relevant goods. Both, chemical chelators such as dimercaptosuccinic acid (DMSA) or ethylendiamine-tetraacetic acid (EDTA) (Fig.1), and natural ones such as desferrioxamine or the penicillin precursor penicillamine are used in medicinal applications as drugs for the treatment of heavy metal intoxication (Foreman, 1953; Walshe, 1956; Smith, 1962; Miller, 1998; Aaseth et al., 2015). Even more important in clinical routine is citrate, which is used to complex and mask calcium in blood samples or stored blood to inhibit the coagulation cascade and to prevent blood clotting.

In addition, EDTA is applied, e.g. in eye drops and contact lens solution where it suppresses the growth of germs. Beyond that, chelators are also used as food additives for preservation. Examples are the citric acid and its salts, referred to as the food additives E330-E333, the isoascorbic acid (E315) and its salt (E316) as well as tartaric acid (E334) and EDTA (E385). Furthermore, chelators are added to detergents to reduce the water hardness by complexing Ca^{2+} and Mg^{2+} ions. The broad field of such detergents includes common laundry and dish detergents, body or hand soaps as well as shampoos, but also detergents for parts cleaning in industrial processes. Additionally, chelators are used in the process of pulp bleaching in paper production. Here, the most commonly used bleaching agent is hydrogen peroxide which is prone to metal catalysed decomposition. Therefore, in order to increase the efficiency and longevity of the hydrogen peroxide, EDTA is usually applied to remove iron, manganese and copper ions from the pulp before bleaching. Likewise, EDTA is used in textile bleaching and cosmetics to prevent oxidative decolouration catalysed by metal ions. Chelators are needed in most processes or products to remove disruptive metal ions or to protect sensitive surfaces from metal encrustations. However, in some applications, metal ions are not disturbing, but rather desirable and chelators are needed to maintain a constant concentration. Therefore, most liquid fertilizers used in agriculture contain EDTA to stabilise Fe^{3+}, Cu^{2+} and Zn^{2+} ions and to keep them soluble in order to increase the bioavailability for plants, which need them as micronutrients.

1.7 Aminopolycarboxylic acid chelators

Although there is a wide variety of natural as well as synthetic chelators with different chemical properties, the APCA EDTA is the most abundantly used and has dominated the market of chelators for decades. After utilization, the largest part of the EDTA ends up in sewage and, since conventional sewage plants do not retain this compound, it eventually finds its way into the groundwater, rivers and seas. According to data submitted from the producers, the European Union estimated that the yearly release of EDTA into the hydrosphere amounted to 266 tonnes and the release as dust into the atmosphere to 11 tonnes in 2004 (Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, 2004). The degradation of EDTA in the environment processes rather slowly, though. Although in aqueous solutions, Fe(III)EDTA-complexes can be degraded photolytically (Kari and Giger, 1995; Kari and Giger, 1996) the degradation of other EDTA-metal complexes by photolysis is rather inefficient. Besides abiotic degradation, biodegradation of EDTA also occurs. However, similar to photolysis, the efficiency of biodegradation depends strongly on the complexed metal and can differ greatly under special conditions. Thus, half-life of EDTA in soil can range from 25 to 71 days (Meers et al., 2005; Guo et al., 2015). In general, the toxicity of EDTA itself for humans is negligible. However, for aquatic organisms, e.g. fish, the uptake of high concentrations of uncomplexed EDTA can cause nutrient deficiency by reducing the absorptions of essential ions (Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, 2004). However, with over-stoichiometric amounts of calcium and magnesium being present in aqueous solutions, EDTA usually occurs as complex under environmental conditions. Nonetheless, the amount of EDTA in the hydrosphere accumulates due to its numerous applications and its rather poor degradability and this might still pose an environmental risk. Although EDTA occurs in waste as well as surface water in complex with the ubiquitously present metal ions, these complexes can always undergo metal exchange reactions (Nowack et al., 2001). Subsequently, interactions with sediment-bound heavy metals cannot be excluded either. In complex with EDTA, such previously bound, toxic heavy metals like lead or cadmium might get solubilized and are then either directly or after degradation of EDTA bioavailable in the hydrosphere. Since it is hard to assess which impacts high EDTA concentration in the hydrosphere might really have, there are global efforts to reduce the amount of EDTA in use. However, EDTA cannot just be omitted in most of its current forms of

application without reducing the utility. Therefore, producers tend to substitute EDTA for other compounds, which possess similar chelating capacities, but are more efficiently biodegradable. Especially other chelators consisting of aminopolycaboxylates have found to be promising surrogates, e.g. nitrilotriacetic acid (NTA), iminodisuccinic acid (IDS), polyaspartic acid or the above mentioned EDDS. EDDS is an isomer of EDTA and exhibits similar chelating capacities as EDTA. However, of the three existing stereoisomers of EDDS, only the [*S*,*S*] isomer is of particular interest as substitute for EDTA, because its degradation is most efficient with a half-life ranging from 3.8 to 7.5 days (Meers et al., 2005; Tandy et al. 2006). Thus, [*S*,*S*]-EDDS would be a sustainable alternative to EDTA. [*S*,*S*]-EDDS can be synthesized chemically using different synthesis pathways: EDDS can either be synthesized as racemic mixture from ethylendiamine and maleic acid (or fumaric acid) or the [*S*,*S*] isomer can be specifically synthesized from L-aspartic acid and 1,2-dibromoethane. However, a racemic mixture is undesirable and the stereospecific synthesis of [*S*,*S*]-EDDS is more expensive and involves the toxic and carcinogenic dibromoethane. Since the actinomycetes *A. japonicum* and some other bacteria of this genus produce [*S*,*S*]-EDDS naturally and stereospecifically, a biotechnological production of the chelating agent is also conceivable. Yet to be an economically interesting alternative to chemical synthesis, biotechnological production needs to be rather high-yielding.

1.8 Aim of the work

Chelating compounds consisting of aminopolycarboxylates like EDTA are of great economical interest. However, since the detection of gene clusters coding for small aminopolycarboxylate metallophores resembling [*S*,*S*]-EDDS by using genome mining tools like antiSMASH was not possible so far, these compounds and their respective gene cluster might have been overlooked in the past. Therefore, the first aim of this work was to investigate the distribution of the potential to produce [*S*,*S*]-EDDS or EDDS-like compounds in actinomycetes and their role for the producer strains.

The second aim of this work was to further optimize the [*S*,*S*]-EDDS production in *A. japonicum* in order to pave a way for the establishment of an economic, biotechnological production of [*S*,*S*]-EDDS. This work focused on the genetic optimization of *A. japonicum* to improve [*S*,*S*]-EDDS production titers under standard fermentation conditions, even in the presence of zinc.

2 Results

2.1 Identification of the novel aminopolycarboxylic acid siderophore gene cluster of ethylendiaminesuccinic acid hydoxyarginine (EDHA)

2.1.1 Discovery of a new [*S*,*S*]-EDDS-like gene cluster

By investigating putative biosynthesis genes in close proximity to binding sites of the Zur regulator, the genes for [*S*,*S*]-EDDS biosynthesis were identified in *Amycolatopsis japonicum* (Spohn et al., 2016). This approach was established because standard bioinformatic tools such as antiSMASH (Weber et al., 2015), which are used to identify biosynthetic gene clusters in genome sequences, could not detect the EDDS biosynthetic genes in the genome of *A. japonicum*. Therefore, an alternative strategy was required to evaluate the potential for the synthesis of aminopolycarboxylic acids (APCA) with an ethylenediamine-moiety in actinobacteria. Hence, a computational screening approach with the amino acid sequence of the [*S*,*S*]-EDDS biosynthesis enzymes AesA-C of *A. japonicum* as query sequence was implemented. The [*S*,*S*]-EDDS biosynthetic genes form an operon, which also includes *aesD* encoding the EDDS efflux pump AesD. Thus, AesA-D were taken as query of the computational search. Using a combination of MultiGeneBlast (Medema et al., 2013) and BLAST, a GenBank database was screened for the [*S*,*S*]-EDDS genes. This search revealed that many actinobacterial genomes contain a [*S*,*S*]-EDDS like operon. However, in some genomes, the newly discovered operon did not only consist of four genes, but included an additional open reading frame (ORF) upstream of *aesA*. The gene product of this ORF showed similarity to the VioC enzyme (Accession number: AAP92493) of *Streptomyces vinaceus*. VioC is involved in the biosynthesis of viomycin, an anti-tuberculosis agent. VioC is an iron dependant oxygenase that catalyses the synthesis of the aproteinogenic amino acid hydroxyarginine from L-arginine and 2-oxoglutarate (Yin and Zabriskie, 2004). Since the newly detected five genes containing operon resembled a combination of the [*S*,*S*]-EDDS biosynthesis genes and a gene coding for a VioC-like enzyme, the product of the respective enzymes is most likely a compound that contains both, a central ethylenediamine-moiety and a hydroxyarginine. So far, several APCAs containing an ethylenediamine-moiety have been described in the literature, e.g. rhizobactin of *Sinorhizobium meliloti*, staphyloferrin B of *Staphylococcus aureus* or histargin of *Streptomyces roseoviridis* (Ogawa et al., 1984; Smith et al., 1985;

Drechsel et al, 1993; Umezawa et al., 1993). There is only one known compound, namely L-681,176 that combines both characteristics, though (Fig. 2). This compound, produced by the actinomycete *Streptomyces* sp. MA5143a, was first discovered during a screening for inhibitors of angiotensin converting enzymes (Hensens and Liesch, 1984; Huang et al., 1984). Since those enzymes are zinc-dependant metalloproteins, they were inhibited by L-681,176 due to its capability to capture and complex bivalent ions (Bünning and Riordan, 1985). Considering that L-681,176 combined features of [*S*,*S*]-EDDS, such as its chelating properties, a central ethylenediamine-unit and a hydroxyarginine, we hypothesized that L-681,176 is the final product of the newly discovered five genes containing EDDS-like cluster. For simplicity, the compound L-681,176 has therefore been renamed ethylenediaminesuccinic acid hydroxyarginine (EDHA) in accordance with the standing nomenclature for natural and synthetic APCAs.

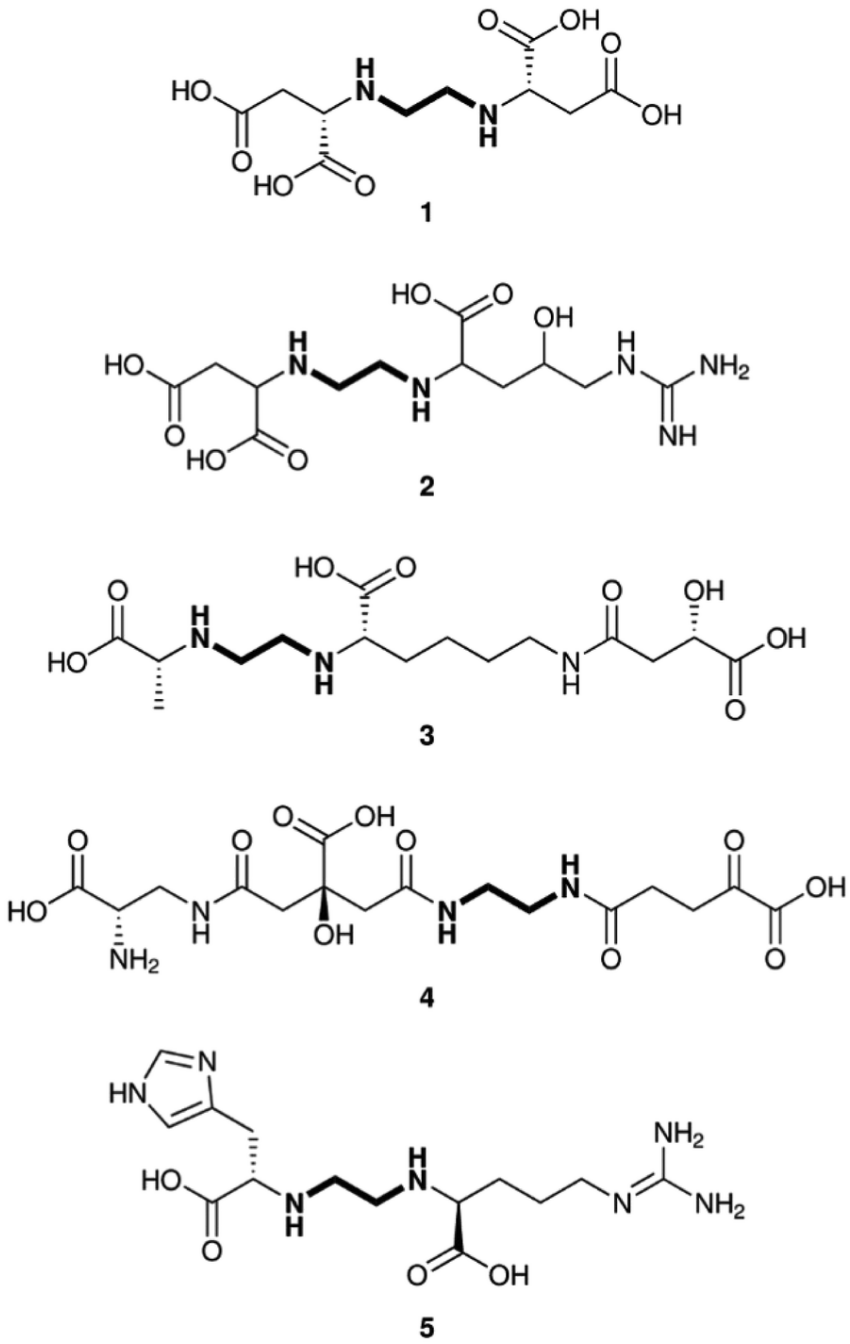

Fig. 2 (publication 1): Chemical structures of natural products containing an ethylenediamine-moiety, (1) [*S*,*S*]-EDDS, (2) L-681,176 or rather EDHA, (3) rhizobactin, (4) staphyloferrin B and (5) histargin. Central ethylenediamine moieties are highlighted in bold.

2.1.2 Genome sequencing and analysis of *Streptomyces* sp. MA5143a

The EDHA operon was found in the genome of several actinobacteria, including mostly pathogenic strains like e.g. *Streptomyces scabies*, *Corynebacterium*

pseudotuberculosis, *Corynebacterium ulcerans* and *Nocardia brasiliensis*, but also in the well-investigated antibiotic producer *Streptomyces avermitilis.* However, none of these strains were referred to in connection with the chelating compound EDHA. The only verified producer of EDHA so far was Streptomyces sp. MA5143a (Hensens and Liesch, 1984; Huang et al., 1984). Yet, no genome sequence was available from this producer. In order to enable a link between the EDHA operon and the EDHA production, the genome of *Streptomyces* sp. MA5143a was sequenced using Illumina HiSeq. This resulted in a total of 7 970 359 reads with about 1.5 giga bases. The GC-content of *Streptomyces* sp. MA5143a is 71.47 %. The draft genome of *Streptomyces* sp. MA5143a comprises 9 838 959 bases and contains 8311 putative protein-coding sequences, 48 tRNAs as well as seven copies of the ribosomal RNA operon. MultiGene-Blast analyses resulted in the identification of the genes MA_5143a_00506 to MA_5143a_00502, whose gene products resembled VioC and the EDDS-enzymes AesA-D. The presence of these *vioC*- and *aesA-D*-like genes in the genome of the EDHA producer confirmed that the EDHA operon is indeed the biosynthesis cluster of this APCA. However, studying the genetic region upstream of the EDHA operon in *Streptomyces* sp. MA5143a led to the discovery of a further operon structure. This operon consisted of four genes, MA_5143a_00507 to MA_5143a_00510, which showed 27-37 % similarity to the genes *appA-D* of *Bacillus subtilis*. This *app* operon encodes a permease specialized for the uptake of tetra- and pentapeptides in *B. subtilis* (Koide and Hoch, 1994). Examination of further genomes that also contain the EDHA operon, revealed *app*-operons to be present upstream of the respective *vioC* genes in either *S. avermitilis*, *S. scabies*, *C. pseudotuberculosis, C. ulcerans* and *N. brasiliensis* (Fig. 3).

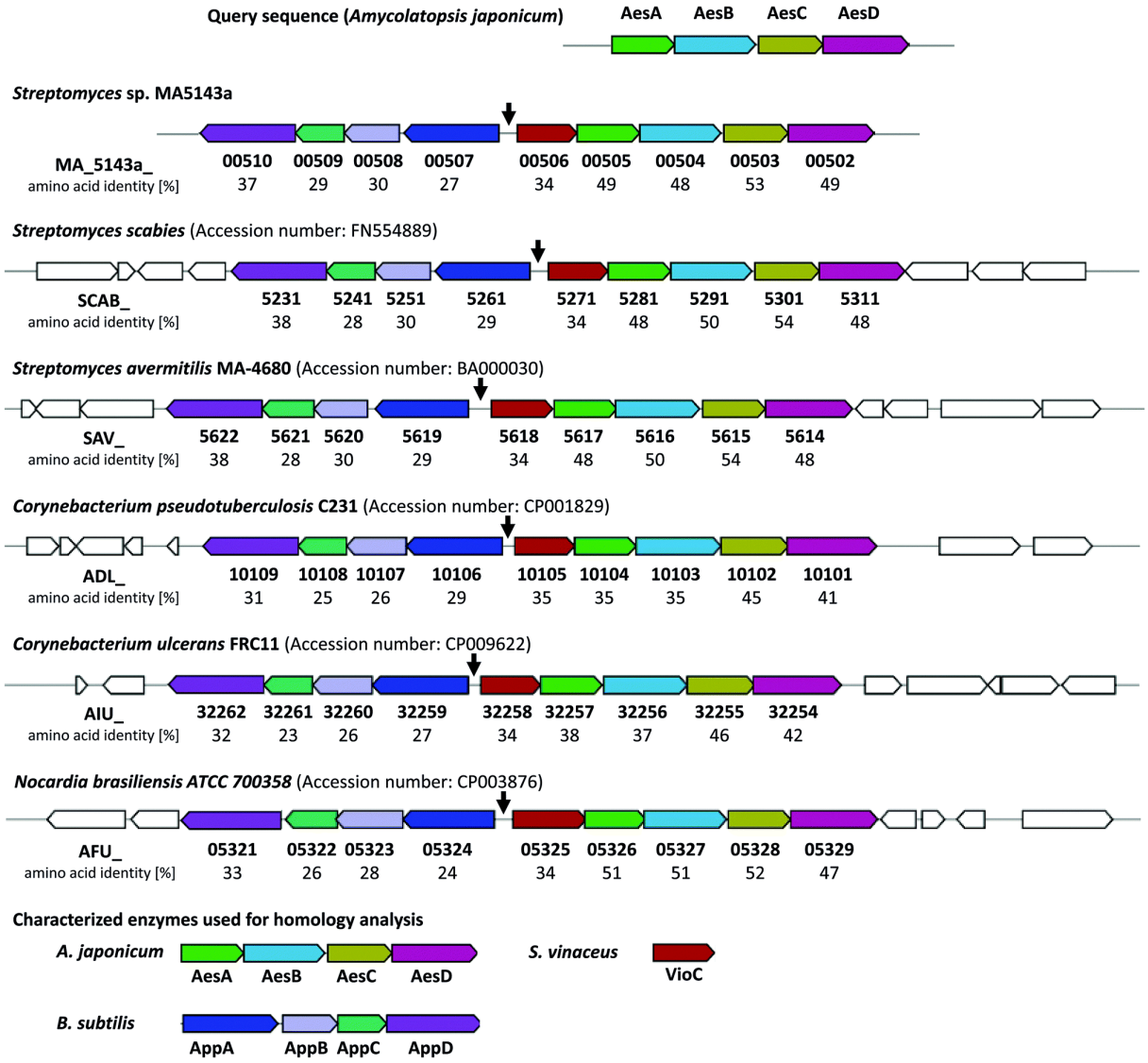

Fig. 3 (publication 1): Gene maps of putative EDHA gene clusters.

Depiction of the genetic loci identified by the *in silico* screening approach. Amino acid sequences of AesA-D of *A. japonicum* were used as query. Similar genes are depicted in the same colour. The locus tags are shown beneath the corresponding gene. The vertical arrows indicate the location of an IdeR binding motif. Percentages of amino acid identity to AppD (P42064), AppC (P42063), AppB (P42062), and AppA (P42061) of *B. subtilis*, VioC (AAP92493) of *S. vinaceus* and AesA (AIG74588), AesB (AIG74589), AesC (AIG74590) and AesD (AIG74591) of *A. japonicum* are shown beneath the corresponding gene encoding the similar gene product.

Taking the direct proximity and genetic co-localization of the EDHA operon to the *app* operon in several strains into account, it seemed like their respective gene products shared a functional connection. Probably the gene products of MA_5143a_00506 to MA_5143a_00504 also serve as oligopeptide uptake system in *Streptomyces* sp. MA5143a and presumably facilitate the re-uptake of EDHA into the cell after successfully complexing ions in the cell environment.

2.1.3 Phylogeny of *Streptomyces* sp. MA5143a

With the complete genome at hand, a Multi Locus Sequence Analysis (MLSA) with 50 housekeeping genes was performed in order to classify *Streptomyces* sp. MA5143a within the genus *Streptomyces*. For this, all strains whose genome sequence was published and whose 16 S rRNA gene sequences showed a 99 % similarity with *Streptomyces* sp. MA5143a were taken into account. In the resulting phylogenetic tree, *Streptomyces* sp. MA5143a formed a monophyletic clade with *Streptomyces torulosus* NRRL B-3889, *Streptomyces neyagawaensis* NRRL B-3092 and *Streptomyces ossamyceticus* NRRL B-3822. Additionally, to the MLSA, DNA-DNA hybridization values between those strains and *Streptomyces* sp. MA5143a were calculated *in silico.* For *S. torulosus* NRRL B-3889 the DNA-DNA hybridization value was about 44.30 %, for *S. neyagawaensis* NRRL B-3092 about 45.20 % and for *S. ossamyceticus* NRRL B-3822 about 46.20 %. Since these values are all below the minimum threshold of 70 % for relatedness between different species, *Streptomyces* sp. MA5143a is presumably a new, yet undescribed species within *Streptomyces*.

Analysing the closest relatives of *Streptomyces* sp. MA5143a revealed that neither the genome of *S. torulosus* NRRL B-3889 nor of *S. neyagawaensis* NRRL B-3092 nor of *S. ossamyceticus* NRRL B-3822 contained an EDHA operon. However, those four strains belonged to a greater clade which also comprises the plant pathogens *Streptomyces scabies* and *Streptomyces europaeiscabiei*, whose genomes both contained the EDHA operon. According to our analysis, most actinobacterial species with the EDHA operon were only distantly related to *Streptomyces* sp. MA5143a.

2.1.4 EDHA production in *Streptomyces* sp. MA5143a and *Streptomyces scabies*

The *in silico* analyses revealed that besides *Streptomyces* sp. MA5143a also other actinobacterial genomes contained an EDHA biosynthetic cluster. In order to proof that these actinobacteria are indeed capable of producing EDHA*, S. scabies* was exemplarily chosen for production tests. For these tests, *S. scabies* was grown in a modified version of the EDHA production medium of Huang et al. (1984) in specifically treated glass flasks (Zwicker et al., 1997) in order to reduce contamination with trace elements. As a control, *Streptomyces* sp. MA5143a was cultured on identical conditions. After five days of cultivation, the supernatants of each culture were analysed by LC-ESI-MS. The molecular weight of EDHA is 349.343 Da. Therefore a mass of $[M + H]^+$ = *m/z* 350 in positive mode was expected. Indeed, a compound with the mass of $[M + H]^+$ = *m/z* 350 could be detected in the supernatant of *S. scabies* as well as in *Streptomyces* sp. MA5143a. To ensure that this compound is indeed EDHA, Cu(II)SO_4 was added to the supernatants and LC-ESI-MS analysis was performed again. Since EDHA complexes bivalent ions like Cu^{2+}, this time an EDHA-^{63}Cu(II)-complex with a mass of $[M + H]^+$ = *m/z* 411 and an EDHA-^{65}Cu(II)-complex with a mass of $[M + H]^+$ = *m/z* 413 were expected in the measurements. In fact, after the copper treatment, in addition to the mass of $[M + H]^+$ = *m/z* 350, the masses of $[M + H]^+$ = *m/z* 411 and $[M + H]^+$ = *m/z* 413 were detectable (Fig. 3 and 4, publication 1). Furthermore, the relative heights of the $[M + H]^+$ = *m/z* 411 and $[M + H]^+$ = *m/z* 413 peaks resemble the natural isotope abundance of ^{63}Cu(II) and ^{65}Cu(II), confirming the hypothesis that *S. scabies* is also capable of synthesizing EDHA.

2.1.5 *In silico* prediction of the EDHA cluster regulation

In bacteria, chelating compounds are commonly produced in response to a lack of certain trace elements. For instance, [*S*,*S*]-EDDS is synthesized in various *Amycolatopsis* species when they have to cope with a zinc depleted environment. On the contrary, [*S*,*S*]-EDDS is not produced if a certain threshold of zinc is exceeded (Spohn et al., 2016). In this case, the so called Zinc Uptake Regulator (Zur) gets activated by intracellular zinc ions. In a zinc-bound state, the Zur regulator binds to the upstream region of the [*S*,*S*]-EDDS biosynthesis genes, where a short

palindromic, putative binding motif is located, and thus inhibits gene transcription. Considering the structural similarity to [*S*,*S*]-EDDS, it can be hypothesised that EDHA is presumably also produced under zinc depleted conditions to serve as zincophore. Hence, EDHA production might also be regulated by Zur. Screening the genome of *Streptomyces* sp. MA5143a led to the discovery of a metalloregulator, which was highly similar (88 % amino acid sequence identity) to the Zur regulator of *S. coelicolor*. In order to determine Zur regulated ORFs in *Streptomyces* sp. MA5143a, its genome was scanned with PatScanUI (Dsouza et al., 1997) for possible Zur binding sites, using the DNA binding motif of Zur_{Sco} as query. Possible Zur binding sites were predicted upstream of genes coding for an ABC-transporter with a high similarity to ZnuABC of *S. coelicolor* and upstream of genes coding for ribosomal proteins. This matched the results obtained for the Zur regulon of *S. coelicolor* where some ribosomal genes are also Zur regulated. However, no Zur binding motif was identified close to the EDHA operon of *Streptomyces* sp. MA5143a, indicating that the transcription of the operon is not zinc-dependent. Considering that iron is the trace element, which is needed most in many organisms, it seems likely that *Streptomyces* sp. MA5143a synthesizes EDHA in response to iron deficiency and that the biosynthesis genes are therefore more likely to be regulated by an iron responsive regulator. In Gram-positive bacteria like *Mycobacterium* spp., *Corynebacterium* spp. and *Streptomyces* spp., proteins of the iron dependent regulatory protein (IdeR) regulator family repress the transcription of genes involved in iron metabolism (Seeboth and Schupp, 1995; Pohl et al., 1999a; Pohl et al., 1999b; Tunca et al., 2007). Similar to Fur regulators, IdeR changes its conformation upon binding ferrous iron when a surplus of free ferrous iron is present in the cytoplasma. IdeR is then able to bind specifically to the corresponding A/T-rich motifs in the promoter of its DNA targets and thus to repress the downstream genes (Lee and Helmann, 2007). When checking the genome of *Streptomyces* sp. MA5143a for a putative IdeR regulator, the ORF MA_5143a_05034 was detected. This ORF encodes for a putative regulator with 97 % amino acid similarity to DmdR1 (Flores and Martin, 2004), which is the IdeR family protein of *S. coelicolor*. To identify the putative regulon of this IdeR protein in *Streptomyces* sp. MA5143a, a PatScanUI analysis was conducted by using the published DNA-binding motif of IdeR of *Mycobacterium tuberculosis* (Gold et al., 2001) as query. This resulted in the identification of 15 ORFs, which are presumably regulated by IdeR. According to

annotation, all of the discovered ORFs encoded either for proteins directly involved in iron uptake or for metalloproteins, e.g. a putative aconitase, which needs iron as cofactor and is known to be Fur regulated in *Escherichia coli* (Cunningham et al., 1997).

Three of the putative IdeR DNA-binding sites were located next to two independent NIS gene clusters (upstream of MA_5143a_03356, MA_5143a_04108 and MA_5143a_04110). Based on the high sequence similarity of one of those clusters to the desferrioxamine siderophore cluster of *S. coelicolor*, *Streptomyces* sp. MA5143a is presumably capable of producing at least one, but more likely two additional chelating compounds besides EDHA. However, not only those two putative siderophore biosynthesis clusters contained IdeR binding motifs, but also the EDHA operon. In the non-coding 5' upstream region of the *vioC*-like gene (MA_5143a_00506), the first gene of the EDHA operon, a highly conserved IdeR-binding motif was detected. This clearly points to an iron-responsive regulation of the EDHA operon by IdeR. Furthermore, an iron-responsive regulation of the EDHA biosynthesis suggests the function of EDHA as a siderophore to facilitate iron uptake in *Streptomyces* sp. MA5143a. For comparison, the genomes of more species containing EDHA clusters were screened for IdeR binding motifs. Thus, the presence of the IdeR binding motif was also discovered in the upstream regions of the *vioC*-like genes in *S. scabies*, *S. avermitilis*, *C. pseudotuberculosis*, *C. ulcerans* and *N. brasiliensis*. This indicates that the biosynthesis of EDHA is iron responsively regulated by the IdeR regulator in all those strains.

2.1.6 *In vivo* verification of the metalloregulation

To verify that the transcription of the EDHA cluster is indeed iron responsively regulated, *Streptomyces* sp. MA5143a was grown under EDHA production conditions as before, but the medium was supplemented with 25 µM of either $Fe(II)SO_4$, $Zn(II)SO_4$, $Ni(II)SO_4$, $Mn(II)SO_4$, $Co(II)SO_4$ or, as control, without additional ions. Furthermore, one culture was supplemented with Fe(III)citrate (60 mg/L) in order to keep the ferrous iron soluble and to avoid oxidation and formation of an insoluble ferric iron complex. Thereafter, the production of EDHA was measured on days 2, 3, 4, 5 and 7 of cultivation. EDHA production comparable to the control without ion

supplementation was detected in the presence of all metal ions except for iron. In presence of Fe(III)citrate almost no production could be detected, while in presence of Fe(II)SO_4 the production of EDHA was significantly lower compared to the control (Fig. 5, publication 1). Similar results were obtained when the experiments were repeated with *S. scabies* (Fig. 3, publication 1).

Additionally, to the measurements of EDHA titers, the transcription profile of the EDHA biosynthesis genes in *Streptomyces* sp. MA5143a was analysed. For this, cells grown in medium supplemented with 25 µM of either Zn(II)SO_4, Ni(II)SO_4, Mn(II)SO_4, Co(II)SO_4 or 60 mg Fe(III)citrate were harvested after 72 h of cultivation and qualitative reverse transcription PCR (RT-PCR) was performed. All five genes of the EDHA operon (MA_5143a_00506 to MA_5143a_00502) were expressed in the presence of zinc, nickel, cobalt and manganese, but no transcript could be observed in cultures containing Fe(III)citrate (Fig. 6, publication 1). These results are in accordance with the EDHA production pattern and prove once more that these strictly iron repressed genes encode for the EDHA cluster. Furthermore, it shows that although EDHA is structurally closely related to the zincophore [*S*,*S*]-EDDS of *A. japonicum*, in *Streptomyces* sp. MA5143a as well as in *S. scabies* and probably also in *S. avermitilis*, *C. pseudotuberculosis*, *C. ulcerans* and *N. brasiliensis*, EDHA instead serves as siderophore.

2.2 Metabolic engineering of *Amycolatopsis japonicum* for optimized production of [*S*,*S*]-EDDS, a biodegradable chelator

APCAs, as e.g. EDTA and its substitute NTA, are used extensively in various industrial branches and thus have a huge annual consumption. However, EDTA, with an annual production/import of up to 10 000 tons (European Chemicals Agency, 2020a) probably the most abundantly used complexing agent in the EU, is believed to be a potential environmental threat due to its inefficient biodegradability. In addition, its substitute NTA, with an annual production/import of up to 1000 tons in the EU (European Chemicals Agency, 2020b), has a higher biodegradability, yet is also possibly carcinogenic to humans (International Agency for Research on Cancer, 1999). As a result, the EDTA isomer [*S*,*S*]-EDDS has become an increasingly popular and environmental friendly alternative in recent years, so far without known health risks. Up to date commercially available [*S*,*S*]-EDDS is only chemically synthesized. However, the chemical synthesis of [*S*,*S*]-EDDS is expensive. Thus, the market price for [*S*,*S*]-EDDS exceeds the price for EDTA by far. In order to make [*S*,*S*]-EDDS more competitive, increased efforts are undertaken to establish a low cost production. Since [*S*,*S*]-EDDS is a natural product of various actinomycetes like *A. japonicum*, a biotechnological production is also possible and highly desirable. Yet, in order to enable an economical biotechnological production, the natural production titer in *A. japonicum* has to be increased and the repression of the biosynthesis in presence of zinc has to be abolished. Since *A. japonicum* can easily be genetically manipulated and has already turned out to be a well usable strain for metabolic engineering and natural product synthesis in the past (Schwarz et al., 2018), it was decided to optimize [*S*,*S*]-EDDS production in this strain.

2.2.1 Increase of [*S*,*S*]-EDDS production by introducing additional copies of the biosynthetic genes *aesA-D* into *A. japonicum*

Firstly, in order to increase the [*S*,*S*]-EDDS production, an additional copy of the [*S*,*S*]-EDDS biosynthesis genes *aesA-D* under control of the native promoter was integrated into the genome of *A. japonicum* wild type. For this, the genes *aesA-D* were cloned into pSET152 (Bierman et al., 1992) (pSET-*aesA-D*) and the newly constructed plasmid was transferred to *A. japonicum* where it integrated into the

genome via the *ΦC31* attachment site. The resulting recombinant strain *A. japonicum* ZRP1 (zinc regulated producer) was then cultivated in a zinc depleted [*S*,*S*]-EDDS production medium and [*S*,*S*]-EDDS production was determined by HPLC. The analyses revealed that *A. japonicum* ZRP1 produced significantly more [*S*,*S*]-EDDS (0.7 g/L) than *A. japonicum* WT (0.3 g/L) (Fig. 4).

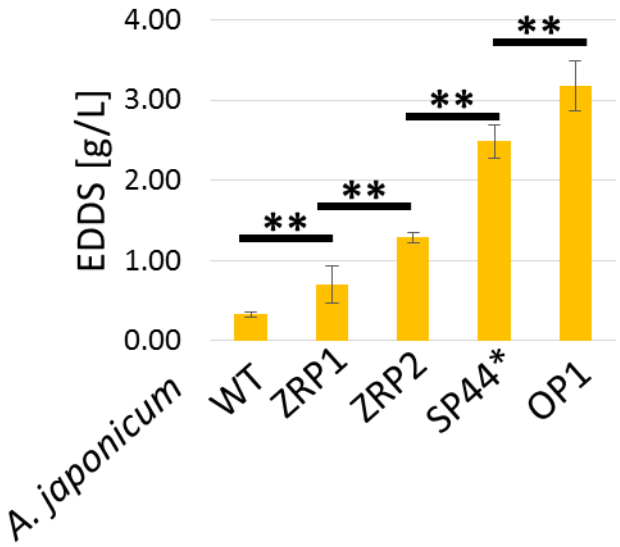

Fig. 4 (publication 2): [*S,S*]-EDDS production titer of different *A. japonicum* strains grown in zinc depleted medium after five days of cultivation.
The [*S,S*]-EDDS production was stepwise increased by adding one copy (*A. japonicum* ZRP1) or two copies (*A. japonicum* ZRP2) of the *aesA-D* genes into the genome of *A. japonicum* WT, exchanging the native promoter (*A. japonicum* SP44*) or combining both (*A. japonicum* OP1). Statistically significant differences are marked with * (**: $p \leq 0.01$, ns: not significant, Man-Whitney U-test, n = 6).

To further increase the [*S*,*S*]-EDDS production, a second plasmid (pMS_*aesA-D*), which contains another copy of the biosynthesis genes, was integrated into *A. japonicum* ZRP1 via the *ΦBT1* attachment site. The resulting modified strain *A. japonicum* ZRP2 produced four times more [*S*,*S*]-EDDS (1.3 g/L) than *A. japonicum* WT when cultivated under zinc depleted conditions (Fig. 4). Thus, by increasing the [*S*,*S*]-EDDS operon copy number a well-producing strain could be developed (Fig. 5).

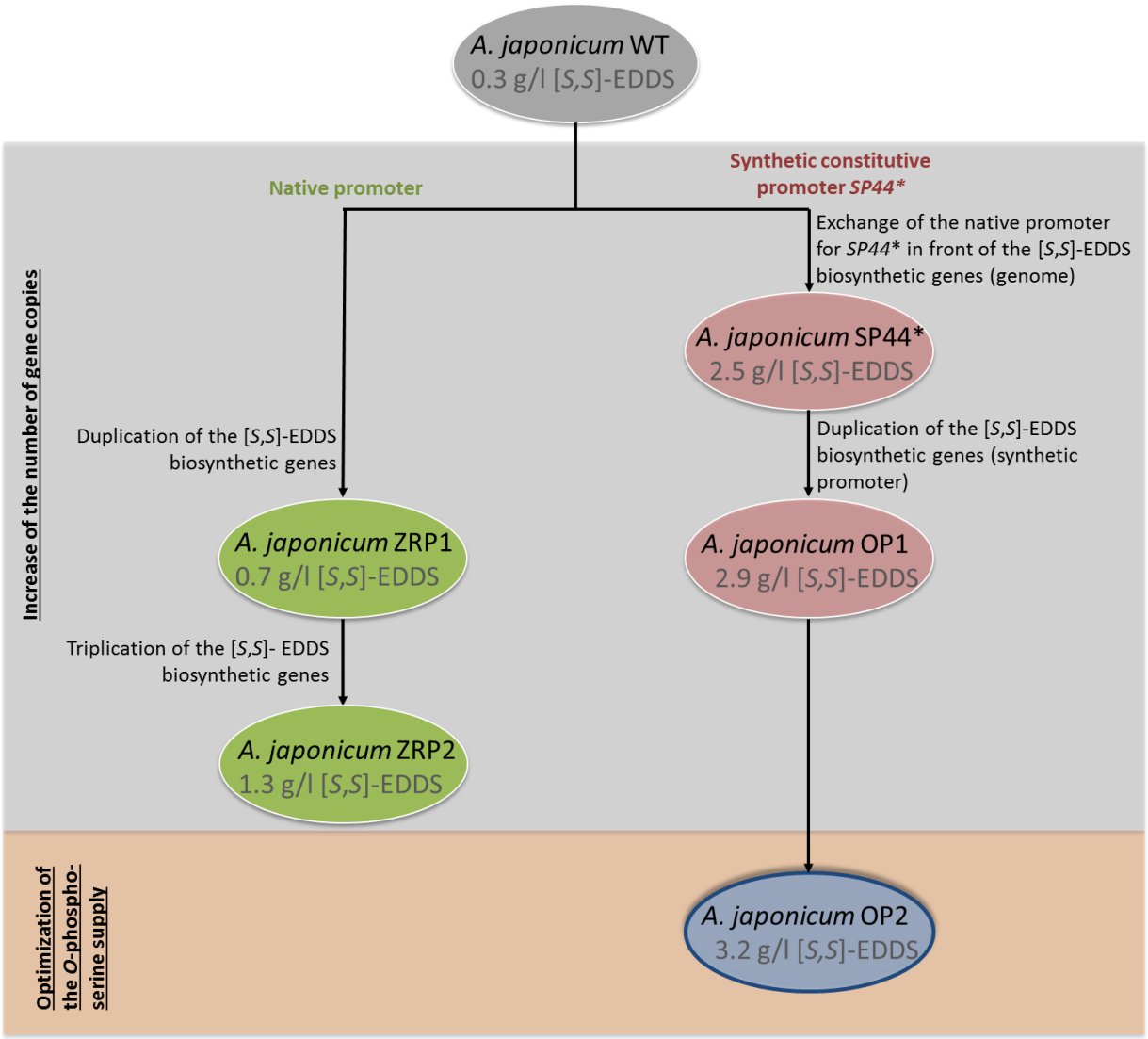

Fig. 5 (publication 2): Genetic engineering steps to optimize the [*S*,*S*]-EDDS production rates in *A. japonicum.*

Average [*S*,*S*]-EDDS production of the strains in zinc depleted SM medium is given.

WT: wild type

ZRP: additional copy of the [*S*,*S*]-EDDS biosynthetic genes *aesA-D* under the control of the native, zinc regulated promoter

OP: the [*S*,*S*]-EDDS biosynthetic genes under the control of the synthetic promoter *SP44**

2.2.2 Glucuronidase activity testing of heterologous promoters

In both strains, *A. japonicum* ZRP1 and *A. japonicum* ZRP2, the additional copies of the [*S*,*S*]-EDDS biosynthesis genes were controlled by the native promoter.

Consequently, although [*S*,*S*]-EDDS production was increased, it was still zinc regulated in the modified strains just as in *A. japonicum* WT. To circumvent this zinc regulation, two main approaches were applicable in principle: either the deletion of the regulator gene *zur* or the exchange of the native promoter with a strong constitutive promoter. As mentioned above, the deletion of *zur in A. japonicum* leads to a zinc deregulated [*S*,*S*]-EDDS producer strain. However, the deletion of *zur* caused undesirable side effects and the deletion mutant suffers from reduced fitness. Therefore, the more specific approach of exchanging the native promoter was pursued in this work. On the one hand this approach targets only the deregulation of the [*S*,*S*]-EDDS biosynthesis genes rather than the whole Zur regulon and on the other hand, it could additionally improve transcription levels of EDDS biosynthetic genes. To prepare the exchange of the native promoter of *aesA-D* for a strong constitutive one, several promoters were considered and finally three promoters were chosen to be tested for their application in *A. japonicum* by using the glucuronidase activity assay: firstly, the *ermE*p* promoter, a widely used constitutive promoter in actinomycetes that derives from *Saccharopolyspora erythraea* (Schmitt-John and Engels, 1992; Bibb et al., 1994; Siegl et al., 2013); secondly, *actII-orf4*p, a native promoter of the actinorhodin biosynthesis cluster of *S. coelicolor* (Uguru et al., 2005) and thirdly, *SP44** (Wang et al., 2013; Bai et al., 2015), a synthetic derivative of the promoter of the *kasO gene*, whose gene product regulates the polyketide synthase type I gene cluster for coelimycin of *S. coelicolor.* For the glucuronidase assay, the chosen promoters were cloned in front of the glucuronidase reporter gene *gusA* into the vector pGus (Myronovskyi et al., 2011). The resulting plasmids pGus_*SP44**, pGus_*ermE*p* and pGus_*actII-orf4*p were then integrated in *A. japonicum* WT. The strain *A. japonicum* pGus_*aesA*p, which contains the native promoter of the [*S*,*S*]-EDDS biosynthesis genes *aesA-D* and a strain containing the unaltered promoterless pGus plasmid (Spohn et al., 2016) were used as controls. A defined amount of cells of each strain was disrupted after 72 h of growth in a medium containing zinc and the glucuronidase activity of each cell extract was measured in Miller units per milligram biomass using a spectrophotometric assay (Table 1, publication 2). As expected, the native promoter was inactive in the presence of zinc, while all other promoters were active. Of the three tested promoters, the *actII-orf4*p promoter of *S. coelicolor* showed the weakest activity and the *ermE*p* promoter showed medium activity. However, under control of the *SP44** promoter the expression of the glucuronidase gene was

highest, such as the glucuronidase activity with the *SP44** promoter was four times higher than with the *ermE*p*.

2.2.3 Construction of a zinc deregulated strain by exchanging the *aesA-D* promoter region

After identification of *SP44** as being the most effective promoter in *A. japonicum,* the native *aesAp* promoter of the [*S*,*S*]-EDDS biosynthesis genes was exchanged for this promoter in the genome of *A. japonicum*. This exchange led to the deletion of the Zur DNA-binding motif including most of the intergenic region upstream of *aesA*. However, an 18 base pair sequence upstream of *aesA* remained unchanged in the newly constructed strain *A. japonicum* SP44* in order to preserve a potential, native ribosome binding site (Fig. 4, publication 2). When the [*S*,*S*]-EDDS production titer of *A. japonicum* SP44* was measured in the zinc depleted [*S*,*S*]-EDDS production medium, it was almost twice that of *A. japonicum* ZRP2, which contained two additional copies of the [*S*,*S*]-EDDS biosynthesis gene (Fig. 4).

2.2.4 Overexpression of the *aesA-D* genes in *A. japonicum* with a constitutive promoter

Since both strategies, introducing additional copies of the [*S*,*S*]-EDDS biosynthesis genes as well as exchanging the native promoter, had an advantageous effect on [*S*,*S*]-EDDS production, a combination of both was pursued in the next step. To achieve this, a plasmid containing the genes *aesA-D* under control of the synthetic promoter *SP44** was integrated into the genome of *A. japonicum* SP44*. The resulting strain *A. japonicum* OP1 produced ten times more [*S,S*]-EDDS than *A. japonicum* WT (3.2 g/L compared to 0.3 g/L) (Fig. 4).

2.2.5 Optimization of the precursor supply

The significant increase of [*S*,*S*]-EDDS production in *A. japonicum* OP1 compared to *A. japonicum* WT is presumably a result of higher transcription rates of the genes

aesA-D due to the stronger promoter and multiple copies of the genes. However, secondary metabolite production is not only linked to transcription rates of biosynthetic genes. Sufficient provision of essential precursor molecules can also play a crucial role in production. Therefore, the optimization of the precursor supply was addressed next. In analogy to the staphyloferrin B biosynthesis in *S. aureus*, which produces a siderophore with a similar ethylenediamine-moiety in its backbone, it is postulated that *A. japonicum* synthesizes [*S*,*S*]-EDDS from *O*-phosphoserine, oxaloacetate and aspartate (Spohn et al., 2016) (Fig. S1, publication 2). In feeding studies with labelled aspartic acid it was previously proven that aspartic acid is one of the building blocks of [*S*,*S*]-EDDS (Cebulla, 1995). However, similar feeding experiments with *O*-phosphoserine were not possible, because *A. japonicum* was not able to take up *O*-phosphoserine efficiently when given as sole carbon source (unpublished data). *O*-phosphoserine is known to be a precursor of serine biosynthesis (Fig. 5, publication 2). As shown in other bacteria like *E. coli* and *Salmonella typhimurium*, important enzymes in serine biosynthesis are the feedback regulated phosphoglycerate dehydrogenase SerA, which oxidizes 3-phosphoglycerate to 3-phosphohydroxypyruvate (Sugimoto and Pizer 1968a; Sugimoto and Pizer 1968b; Schuller et al., 1995), and the phosphoserine transaminase SerC (Pizer, 1963; Umbarger et al., 1963), which builds *O*-phosphoserine from 3-phosphohydroxypyruvate. To verify whether the *O*-phosphoserine production occurs in a similar way in *A. japonicum* and whether *O*-phosphoserine is indeed one of the [*S*,*S*]-EDDS building blocks, an *A. japonicum* ***ΔserC*** deletion mutant was constructed. Under [*S*,*S*]-EDDS production conditions, this mutant showed severe growth retardation compared to the wild type. Additionally, no [*S*,*S*]-EDDS production was measurable in the mutant, not even when grown long enough to reach a comparable biomass as *A. japonicum* wild type during [*S*,*S*]-EDDS production. Growth deficiency could only be reversed by feeding serine to the mutant. However, in contrast to that, [*S*,*S*]-EDDS production could not be regained by feeding serine, but only by introducing a plasmid encoded *serC*. This confirmed that *O*-phosphoserine is indeed an essential precursor for the [*S*,*S*]-EDDS biosynthesis.

After *O*-phosphoserine was proven to be a precursor of the [*S*,*S*]-EDDS biosynthesis, the next step aimed at increasing the *O*-phosphoserine supply by overexpressing the respective biosynthesis genes. For this, the genes *serC* and *serA* of *A. japonicum*

were amplified and cloned into the integrative plasmid pRM4. The plasmid was then integrated into *A. japonicum* OP1. The resulting strain *A. japonicum* OP2 thus contained an additional copy of its *serA* and *serC* genes under control of the constitutive promoter *ermE*p*. When this strain was grown under [*S*,*S*]-EDDS production conditions in zinc depleted medium, no significantly increased [*S*,*S*]-EDDS production was measureable in *A. japonicum* OP2 (3.1 g/L) compared to *A. japonicum* OP1 (2.9 g/L). A significant difference was observed when both strains were grown in a medium containing zinc. While *A. japonicum* OP1 produced only about 2.2 g/L [*S*,*S*]-EDDS in the presence of zinc, *A. japonicum* OP2 produced 3.3 g/L (Fig. 6), even though the [*S*,*S*]-EDDS biosynthesis genes are under the control to the *SP44** promoter in both strains.

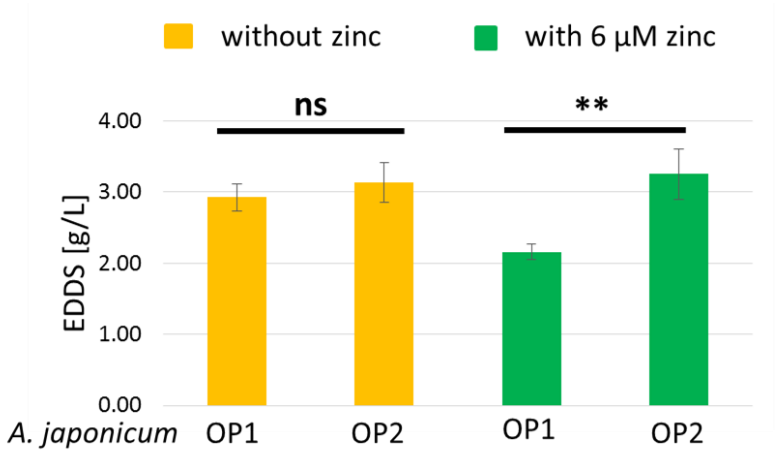

Fig. 6 (publication 2): [*S*,*S*]-EDDS production titers of the *A. japonicum* strains OP1 and OP2.

Cells were grown for five days in either zinc depleted or zinc supplemented (6 µmol/L) SM-medium.

A. japonicum OP1 contains additional copies of the [*S,S*]-EDDS genes (*aesA-D*) under the control of the constitutive promoter *SP44**; *A. japonicum* OP2 contains in comparison to *A. japonicum* OP1 an additional copy of the *O*-phosphoserine biosynthesis genes *serA* and *serC* under control of the *ermEp**. Statistically significant differences are marked with * (**: $p \leq 0.01$, ns: not significant, Man-Whitney U-test, n = 6).

In *A. japonicum* OP2 the constitutive expression of the *O*-phosphoserine biosynthesis genes *serA* and *serC* due to the plasmid encoded copies obviously prevented this decrease in the [*S*,*S*]-EDDS production, which is observable in *A. japonicum* OP1 when comparing production in zinc containing medium to production in zinc depleted medium. This indicated that not only the genes *aesA-D,* but also the formation of its precursor *O*-phosphoserine is affected by zinc. However, no Zur-binding motif could be detected in the upstream regions of *serA* and *serC.* Additionally, when the strain *A. japonicum Δzur* OP1 was constructed and grown in either zinc supplemented or zinc depleted medium, [*S*,*S*]-EDDS production was lower (Fig. 7, unpublished data) in the presence of zinc similarly as in *A. japonicum* OP1. This suggests that there are more mechanisms influencing the zinc metabolism in *A. japonicum* than only the well-known Zur regulator.

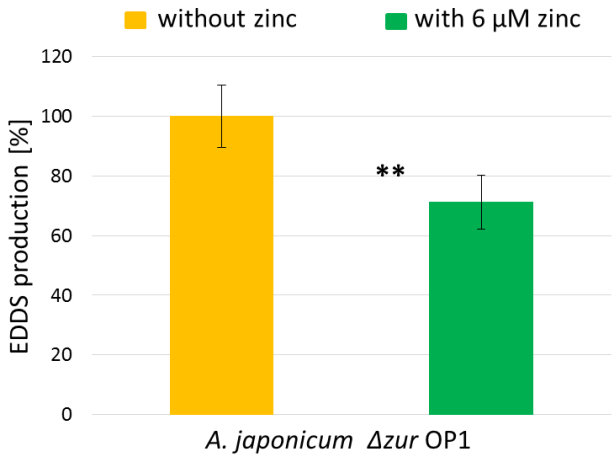

Fig. 7: Comparative [*S*,*S*]-EDDS production of *A. japonicum Δzur* OP1 in percentage. The strain was grown for five days in either zinc depleted or zinc supplemented (6 µmol/L) SM-medium. [*S*,*S*]-EDDS production of *A. japonicum Δzur* OP1 in zinc depleted medium was set as 100 %. Statistically significant differences are marked with * (**: $p \leq 0.01$, Man-Whitney U-test, n = 6).

2.2.6 Scale-up of the [*S*,*S*]-EDDS overproducing strain OP2

The newly generated strain *A. japonicum* OP2 turned out to be an exceedingly good [*S*,*S*]-EDDS producer even in the presence of zinc. Therefore, this strain was chosen to be also tested in large-scale biotechnological production in cooperation with the

Slovenian company Acies Bio (Tehnološki park 21, 1000 Ljubljana, Slovenija). By using *A. japonicum* WT under low zinc conditions (Zwicker et al., 1997) and the zinc deregulated mutant *A. japonicum Δzur* in a zinc containing environment in fed-batch fermentation, a titer of about 16 g/L of [*S*,*S*]-EDDS could be achieved in previous productions (Fig. S3, publication 2). Due to a very long incubation time of about 900 h, this fermentation is unprofitable, though. Subsequently, the best performing strain *A. japonicum* OP2 was set up in a pH-controlled batch bioprocess in order to assess the potential of the newly generated [*S*,*S*]-EDDS producers for biotechnological production. In this bioprocess, carried out in a 10 L bioreactor with 7 L working volume of nutrient-rich, low-cost industrial medium, production of [*S*,*S*]-EDDS reached up to a titer of about 9.8 g/L in 135 h (Fig. 7, publication 2). The thereof calculated [*S,S*]-EDDS production rate was 4.3 mg/h/g dry cell weight.

3 Discussion

3.1 Identification of the EDHA cluster

In order to investigate how widespread the capability to produce the zincophore [*S*,*S*]-EDDS is distributed within actinobacteria, a MultiGeneBlast search using the sequences of the [*S*,*S*]-EDDS biosynthesis enzymes AesA-C was conducted. Thus, various bacteria harboring [*S*,*S*]-EDDS biosynthesis genes similar to *aesA-C,* including the transporter gene *aesD*, were identified. However, in many of the discovered species there was an additional biosynthesis gene upstream of this operon. This additional biosynthesis gene, encoding a VioC-like enzyme, forms probably a polycistronic unit with the four homologous [*S*,*S*]-EDDS biosynthesis genes due to its genetic arrangement and similar iron repressed transcription profile. VioC catalyses the synthesis of the aproteinogenic amino acid hydroxyarginine in the viomycin biosynthesis (Yin and Zabriskie, 2004). Given the high similarity to AesA-D of the [*S*,*S*]-EDDS biosynthesis*,* as well as to VioC of the viomycin biosynthesis, the product of this newly discovered cluster was expected to resemble [*S*,*S*]-EDDS extended with a hydroxyarginine. A compound produced by *Streptomyces* sp. MA5143a, namely ethylenediaminesuccinic acid hydoxyarginine (EDHA), fulfilled this specification. Through subsequent production experiments and transcription profiling in *Streptomyces* sp. MA5143a, this so called EDHA operon could be linked to the production of EDHA.

Due to both, the highly similar biosynthetic gene clusters and the structural similarity of [*S*,*S*]-EDDS and EDHA, it was hypothesized that the transcription of the EDHA cluster in *Streptomyces scabies* and in *Streptomyces* sp. MA5143a was zinc regulated as demonstrated for the [*S,S*]-EDDS biosynthetic gene cluster, However, a zinc-repression of the EDHA production could not be observed. Instead, transcription of the EDHA genes and production of the compound was inhibited in the presence of iron. Additionally, a putative IdeR binding motive in the non-coding region upstream of the EDHA operon was discovered *in silico*. Similar to regulators of the Fur family, IdeR regulators are known to be involved in controlling the biosynthesis of siderophores and iron uptake systems (Hantke, 2001). However, while Fur regulators are preferably used by Gram-negative bacteria to regulate iron uptake mechanisms, Gram-positives with high GC-content usually possess IdeR regulators or their homologue DtxR. Considering these findings, it can be concluded that EDHA

functions as a siderophore in *Streptomyces scabies* as well as in *Streptomyces* sp. MA5143a and its production is probably downregulated by IdeR when a certain threshold of iron is exceeded. According to a phylogenetic analysis with actinomycetes genomes, the EDHA operon is present in non-pathogenic species such as *Streptomyces avermitilis* as well as several pathogenic bacteria like e.g. *Streptomyces scabies* (potato pathogen), *Corynebacterium pseudotuberculosis* (sheep and goat pathogen) as well as *Nocardia brasiliensis* and *Corynebacterium ulcerans* (human pathogens). Thus, the EDHA operon could be detected in different genera of actinobacteria, which are only distantly related to each other and species, which live in different ecological niches, showing that the ability to produce EDHA production is quite widespread.

3.2 One strain, many siderophores – a common feature

The EDHA operon is not the only IdeR/iron-regulated metallophore cluster in *Streptomyces* sp. MA5143a. Besides the EDHA operon, also a desferrioxamin-like cluster and another NRPS cluster with an IdeR binding motif were identified suggesting that *Streptomyces* sp. MA5143a has the potential to produce more than one siderophore. This capability is quite common in bacteria, but the conditions under which the individual compounds are produced have not yet been understood. One reason to maintain multiple siderophore cluster might be that siderophore piracy, meaning the uptake and use of siderophores by non-producers (xenophore uptake), is also rather common in bacteria (Luckey et al., 1972; Granger and Price, 1999; Loper and Henkels, 1999; Yamanaka et al., 2005; D'Onofrio et al., 2010; Tanabe at al., 2011; Cordero et al., 2012; Miethke et al., 2013; Endicott et al., 2017). The ability to produce multiple siderophores might therefore increase the possibility of at least one siderophore being inaccessible for competitors within a bacterial community consisting of pirating/cheating non-producers and producers. Especially for pathogenic bacteria, which compete not only with other bacteria, but also have to evade the host's immune response, the capability of producing different siderophores might allow context-specific advantages. Thus, being able to switch from siderophores with a high iron affinity like enterobactin, which easily outcompetes siderophores of other bacteria, but can be detected by the innate immune system

(Flo et al., 2004), to a siderophore like salmochelin with a lower affinity, which is therefore less competitive, but stealthier (Abergel et al., 2006; Fischbach et al., 2006) is probably beneficial and is probably the reason for the existence of multiple siderophore clusters in the genome. In *Escherichia coli* for example expression of the salmochelin cluster can lead to increased virulence, because in contrast to enterobactin, the C-glycosylated derivative salmochelin is not detected by the mammalian innate immune response (Fischbach et al., 2006). Another possible reason for the synthesis of structurally and chemically different siderophores is that their combination allows the microorganisms to provide iron under a wide range of ecological conditions (McRose et al., 2018). Produced simultaneously, siderophores can have a synergistic effect, like in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, which produce siderophores of different hydrophobicity (Gobin et al., 1996; Lane et al., 1998). It has also been shown for several marine siderophore producers that iron is shuttled from siderophores of high hydrophobicity to those of low hydrophobicity (Martinez et al., 2003; Homann et al., 2009; Gauglitz et al., 2014). Furthermore, some bacteria not only produce siderophores of different hydrophobicity, but also with different iron affinities. Iron mobilization from iron oxide goethite for example has been shown to be more efficient if not only one, but both, a weak organic ligand oxalate as well as a strong hydroxamate siderophore like desferrioxamine are present (Cheah et al., 2003; Reichard et al., 2007). Further examples for bacteria, which produce siderophores with different iron affinities, are e.g. *Burkholderia cepacia* ATCC25416 producing the hydroxamate siderophore ornibactin (Meyer et al., 1995), as well as cepabactin and pyochelin (Meyer et al., 1993). *Pseudomonas aeruginosa* PAO1, synthesizes pyoverdine (Cox and Adams, 1985) with a high iron affinity (pFe = 27) (Albrecht-Gary et al., 1994) and the lower affinity siderophore pyochelin (Cox and Graham, 1979) (pFe = 16) (Brandel et al., 2012). *Azotobacter vinelandii* uses azotobactin (Bulen and LeComte, 1962; Page et al., 1991), which is structurally related to pyoverdine, and vibrioferrin (pFe = 18.4) (Baars et al., 2018), an α-hydroxycarboxylate siderophore, as well as several structurally related catechol siderophores (Corbin et al., 1969; Cornish et al., 1995; Cornish et al., 1998) with different iron affinities. Synthesizing siderophores is beneficial in iron depleted environments. However, it can be a metabolically costly trait, as demonstrated in an experiment by Griffin et al. (2004), in which a pyoverdine producing wild type was outcompeted by a non-producing mutant of *Pseudomonas*

aeruginosa in an iron rich environment. Synthesizing high affinity siderophores might be more cost intensive than producing low affinity siderophores. Therefore, bacteria maybe distinguish between the use of first line siderophores and costlier second line siderophores. As second line siderophores are assumed to be more “expensive” in their production, they might only be produced during severe iron limitation and in lower amounts to work synergistically with less “expensive” siderophores. In *A. vinelandii* for example Baars et al. (2016) observed that the siderophores with a lower iron affinity are usually produced in higher amounts compared to those with a high affinity. Additionally, *P. aeruginosa* was shown to rely on the production of pyoverdine in a highly iron-limited environment, but switched to the production of pyochelin when iron was relatively more accessible (Dumas et al., 2013). It was assumed that this switch in production was made in response to the prevalent environmental conditions in order to optimize the cost-to-benefit ratio of the siderophore synthesis in *P. aeruginosa.* The production of pyoverdine, the siderophore with the higher affinity to iron, is considered to be more “expensive” than the production of pyochelin due to higher the number of biosynthesis genes involved, the number of nucleotides and amino acids used for synthesis, as well as the higher molecular mass of the siderophore (Dumas et al., 2013). Taking this in account, there are many possible explanations why the genome of *Streptomyces* sp. MA5143a contains more than one putative siderophore cluster and how these might interact which each other. The production of EDHA could be more cost-efficient than that of desferrioxamine due to its lower molecular weight. Therefore, it could also serve as first-line siderophore. However, EDHA could also be used to act synergistically with other siderophores produced by *Streptomyces* sp MA5143a.

3.3 Comparison of the EDHA and [*S*,*S*]-EDDS biosynthesis cluster

Due to a similar gene organization of the biosynthesis gene cluster EDHA and [*S*,*S*]-EDDS as well as due to the similarity of their structures, it seemed likely that EDHA might also function as a zincophore. Both compounds form complexes with different trace elements *in vitro*. However, EDHA and [*S*,*S*]-EDDS seem to have evolved to fulfill different functions in the respective producers, as evidenced by the IdeR/iron regulated biosynthesis of the former and the Zur/zinc regulated biosynthesis of the

latter. Even though the IdeR/DtxR regulator family and the Zur/Fur like regulators have some structural similarity, as they both feature an N-terminal DNA binding site with a helix-turn-helix motif and have a domain for metal-binding as well as dimerization, they neither share any sequence similarity nor binding motives (Hantke, 2001). Furthermore, not only the regulation of EDHA synthesis differs from that of [*S*,*S*]-EDDS synthesis, but also the two compounds are probably taken up by different systems. In Gram-positive bacteria, siderophore uptake is often mediated by ABC transporter systems, e.g. the SirABC system in *Staphylococcus aureus,* which mediates the uptake of the APCA siderophore staphyloferrin B (Cheung et al., 2009). In close proximity to the EDHA biosynthesis genes, another operon-like structure consisting of four putative transporter genes was detected. These four genes, *appA-D,* with opposite transcription direction to the EDHA biosynthesis genes are only separated from the EDHA operon by a small intergenic region containing the putative IdeR binding motif. They exhibited similarity (27 % - 37 %) to AppA-D, an ABC transporter system, of *Bacillus subtilis* and were strictly conserved in species that contained the EDHA cluster. In *B. subtilis* the AppA-D system is able to transport tetra- and pentapeptides, but no tripeptides and probably also no dipeptides, for which *B. subtilis* has specific dipeptide and tripeptide transporters (Koide and Hoch, 1994; Levdikov et al., 2005). Although the genome of *A. japonicum* contains several *appA-D* like systems, none of those is in close proximity to the [*S*,*S*]-EDDS biosynthesis genes. Assuming a similar specialization of the transporter systems for *Streptomyces* sp. MA5143a, it is likely that the AppA-D-like system is also particularly suitable for the transport of tetra- and pentapeptides, but not for smaller compounds. This would make it probably a fitting uptake system for EDHA, which is more aminoacid-like than [*S*,*S*]-EDDS, due to the hydroxyarginine extension. Additionally, EDHA contains not only two, but five nitrogen atoms and is larger than [*S*,*S*]-EDDS. Thus an oligopeptide transporter like AppA-D is probably suitable for EDHA while it might be ineffectual for the smaller [*S*,*S*]-EDDS. However, since no *appA-D* homologue could be identified next to the [*S*,*S*]-EDDS-biosynthesis genes, this might indicate that *A. japonicum* uses a different kind of ABC transport system for the [*S*,*S*]-EDDS import. It is reasonable to expect that the transcription of the genes coding for the metallophore uptake system will be regulated by the same regulator as the transcription of the biosynthesis genes. Since the putative IdeR binding motif is located in the small intergenic region between both, the EDHA operon as well the

appA-D operon, the regulator may function bi-directionally and regulate both. Thus, to prove that the AppA-D-like system of *Streptomyces* sp. MA5143a might function a EDHA uptake system, iron repressed expression of the *appA-D* genes must be confirmed in the future. For *A. japonicum*, a zinc/Zur repressed ABC-transporter system has been identified (Spohn et al., 2016). However, due to its similarity to the widely spread zinc uptake system ZnuABC of other bacteria, which are not capable of producing [*S*,*S*]-EDDS, it is inconclusive whether this is as specialized metallophore uptake system.

3.4 Generation of an [*S*,*S*]-EDDS overproducing strain for biotechnological production

Although the chemical synthesis of [*S*,*S*]-EDDS has been established for decades and the advantages of the compound over EDTA are well known, EDTA still dominates the market. While EDTA has an annual production/import of up to 10 000 tons in the EU, EDDS production/import is only about 100 tons per year (European Chemicals Agency, 2020c). The main reason for EDDS still remaining a niche product, is probably the high market price, as EDDS costs multiple times as much as EDTA. However, if the establishment of an economical and sustainable biotechnological production of [*S*,*S*]-EDDS is achieved, [S,S]-EDDS could replace EDTA in many industrial applications. To enable the biotechnological production of [*S*,*S*]-EDDS while avoiding the disadvantages of a reduced fitness due to the deletion of the Zur regulator, the overproduction strain *A. japonicum* OP2 was generated. In this strain, multiple copies of the biosynthesis genes *aesA-D* under the control of a strong constitutive promoter in addition with an enhanced supply of the precursor *O*-phosphoserine resulted in a production ten times higher than in the wild type. In a bioprocess, *A. japonicum* OP2 produced up to 9.8 g/L [*S,S*]-EDDS in 135 h fermentation time. This is a significant improvement compared to previous experiments with *A. japonicum* Δ*zur*, where a fed-batch fermentation for 900 h was needed to yield 16.3 g/L [*S,S*]-EDDS (Fig. S3, publication 2). For *A. japonicum* OP2 volumetric [*S,S*]-EDDS productivity was highest in the first 70 h of bioprocess reaching 80 mg/L/h, while *A. japonicum* Δ*zur* yielded only 20 mg/L/h in minimal medium (Fig. S1). These results demonstrate that *A. japonicum* OP2 has an

excellent potential for further development of an economically viable [*S*,*S*]-EDDS bioprocess. However, additional optimization steps, such as fermentation in cheaper media, will be needed to contribute to reduced production costs.

3.5 Zinc influence on the *O*-phosphoserine production

The evaluation of the effects observed during the optimization process has provided new insights into the Zur/zinc influence on the [*S*,*S*]-EDDS biosynthesis. In the strain *A. japonicum* OP1 the native promoter of the [*S*,*S*]-EDDS biosynthesis was exchanged for the synthetic promoter *SP44** to overcome the Zur mediated zinc repression of the [*S*,*S*]-EDDS biosynthesis. Additionally, a plasmid encoded copy of the [*S*,*S*]-EDDS biosynthesis genes under control of the same synthetic promoter was introduced to further improve production. Elimination of the Zur binding motif in the native promoter area by introducing the strong, synthetic promoter was supposed to have a double beneficial effect: increasing the [*S*,*S*]-EDDS production on the one hand, as well as establishing a zinc deregulated, constitutive biosynthesis. These steps led to a significant improvement of the [*S*,*S*]-EDDS production of the strain *A. japonicum* OP1 compared to previous strains. Furthermore, the strain was producing [*S*,*S*]-EDDS when grown in the presence of zinc, unlike the wild type. However, when the [*S*,*S*]-EDDS production titers of the strain *A. japonicum* OP1 in either zinc depleted or zinc supplemented medium were compared more closely, a lower [S,S]-EDDS production was observed in the presence of zinc, indicating that zinc still negatively affects the production.

The strain *A. japonicum* OP1 was further optimized by introducing an additional copy of the two *O*-phosphoserine/serine biosynthesis genes *serA* and *serC* under control of another constitutive promoter, resulting in *A. japonicum* OP2. The introduction of additional copies of *serA a*nd *serC* was supposed to increase the intracellular concentration of the essential precursor *O*-phosphoserine and thus to further improve [*S*,*S*]-EDDS production. Unexpectedly, the [*S*,*S*]-EDDS production titers of *A. japonicum* OP2 were similar to those of *A. japonicum* OP1, when both strains were grown in zinc depleted medium. In zinc supplemented medium, production titers of both strains differed significantly, though. Whereas *A. japonicum* OP1 produced considerably less [*S*,*S*]-EDDS in the presence of zinc, production titers of *A. japonicum* OP2 were similar when grown in either zinc depleted or zinc

supplemented medium. Thus, overexpression of *serA* and *serC* eliminated the negative effect of zinc on the [*S*,*S*]-EDDS production observed in *A. japonicum* OP1.

Since the overexpression of *serA* and *serC* under control of a synthetic promoter resulted in a completely zinc deregulated [S,S]-EDDS production in *A. japonicum* OP2, it can be hypothesized that the *O*-phosphoserine biosynthesis is probably influenced by zinc and/or a zinc responsive regulator. Considering Zur being the major zinc-sensing regulator, which also controls the [*S*,*S*]-EDDS biosynthesis genes in *A. japonicum* WT, the regulator seemed to be a promising candidate to be also involved in regulating the *O*-phosphoserine biosynthesis genes. To investigate whether *serA* and *serC* were regulated by Zur, the upstream regions of respective genes were screened for the deduced $Zur_{A.ja}$ binding motif. However, no Zur binding motives could be identified in the respective upstream regions. This indicates that Zur is not (directly) involved in the regulation of *serA* and *serC.* Furthermore, an *A. japonicum Δzur* OP1 mutant was generated to research any indirect influence of Zur on *serA* and *serC.* However, when tested, the mutant produced less [*S*,*S*]-EDDS in the presence of zinc than in the absence of zinc. Thus, *A. japonicum Δzur* OP1 exhibited a similar production profile as *A. japonicum* OP1. These results suggest that Zur is not responsible for the lower [*S*,*S*]-EDDS production of the strain *A. japonicum* OP1 in presence of zinc.

The additional copy of *serA* and *serC* in *A. japonicum* OP2 presumably increased the availability of the essential [*S*,*S*]-EDDS precursor *O*-phosphoserine. However, an effect could only be observed in a medium containing zinc, as [*S*,*S*]-EDDS production titers remained at the same level in this mutant in the presence as well as in the absence of zinc, contrary to production in *A. japonicum* OP1. This indicates that *O*-phosphoserine production displays a bottleneck in [*S*,*S*]-EDDS production in *A. japonicum* OP1. It is only limiting in zinc supplemented medium, though. The reason for this observation remains unclear. Based on the results obtained so far, two hypotheses can be advanced in order to explain the lingering influence of zinc on the [*S*,*S*]-EDDS production in the strain *A. japonicum* OP1: Hypothesis A involves another, yet unknown zinc sensing regulator in *A. japonicum* that directly or indirectly influences the *O*-phosphoserine biosynthesis. Hypothesis B though, focuses on the general effects zinc deficiency has on the bacterial metabolism, especially on zinc dependant metalloenzymes. Inactivity of those enzymes due to zinc deficiency

presumably leads to severe changes in the overall growth and metabolic fluxes in *A. japonicum.* As a result of the metabolic flux changes, the availability of the precursor *O*-phosphoserine presumably also varies and influences the [*S*,*S*]-EDDS production. In the following passages, both hypotheses shall be discussed independently from each other in more detail.

3.5.1 Lrp regulators and their role in amino acid and secondary metabolite biosynthesis

As mentioned above, hypothesis A involves another zinc sensing regulator, which directly or indirectly influences the *O*-phosphoserine biosynthesis. In *Escherichia coli*, the *O*-phosphoserine biosynthesis genes *serA* and *serC* are regulated by a leucine-responsive regulatory protein (Lrp) (Tuan et al., 1990; Rex et al., 1991; Chen et al., 1997; Man et al., 1997; Chen and Newman, 1998; Hung et al, 2002). The Lrp regulators are widely spread throughout bacteria as well as archaea. They are mainly involved in the regulation of amino acid metabolism (Brinkman et al., 2003; Peeters and Charlier, 2010). Based on genome annotation, *A. japonicum* possesses about two dozen Lrp-like regulator genes. It can be hypothesised that in *A. japonicum* the transcription of the *O*-phosphoserine/serine biosynthesis genes *serA* and s*erC* is regulated by one of these Lrp-like regulators. It was recently discovered that in some actinobacteria Lrp family proteins are not only involved in the regulation of amino acid biosynthesis, but also in the production of secondary metabolites (Liu et al., 2017a; Liu et al., 2017b; Xu et al., 2020). SACE_Lrp, a regulator of *Saccaropolyspora erythraea,* indirectly influences the erythromycin production by controlling the metabolism of branched-chain amino acids. After deletion of this regulator, the uptake of branched-chain amino acids from the medium, as well as their intracellular catabolism was promoted, although growth rate was not affected. Compared to the wild type, a 19 % increase in erythromycin production was observed in the SACE_Lrp deletion mutant. The more efficient uptake of branched amino acids in the deletion mutant resulted presumably in an increased production of propionyl-CoA and methylmalonyl-CoA, both building blocks of the polyketide erythromycin. (Liu et al., 2017a). An Lrp regulator with high similarity to SACE_Lrp of *S. erythraea* influences not only morphological differentiation, but also actinorhodin production in *Streptomyces coelicolor* (Liu et al., 2017b). More recently, the regulator SLCG_Lrp has been reported to directly activate the transcription of lincomycin A biosynthesis genes in *Streptomyces lincolnensis*. Additionally, the SLCG_Lrp regulator indirectly

affects the production by regulating an amino acid exporter, which controls the intracellular concentration of the lincomycin A precursors proline and cysteine (Xu et al., 2020). All these examples show that the production of secondary metabolites can be influenced by Lrp regulators, although the molecular mechanisms differ considerably. Further research is needed to determine whether the serine biosynthesis genes of *A. japonicum* are also regulated by a member or the Lrp family and whether there is a similar interaction between primary and secondary metabolism in *A. japonicum* as in *S. erythraea*. The transcription of *serC* and *serA* may be slightly downregulated in the presence of zinc, as no *O*-phosphoserine is required for [*S,S*]-EDDS production. This regulation might be due to an Lrp regulator interacting with another yet unknown zinc-sensing regulator.

3.5.2 Effects of zinc deficiency on the proteome and on the metabolic fluxes

Another possible explanation could be that there is no zinc responsive regulation of *O*-phosphoserine biosynthesis at all, neither by Zur nor by any other zinc regulator. Transcriptomic analysis did not reveal a significant influence of zinc on the transcription levels of *serA* and *serC* so far (unpublished data), leading to hypothesis B: When *A. japonicum* suffers from zinc deficiency, the bacterial proteome and thus metabolic fluxes undergo severe changes, which indirectly also affect *O*-phosphoserine availability.

Based on bioinformatic analysis, about 6 % of the bacterial proteome consists of putative zinc binding metalloproteins (Andreini et al., 2009), which are probably effected by a zinc deficiency. Unfortunately, little is known about most of these proteins regarding their regulation or functionality. Since there are not many well-studied examples throughout bacteria, it is almost impossible to predict the changes of the bacterial zinc proteome during zinc limitation. However, when *A. japonicum* WT is cultivated in zinc depleted minimal medium, biomass production within three days of cultivation is lower (12.8 ± 4.9 g/L dry cell weight, n = 3) compared to cultivation in zinc containing medium (19.7 ± 2.7 g/L dry cell weight, n = 3). This indicates that the cells do indeed undergo metabolic changes, probably caused by the altered zinc proteome. An exception where zinc limitation and its effect on zinc proteins is well documented are ribosomal (metallo)proteins. During investigations of the Zur

regulons, a number of paralogous genes encoding for ribosomal proteins were discovered in many bacteria (Lindsay and Foster, 2001; Macia et al., 2007; Owen et al., 2007; Li et al., 2009; Smith et al., 2009; Dowd et al., 2012; Pawlik et al., 2012; Lim et al., 2013; Eckelt et al., 2014; Gilston et al., 2014; Mazzon et al., 2014; Mortensen et al., 2014; Sheng et al., 2015). The Zur/zinc repressed genes code for non-zinc-requiring paralogues of the ribosomal proteins L31, L33, L36, and S14. These ribosomal proteins, which are usually expressed under zinc replete conditions, contain a zinc binding motif (CxxC motif) in contrast to their Zur regulated paralogues (Makarova et al., 2001). In *B. subtilis*, the Zur-repressed ribosomal protein paralogues are expressed under zinc limited conditions. They are able to take over the function of the cognate zinc binding ribosomal proteins (Nanamiya et al., 2004; Akanuma et al., 2006; Gabriel and Helmann, 2009; Nanamiya and Kawamura, 2010). As a consequence, the zinc containing ribosomal proteins are released from the ribosome into the cytoplasm. Hence, it is hypothesized that the bound zinc can be mobilized and redistributed. Due to the high amount of ribosomal proteins present in the cell, ribosomal proteins probably account for a large proportion of the intracellular zinc pool. Thus, the ribosomal proteins might serve as emergency reservoir for zinc (Panina et al., 200; Akanuma et al., 2006; Gabriel and Helmann, 2009).

Having zinc independent substitutes for essential, zinc requiring proteins, e.g. as ribosomal proteins, is one failsafe design to cope with severe zinc deficiency. It is not the only option, to prevent a kind of metabolic blackout in case of zinc deficiency, though. For some metalloenzymes it has been shown that they can retain their structural integrity and activity using alternate divalent metal ions. However, there is probably not a failsafe design for every, zinc requiring enzyme. In cases where neither enzyme substitution nor alternate metalation occurs, enzymatic reactions catalysed by zinc metalloenzymes are probably jeopardized. Hesketh et al. (2009) for example hypothesized that a zinc-dependant biosynthetic enzyme, which cannot be functional without zinc, might play a crucial role in undecylprodigiosin (Red) and actinorhodin (Act) production in *S. coelicolor* and causes the antibiotic production to stop when the production medium is depleted of zinc. For Red as well as Act production it was shown that intracellular zinc concentration affected production levels. Act production was even reported to be barely detectable in minimal medium without supplementation of zinc (Hesketh et al., 2009). Although the molecular context is not fully elucidated yet, the Zur regulator does not seem to be directly

involved in regulating the Act or Red biosynthesis. However, culture conditions leading to the expression of the zincophore cluster coelibactin, which is regulated by the zinc-sensing Zur and the MarR-like regulator AbsC, correlated with the downregulation of the Act and Red biosynthesis in *S. coelicolor*. Although none of the enzymes involved in the Red biosynthesis has been reported to be zinc-dependant, a polyketide cyclase/dehydratase encoded by *actVII* (SCO5090), which is essential in the Act biosynthesis, was predicted to need zinc for activity (Fernandez-Moreno et al., 1992).

Even though the final proof is still lacking, Act biosynthesis seems to be an example for zinc deficiency causing a change in the metabolic production profile due to non-functional zinc metalloproteins. Similar to Act production of *S. coelicolor*, non-functional zinc proteins might affect the secondary metabolism in *A. japonicum* and the [*S*,*S*]-EDDS-production. However, in the case of [*S*,*S*]-EDDS-production, zinc deficiency seems to have a positive effect on the production. In zinc depleted [*S*,*S*]-EDDS production medium, [*S*,*S*]-EDDS production by *A. japonicum* is higher, although the fitness of the strain declines due to zinc limitation. Since this effect can be compensated by overexpression of the *serA* and *serC* genes, *O*-phosphoserine merely seems to be a limiting resource under optimal/zinc supplemented growth conditions. Under these improved growth conditions, more metabolic pathways, e.g. antibiotic production, may compete for *O*-phosphoserine and the immediate downstream product serine. As a consequence, the availability of *O*-phosphoserine for [*S*,*S*]-EDDS synthesis might be significantly lower under zinc-saturated growth conditions. Under zinc depleted conditions, some metabolite pathways may be blocked and [*S*,*S*]-EDDS production is likely benefiting from released (*O*-phosphoserine/serine) resources.

Regardless of the reason for the lower [*S*,*S*]-EDDS production in mutant strains such as *A. japonicum* OP1 when cultivated in the presence of zinc, it has been shown that merely overexpressing the biosynthesis genes of secondary metabolite clusters is not a sufficient method to achieve high levels of production. An undersupply of precursors is often the bottleneck that needs to be eliminated. Therefore, an in-depth knowledge of the interaction of pleiotropic regulators of the primary and secondary metabolism, e.g. Lrp-regulators or Zur, is absolutely necessary. Overexpression or deletion of such global players may have beneficial effects on genes necessary for

the production of the desired secondary metabolites. However, at the same time a multitude of unrelated genes can also be affected. A deletion of the Zur-regulator in *A. japonicum* will probably impact the entire metabolism, and interfere, for example, with ribosome biogenesis and protein biosynthesis, as well as spore formation. The deletion of the Zur-regulator in *A. japonicum* allowed to study the regulation of [S,S]-EDDS biosynthesis and led to the construction of the first “zinc deregulated” [*S*,*S*]-EDDS producer. Since this mutant suffered from reduced physical fitness, it was not an ideal candidate for further metabolic engineering, though. In order to optimize the production of a secondary metabolite, it is therefore essential to consider the cell as a whole and to decide individually from case to case which screws need to be turned to achieve the greatest effect. This requires detailed knowledge of the regulation and metabolic fluxes.

4 References

4.1 References of Figures

Fig. 1 was taken from publication 2 (Fig. 1)

Fig. 2 was taken from publication 1 (Fig. 1)

Fig. 3 was taken from publication 1 (Fig. 2)

Fig. 4 was taken from publication 2 (Fig. 2)

Fig. 5 was taken from publication 2 (Fig. 3)

Fig. 6 was taken from publication 2 (Fig. 6)

Fig. 7 contains yet unpublished data

4.2 Web references

Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (2004) *Tetrasodium ethylenediaminetetraacetate (Na4EDTA),* European Chemicals Bureau, accessed 01.09.20 <https://echa.europa.eu/documents/10162/1fea0222-45e6-41f8-8fc7-ff645458bbed>

European Chemicals Agency, *Substance Infocard Edetic acid,* accessed 01.09.2020a <https://echa.europa.eu/de/substance-information/-/substanceinfo/100.000.409>

European Chemicals Agency, *Substance Infocard Nitrilotriacetic acid,* accessed 01.09.2020b <https://echa.europa.eu/de/substance-information/-/substanceinfo/100.004.869>

European Chemicals Agency, *Substance Infocard N,N'-1,2-ethanediylbis-L-aspartic acid,* accessed 01.09.2020c <https://echa.europa.eu/de/substance-information/-/substanceinfo/100.103.639>

International Agency for Research on Cancer (1999) *IARC monographs on the evaluation of carcinogenic risks to humans - Volume 73 Some Chemicals that Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances,* accessed 01.09.20 <https://publications.iarc.fr/91>

4.3 Literature references

Aaseth, J., Skaug, M. A., Cao, Y., Andersen, O. (2015) Chelation in metal intoxication—principles and paradigms. *J. Trace Elem. Med. Biol.*, **31**, 260-266.

Achouiti, A., Vogl, T., Endeman, H., Mortensen, B. L., Laterre, P. F., Wittebole, X., van Zoelen, M. A. D., Zhang, Y., Hoogerwerf, J. J., Florquin, S., Schultz, M. J., Grutters, J. C., Biesma, D. H., Roth, J., Skaar, E. P., van't Veer, C., de Vos, A. F., van der Poll, T. (2014) Myeloid-related protein-8/14 facilitates bacterial growth during pneumococcal pneumonia. *Thorax*, **69**, 1034-1042.

Ahn, B.E., Cha, J., Lee, E.J., Han, A.R., Thompson, C.J., Roe, J.H. (2006) Nur, a nickel-responsive regulator of the Fur family, regulates superoxide dismutases and nickel transport in *Streptomyces coelicolor*. *Mol. Microbiol.,* **59**, 1848-1858.

Akanuma, G., Nanamiya, H., Natori, Y., Nomura, N., & Kawamura, F. (2006) Liberation of zinc-containing L31 (RpmE) from ribosomes by its paralogous gene product, YtiA, in *Bacillus subtilis*. *J. Bacteriol.*, **188**, 2715-2720.

Albrecht-Gary, A. M., Blanc, S., Rochel, N., Ocaktan, A. Z., Abdallah, M. A. (1994) Bacterial iron transport: coordination properties of pyoverdin PaA, a peptidic siderophore of *Pseudomonas aeruginosa*. *Inorg. Chem.*, **33**, 6391-6402.

Andreini, C., Bertini, I. and Rosato, A. (2009) Metalloproteomes: a bioinformatic approach. *Acc. Chem. Res.*, **42**, 1471-1479.

Andreini, C., Bertini, I., Cavallaro, G., Holliday, G. L., Thornton, J. M. (2008) Metal ions in biological catalysis: from enzyme databases to general principles. *J. Biol. Inorg. Chem*., **13**, 1205-1218.

Baars, O., Zhang, X., Gibson, M. I., Stone, A. T., Morel, F. M., Seyedsayamdost, M. R. (2018) Crochelins: siderophores with an unprecedented iron-chelating moiety from the nitrogen-fixing bacterium *Azotobacter chroococcum*. *Angewandte Chemie*, **130**, 545-550.

Baars, O., Zhang, X., Morel, F. M., Seyedsayamdost, M. R. (2016) The siderophore metabolome of *Azotobacter vinelandii*. *Appl. Environ. Microbiol.*, **82**, 27-39.

Bagg, A. and Neilands, J.B. (1987) Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry,* **26**, 5471-5477.

Bai, C., Zhang, Y., Zhao, X., Hu, Y., Xiang, S., Miao, J., Lou, C., Zhang, L. (2015) Exploiting a precise design of universal synthetic modular regulatory elements to unlock the microbial natural products in *Streptomyces*. *PNAS*, **112**, 12181-12186.

Bibb, M. J., White, J., Ward, J. M., Janssen, G. R. (1994) The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. *Mol. Microbiol.*,**14**, 533-545.

Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N., Schoner, B. E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene,* **116**, 43-49.

Bobrov, A. G., Kirillina, O., Fetherston, J. D., Miller, M. C., Burlison, J. A., Perry, R. D. (2014) The *Yersinia pestis* siderophore, yersiniabactin, and the ZnuABC system both contribute to zinc acquisition and the development of lethal septicaemic plague in mice. *Mol. Microbiol.*, **93**, 759-775.

Botella, H., Peyron, P., Levillain, F., Poincloux, R., Poquet, Y., Brandli, I., Wang, C., Tailleux, L., Tilleul, S., Charrière, G. M., Waddell, S. J., Foti, M., Lugo-Villarino, G., Gao, Q., Maridonneau-Parini, I., Butcher, P. D., Ricciardi Castagnoli, P., Gicquel, B., de Chastellier, C., Neyrolles, O. (2011) Mycobacterial P1-type ATPases mediate resistance to zinc poisoning in human macrophages. *Cell Host Microbe*, **10**, 248-259.

Brandel, J., Humbert, N., Elhabiri, M., Schalk, I. J., Mislin, G. L., Albrecht-Gary, A. M. (2012) Pyochelin, a siderophore of *Pseudomonas aeruginosa*: physicochemical characterization of the iron (III), copper (II) and zinc (II) complexes. *Dalton Trans.*, **41**, 2820-2834.

Brinkman, A. B., Ettema, T. J., De Vos, W. M., Van Der Oost, J. (2003) The Lrp family of transcriptional regulators. *Mol. Microbiol.*, **48**, 287-294.

Bulen, W. A. and LeComte, J. R. (1962) Isolation and properties of a yellow-green fluorescent peptide from *Azotobacter* medium. *Biochem. Biophys. Res. Commun.*, **9**, 523-528.

Bünning, P. and Riordan, J. F. (1985) The functional-role of zinc in angiotensin converting enzyme - implications for the enzymemechanism. *J. Inorg. Biochem*., **24**, 183-198.

Bütof, L., Schmidt-Vogler, C., Herzberg, M., Grosse, C., Nies, D.H. (2017) The components of the unique Zur regulon of *Cupriavidus metallidurans* mediate cytoplasmic zinc handling. *J. Bacteriol*., **199**, 20.

Cebulla, I. (1995) Gewinnung komplexbildender Substanzen mittels *Amycolatopsis orientalis*. *University of Tübingen*, PhD Thesis.

Challis, G. L. (2005) A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases. *ChemBioChem*, **6**, 601-611.

References

Cheah, S. F., Kraemer, S. M., Cervini-Silva, J., Sposito, G. (2003) Steady-state dissolution kinetics of goethite in the presence of desferrioxamine B and oxalate ligands: implications for the microbial acquisition of iron. *Chem. Geol.*, **198**, 63-75.

Chen, C. and Newman, E. B. (1998) Comparison of the Sensitivities of Two *Escherichia coli* Genes to In Vivo Variation of Lrp Concentration. *J. Bacteriol.*, **180**, 655-659.

Chen, L., Liu, T. and Ma, C. A. (2009) Metal complexation and biodegradation of EDTA and *S,S*-EDDS: a density functional theory study. *J. Phys. Chem. A,* **114**, 443-454.

Cheung, J., Beasley, F. C., Liu, S., Lajoie, G. A., Heinrichs, D. E. (2009). Molecular characterization of staphyloferrin B biosynthesis in *Staphylococcus aureus*. *Mol. Microbiol*., **74**, 594-608.

Choi, S.Y. and Bird, A.J. (2014) Zinc'ing sensibly: controlling zinc homeostasis at the transcriptional level. *Metallomics,* **6**, 1198-1215.

Corbin, B. D., Seeley, E. H., Raab, A., Feldmann, J., Miller, M. R., Torres, V. J., Anderson, K. L., Dattilo, B. M., Dunman, P. M., Gerads, R., Caprioli, R. M., Nacken, W., Chazin, W. J., Skaar, E. P. (2008) Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science*, **319**, 962-965.

Corbin, J. L. and Bulen, W. A. (1969) Isolation and identification of 2,3-dihydroxybenzoic acid and. N2, N6-di(2,3-dihydroxybenzoyl)-l-lysine formed by iron-deficient *Azotobacter vinelandii*. *Biochemistry*, **8**, 757-762.

Cordero, O. X., Ventouras, L. A., DeLong, E. F., Polz, M. F. (2012) Public good dynamics drive evolution of iron acquisition strategies in natural bacterioplankton populations. *PNAS*, **109**, 20059-20064.

Cornish, A. S. and Page, W. J. (1995) Production of the triacetecholate siderophore protochelin by *Azotobacter vinelandii*. *BioMetals*, **8**, 332-338.

Cornish, A. S. and Page, W. J. (1998) The catecholate siderophores of *Azotobacter vinelandii*: their affinity for iron and role in oxygen stress management. *Microbiology*, **144**, 1747-1754.

Cox, C. D. and Adams, P. (1985) Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. *Infect. Immun.*, **48**, 130-138.

Cox, C. D. and Graham, R. I. (1979) Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. *J. Bacteriol.*, **137**, 357-364.

Cunningham, L., Gruer, M. J., and Guest, J. R. (1997) Transcriptional regulation of the aconitase genes (*acnA* and *acnB*) of *Escherichia coli*. *Microbiology*, **143**, 3795-3805.

Diaz-Mireles, E., Wexler, M., Sawers, G., Bellini, D., Todd, J.D., Johnston, A.W.B. (2004) The Fur-like protein Mur of *Rhizobium leguminosarum* is a Mn^{2+}-responsive transcriptional regulator. *Microbiology,* **150**, 1447-1456.

D'Onofrio, A., Crawford, J. M., Stewart, E. J., Witt, K., Gavrish, E., Epstein, S., Clardy, J., Lewis, K. (2010) Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem. Biol.*, **17**, 254-264.

Dowd, G. C., Casey, P. G., Begley, M., Hill, C., Gahan, C. G. (2012) Investigation of the role of ZurR in the physiology and pathogenesis of *Listeria monocytogenes*. *FEMS Microbiol. Lett.*, **327***,* 118-125.

Drechsel, H., Freund, S., Nicholson, G., Haag, H., Jung, O., Zähner, H., Jung, G. (1993) Purification and chemical characterization of staphyloferrin B, a hydrophilic siderophore from staphylococci. *Biometals*, **6**, 185-192.

Dsouza, M., Larsen, N. and Overbeek, R. (1997) Searching for patterns in genomic data, *Trends Genet*., **13**, 497-498.

Dumas, Z., Ross-Gillespie, A. and Kümmerli, R. (2013) Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. *Proc. Royal Soc. B*, **280**, 20131055.

Eckelt, E., Jarek, M., Frömke, C., Meens, J., Goethe, R. (2014) Identification of a lineage specific zinc responsive genomic island in *Mycobacterium avium* ssp. *paratuberculosis*. *BMC genomics*, **15**, 1076.

Endicott, N. P., Lee, E. and Wencewicz, T. A. (2017) Structural basis for xenosiderophore utilization by the human pathogen *Staphylococcus aureus*. *ACS Infect. Dis.*, **3**, 542-553.

Fernandez-Moreno, M.A.*,* Martínez, E.*,* Boto, L.*,* Hopwood, D.A.*,* Malpartida, F. (1992*)* Nucleotide sequence and deduced functions of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin*. J. Biol. Chem.,* **26,***:* 19278-19290*.*

Fischbach, M. A., Lin, H., Zhou, L., Yu, Y., Abergel, R. J., Liu, R. D., Raymond, K. N., Wanner, B. L., Strong, R. K., Walsh, C. T., Aderem, A., Smith, K. D. (2006) The pathogen-associated *iroA* gene cluster mediates bacterial evasion of lipocalin 2. *PNAS*, **103**, 16502-16507.

Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., Akira, S., Aderem, A. (2004) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature*, **432**, 917-921.

References

Flores, F. J. and Martín, J. F. (2004) Iron-regulatory proteins DmdR1 and DmdR2 of *Streptomyces coelicolor* form two different DNA-protein complexes with iron boxes. *Biochem. J.*, **380**, 497-503.

Foreman, H. (1953) The use of chelating agents for accelerating excretion of radioelements. *J. Am. Pharm. Assoc.*, **42**, 629-632.

Gabriel, S. E. and Helmann, J. D. (2009) Contributions of Zur-controlled ribosomal proteins to growth under zinc starvation conditions. *J. Bacteriol.*, **191**, 6116-6122.

Gaddy, J. A., Radin, J. N., Loh, J. T., Piazuelo, M. B., Kehl-Fie, T. E., Delgado, A. G., Ilca, F. T., Peek, R. M., Cover, T. L., Chazin, W. J., Skaar, E. P., Algood, H. M. S. (2014) The host protein calprotectin modulates the *Helicobacter pylori cag* type IV secretion system via zinc sequestration. *PLoS Pathog.*, **10**, e1004450.

Gauglitz, J. M., Iinishi, A., Ito, Y., Butler, A. (2014) Microbial tailoring of acyl peptidic siderophores. *Biochemistry*, **53**, 2624-2631.

Gilston, B. A., Wang, S., Marcus, M. D., Canalizo-Hernández, M. A., Swindell, E. P., Xue, Y., Mondragón, A., O'Halloran, T. V. (2014) Structural and mechanistic basis of zinc regulation across the *E. coli* Zur regulon. *PLoS Biol.*, **12**, e1001987.

Gobin, J. and Horwitz, M. A. (1996) Exochelins of *Mycobacterium tuberculosis* remove iron from human iron-binding proteins and donate iron to mycobactins in the *M. tuberculosis* cell wall. *J. Exp. Med.*, **183**, 1527-1532.

Gold, B., Rodriguez, G. M., Marras, S. A., Pentecost, M., Smith, I. (2001) The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. *Mol. Microbiol.*, **42**, 851-865.

Granger, J. and Price, N. M. (1999) The importance of siderophores in iron nutrition of heterotrophic marine bacteria. *Limnol. Oceanogr.*, **44**, 541-555.

Griffin, A. S., West, S. A. and Buckling, A. (2004) Cooperation and competition in pathogenic bacteria. *Nature*, **430**, 1024-1027.

Günter-Seeboth, K. and Schupp, T. (1995) Cloning and sequence analysis of the *Corynebacterium diphtheriae dtxR* homologue from *Streptomyces lividans* and *S. pilosus* encoding a putative iron repressor protein. *Gene*, **166**, 117-119.

Guo, X., Wei, Z. and Wu, Q. (2015) Degradation and residue of EDTA used for soil repair in heavy metal-contaminated soil. *Transactions of the Chinese Society of Agricultural Engineering*, **31**, 272-278.

Haase, H. and Rink, L. (2014) Multiple impacts of zinc on immune function. *Metallomics*, **6**, 1175-1180.

Hantke, K. (1981) Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol. Gen. Genet.,* **182**, 288-292.

Hantke, K. (2001) Iron and metal regulation in bacteria. *Curr*. *Opin*. *Microbiol*., **4**, 172-177.

Hensens, O. D. and Liesch, J. M. (1984) Structure elucidation of angiotensin converting enzyme inhibitor L-681,176 from *Streptomyces* sp. MA 5143a. *J. Antibiot*., **37**, 466-468.

Hesketh, A., Kock, H., Mootien, S., Bibb, M. (2009) The role of *absC*, a novel regulatory gene for secondary metabolism, in zinc-dependent antibiotic production in *Streptomyces coelicolor* A3 (2). *Mol. Microbiol.*, **74**, 1427-1444.

Homann, V. V., Edwards, K. J., Webb, E. A., Butler, A. (2009) Siderophores of *Marinobacter aquaeolei*: petrobactin and its sulfonated derivatives. *BioMetals*, **22**, 565-571.

Hood, M. I., Mortensen, B. L., Moore, J. L., Zhang, Y., Kehl-Fie, T. E., Sugitani, N., Chazin, W. J., Caprioli, R. M., Skaar, E. P. (2012) Identification of an *Acinetobacter baumannii* zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration. *PLoS Pathog.*, **8**, e1003068.

Huang, D.-L., Tang, D.-J., Liao, Q., Li, H.-C., Chen, Q., He, Y.-Q., Feng, J.-X., Jiang, B.-L., Lu, G.-T., Chen, B., Tang, J.-L. (2008) The Zur of *Xanthomonas campestris* functions as a repressor and an activator of putative zinc homeostasis genes via recognizing two distinct sequences within its target promoters. *Nucleic Acids Res*., **36**, 4295-4309.

Huang, D.-L., Tang, D.-J., Liao, Q., Li, H.-C., Chen, Q., He, Y.-Q., Feng, J.-X., Jiang, B.-L., Lu, G.-T, Tang, J.-L. (2009) The Zur of *Xanthomonas campestris* is involved in hypersensitive response and positively regulates the expression of the *hrp* cluster via *hrpX* but not *hrpG*. *Mol. Plant-Microbe Interact*., **22**, 321–329.

Huang, L., Rowin, G., Dunn, J., Sykes, R., Dobna, R., Mayles, B. A., Gross, D. M., Burg, R. W. (1984) Discovery, purification and characterization of the angiotensin converting enzyme-inhibitor, L-681,176, produced by *Streptomyces* sp. MA 5143a, *J. Antibiot*., **37**, 462-465.

Hung, S. P., Baldi, P. and Hatfield, G. W. (2002) Global Gene Expression Profiling in *Escherichia coli* K12: the effects of leucine-responsive regulatory protein. *J. Biol. Chem.*, **277**, 40309-40323.

Kallifidas, D., Pascoe, B., Owen, G. A., Strain-Damerell, C. M., Hong, H. J., Paget, M. S. (2010) The zinc-responsive regulator Zur controls expression of the coelibactin gene cluster in *Streptomyces coelicolor*. *J. Bacteriol.,* **192**, 608-611.

References

Kari, F. G. and Giger, W. (1995) Modeling the photochemical degradation of ethylenediaminetetraacetate in the River Glatt. *Environ. Sci. Technol.*, **29**, 2814-2827.

Kari, F. G. and Giger, W. (1996) Speciation and fate of ethylenediaminetetraacetate (EDTA) in municipal wastewater treatment. *Water Res.*, **30**, 122-134.

Koide, A. and Hoch, J. A. (1994) Identification of a second oligopeptide transport system in *Bacillus subtilis* and determination of its role in sporulation. *Mol. Microbiol.*, **13**, 417-426.

Lane, S. J., Marshall, P. S., Upton, R. J., Ratledge, C. (1998) Isolation and characterization of carboxymycobactins as the second extracellular siderophores in *Mycobacterium smegmatis*. *BioMetals*, **11**, 13-20.

Lee, J. W. and Helmann, J. D. (2007) Functional specialization within the Fur family of metalloregulators. *BioMetals*, **20**, 485-499.

Levdikov, V. M., Blagova, E. V., Brannigan, J. A., Wright, L., Vagin, A. A., Wilkinson, A. J. (2005) The structure of the oligopeptide-binding protein, AppA, from *Bacillus subtilis* in complex with a nonapeptide. *J. Mol. Biol.*, **345**, 879-892.

Lhospice, S., Gomez, N. O., Ouerdane, L., Brutesco, C., Ghssein, G., Hajjar, C., Liratni, A., Wang, S., Richaud, P., Bleves, S., Ball, G., Borezée-Durant, Lobinski, R., Pignol, D., Arnoux, P., Voulhoux, R. (2017) *Pseudomonas aeruginosa* zinc uptake in chelating environment is primarily mediated by the metallophore pseudopaline. *Sci. Rep.*, **7**, 1-10.

Li, Y., Qiu, Y., Gao, H., Guo, Z., Han, Y., Song, Y., Du, Z., Wang, X., Zhou, D., Yang, R. (2009) Characterization of Zur-dependent genes and direct Zur targets in *Yersinia pestis*. *BMC Microbiol.*, **9**, 128.

Lim, C. K., Hassan, K. A., Penesyan, A., Loper, J. E., Paulsen, I. T. (2013) The effect of zinc limitation on the transcriptome of *Pseudomonas protegens* Pf-5. *Environ. Microbiol.*, **15**, 702-715.

Lindsay, J. A. and Foster, S. J. (2001) *zur*: a Zn^{2+}-responsive regulatory element of *Staphylococcus aureus* The GenBank accession number for the sequence reported in this paper is AF101263. *Microbiology*, **147**, 1259-1266.

Liu, J., Chen, Y., Wang, W., Ren, M., Wu, P., Wang, Y., Wu, H., Li, C., Weaver, D., Zhang, L., Zhang, B. (2017a) Engineering of an Lrp family regulator SACE_Lrp improves erythromycin production in *Saccharopolyspora erythraea*. *Metab. Eng.*, **39**, 29-37.

Liu, J., Li, J., Dong, H., Chen, Y., Wang, Y., Wu, H., Li, C., Weaver, D., Zhang, L., Zhang, B. (2017b) Characterization of an Lrp/AsnC family regulator SCO3361,

controlling actinorhodin production and morphological development *in Streptomyces coelicolor*. *Appl. Microbiol. Biotechnol.*, **101**, 5773-5783.

Loper, J. E. and Henkels, M. D. (1999) Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Appl. Environ. Microbiol.*, **65**, 5357-5363.

Lucarelli, D., Russo, S., Garman, E., Milano, A., Meyer-Klaucke, W., Pohl, E. (2007) Crystal structure and function of the zinc uptake regulator FurB from *Mycobacterium tuberculosis*. *J. Biol. Chem.,* **282**, 9914-9922.

Luckey, M., Pollack, J. R., Wayne, R., Ames, B. N., Neilands, J. B. (1972) Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderochromes as iron carriers. *J. Bacteriol.*, **111**, 731-738.

Ma, Z., Gabriel, S.E. and Helmann, J.D. (2011) Sequential binding and sensing of Zn^{2+} by *Bacillus subtilis* Zur. *Nucleic Acids Res*., **39**, 9130-9138.

Maciąg, A., Dainese, E., Rodriguez, G. M., Milano, A., Provvedi, R., Pasca, M. R., Smith, I., Palù, G., Riccardi, G., Manganelli, R. (2007) Global analysis of the *Mycobacterium tuberculosis* Zur (FurB) regulon. *J. Bacteriol*., **189**, 730-740.

Majer, J., Jokl, V., Dvorakova, E., Juracova, M. (1968) New complexones. XIII. Potentiometric and electrophoretic study of ethylenediamine-N, N′-disuccinic acid and its metal chelates. *Chem. Pap*., **22**, 415-423.

Makarova, K. S., Ponomarev, V. A., Koonin, E. V. (2001) Two C or not two C: recurrent disruption of Zn-ribbons, gene duplication, lineage-specific gene loss, and horizontal gene transfer in evolution of bacterial ribosomal proteins. *Genome Biol.*, **2,** research0033.1.

Man, T. K., Pease, A. J. and Winkler, M. E. (1997) Maximization of transcription of the *serC* (pdxF)-aroA multifunctional operon by antagonistic effects of the cyclic AMP (cAMP) receptor protein-cAMP complex and Lrp global regulators of *Escherichia coli* K-12. *J. Bacteriol.*, **179**, 3458-3469.

Martinez, J. S., Carter-Franklin, J. N., Mann, E. L., Martin, J. D., Haygood, M. G., Butler, A. (2003) Structure and membrane affinity of a suite of amphiphilic siderophores produced by a marine bacterium. *PNAS*, **100**, 3754-3759.

Mazzon, R. R., Braz, V. S., da Silva Neto, J. F., do Valle Marques, M. (2014) Analysis of the *Caulobacter crescentus* Zur regulon reveals novel insights in zinc acquisition by TonB-dependent outer membrane proteins. *BMC Genomics*, **15**, 734.

McRose, D. L., Seyedsayamdost, M. R. and Morel, F. M. (2018) Multiple siderophores: bug or feature? *J. Biol. Inorg. Chem*., **23**, 983-993.

References

Medema, M. H., Takano, E. and Breitling, R. (2013) Detecting sequence homology at the gene cluster level with MultiGeneBlast, *Mol. Biol. Evol*., **30**, 1218-1223.

Meers, E., Ruttens, A., Hopgood, M. J., Samson, D., Tack, F. M. G. (2005) Comparison of EDTA and EDDS as potential soil amendments for enhanced phytoextraction of heavy metals. *Chemosphere,* **58**, 1011-1022.

Meiwes, J., Fiedler, H.P., Zähner, H., Konetschnyrapp, S., Jung, G. (1990) Production of desferrioxamine E and new analogs by directed fermentation and feeding fermentation. *Appl. Microbiol. Biotechnol.,* **32**, 505-510.

Meyer, J. M., Hohnadel, D. and Hall, F. (1989) Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. *Microbiology*, **135**, 1479-1487.

Meyer, J. M., Van Van, T., Stintzi, A., Berge, O., Winkelmann, G. (1995) Ornibactin production and transport properties in strains of *Burkholderia vietnamiensis* and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*). *BioMetals*, **8**, 309-317.

Miethke, M., Kraushaar, T. and Marahiel, M. A. (2013) Uptake of xenosiderophores in *Bacillus subtilis* occurs with high affinity and enhances the folding stabilities of substrate binding proteins. *FEBS Lett.*, **587**, 206-213.

Miller, A. L. (1998) Dimercaptosuccinic acid (DMSA), a non-toxic, water-soluble treatment for heavy metal toxicity. *Alternative medicine review: a journal of clinical therapeutic*, **3**, 199-207.

Moreau, G.B., Qin, A.P. and Mann, B.J. (2018) Zinc acquisition mechanisms differ between environmental and virulent *Francisella* species. *J. Bacteriol*., **200**, 16.

Mortensen, B. L., Rathi, S., Chazin, W. J., Skaar, E. P. (2014) *Acinetobacter baumannii* response to host-mediated zinc limitation requires the transcriptional regulator Zur. *J. Bacteriol*., **196**, 2616-2626.

Myronovskyi, M., Welle, E., Fedorenko, V., Luzhetskyy, A. (2011) β-Glucuronidase as a sensitive and versatile reporter in actinomycetes. *Appl. Environ. Microbiol.*, **77**, 5370-5383.

Nanamiya, H. and Kawamura, F. (2010) Towards an elucidation of the roles of the ribosome during different growth phases in *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.*, **4**, 451-61.

Nanamiya, H., Akanuma, G., Natori, Y., Murayama, R., Kosono, S., Kudo, T., Kobayashi , K., Ogasawara , N., Park, S.-M., Ochi, K., Kawamura, F. (2004) Zinc is a key factor in controlling alternation of two types of L31 protein in the *Bacillus subtilis* ribosome. *Mol. Microbiol.,* **52***,* 273-283*.*

Napolitano, M., Rubio, M.A., Santamaria-Gomez, J., Olmedo-Verd, E., Robinson, N.J., Luque, I. (2012) Characterization of the response to zinc deficiency in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol*., **194**, 2426-2436.

Neal, J.A. and Rose, N.J. (1968) Stereospecific ligands and their complexes. I. A cobalt(III)complex of ethylenediaminedisuccinic acid. *Inorg. Chem.,* **7**, 2405-2412.

Nishikiori, T., Okuyama, A., Naganawa, H., Takita, T., Hamada, M., Takeuchi, T., Aoyagi, T., Umezawa, H. (1984) Production by actinomycetes of (*S,S*)-*N,N*-ethylenediamine-disuccinic acid, an inhibitor of phospholipase c. *J. Antibiot.,* **37**, 426-427.

Nowack, B., Kari, F. G. and Krüger, H. G. (2001) The remobilization of metals from iron oxides and sediments by metal-EDTA complexes. *Water Air Soil Pollut.*, **125**, 243-257.

O'Brien, I.G. and Gibson, F. (1970) Structure of enterochelin and related 2,3-dihydroxynbenzoylserine conjugates from *Escherichia coli*. *Biochim. Biophys. Acta - Gen. Subj*., **215**, 393-402.

Ogawa, K., Naganawa, H., Iinuma, H., Aoyagi, T., Umezawa, H. (1984) The structure of histargin, *J. Antibiot*., **37**, 984-987.

Ong, C. L. Y., Gillen, C. M., Barnett, T. C., Walker, M. J., McEwan, A. G. (2014) An antimicrobial role for zinc in innate immune defense against group A S*treptococcus*. *J. Infect. Dis.*, 209, 1500-1508.

Owen, G. A., Pascoe, B., Kallifidas, D., Paget, M. S. (2007) Zinc-responsive regulation of alternative ribosomal protein genes in *Streptomyces coelicolor* involves Zur and σR. *J. Bacteriol*., **189**, 4078-4086.

Page, W. J., Collinson, S. K., Demange, P., Dell, A., Abdallah, M. A. (1991) *Azotobacter vinelandii* strains of disparate origin produce azotobactin siderophores with identical structures. *Biology of metals*, **4**, 217-222.

Panina, E. M., Mironov, A. A., Gelfand, M. S. (2003) Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. *PNAS*, **100**, 9912-9917.

Patzer, S.I. and Hantke, K. (1998) The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Mol. Microbiol.,* **28**, 1199-1210.

Pawlik, M. C., Hubert, K., Joseph, B., Claus, H., Schoen, C., Vogel, U. (2012) The zinc-responsive regulon of *Neisseria meningitidis* comprises 17 genes under control of a Zur element. *J. Bacteriol*., **194**, 6594-6603.

References

Peeters, E. and Charlier, D. (2010) The Lrp family of transcription regulators in archaea. *Archaea*, **2010**, 750457.

Pelludat, C., Rakin, A., Jacobi, C. A., Schubert, S., Heesemann, J. (1998) The yersiniabactin biosynthetic gene cluster of *Yersinia enterocolitica*: organization and siderophore-dependent regulation. *J. Bacteriol.*, **180**, 538-546.

Pizer, L. I. (1963) The pathway and control of serine biosynthesis in *Escherichia coli*. *J. Biol. Chem.,* **238**, 3934-3944.

Pohl, E., Holmes, R. K. and Hol, W. G. (1999a) Crystal structure of the iron-dependent regulator (IdeR) from *Mycobacterium tuberculosis* shows both metal binding sites fully occupied. *J. Mol. Biol*, **285**, 1145-1156.

Pollack, J.R., and Neilands, J.B. (1970) Enterobactin, an iron transport compound from *Salmonella typhimurium*. *Biochem. Biophys. Res. Commun.* **38**, 989-992.

Reichard, P. U., Kretzschmar, R. and Kraemer, S. M. (2007) Dissolution mechanisms of goethite in the presence of siderophores and organic acids. *Geochim. Cosmochim. Acta*, **71**, 5635-5650.

Rex, J. H., Aronson, B. D. and Somerville, R. L. (1991) The *tdh* and *serA* operons of *Escherichia coli*: mutational analysis of the regulatory elements of leucine-responsive genes. *J. Bacteriol.*, **173**, 5944-5953.

Schmidt, C., Schwarzenberger, C., Gross, C., Nies, D.H. (2014) FurC regulates expression of *zupT* for the central zinc importer ZupT of *Cupriavidus metallidurans*. *J. Bacteriol.*, **196**, 3461-3471.

Schmitt-John, T. and Engels, J. W. (1992) Promoter constructions for efficient secretion expression in *Streptomyces lividans*. *Appl. Microbiol. Biotechnol.,* **36**, 493-498.

Schröder, J., Jochmann, N., Rodionov, D.A., Tauch, A. (2010) The Zur regulon of *Corynebacterium glutamicum* ATCC 13032. *BMC Genomics*,**11**, 12.

Schuller, D. J., Grant, G. A. and Banaszak, L. J. (1995) The allosteric ligand site in the V max-type cooperative enzyme phosphoglycerate dehydrogenase. *Nat. Struct. Biol.,* **2**, 69-76.

Sein-Echaluce, V.C., Pallares, M.C., Lostao, A., Yruela, I., Velazquez-Campoy, A., Peleato, M.L., Fillat, M. F. (2018) Molecular basis for the integration of environmental signals by FurB from *Anabaena* sp. PCC 7120. *Biochem. J.*, **475**, 151-168.

Sheng, Y., Fan, F., Jensen, O., Zhong, Z., Kan, B., Wang, H., Zhu, J. (2015) Dual zinc transporter systems in *Vibrio cholerae* promote competitive advantages over gut microbiome. *Infect. Immun*., **83**, 3902-3908.

Shin, J.H., Jung, H.J., An, Y.J., Cho, Y.B., Cha, S.S., Roe, J.H. (2011) Graded expression of zinc-responsive genes through two regulatory zinc-binding sites in Zur. *Proc. Natl. Acad*. Sci. U.S.A., **108**, 5045-5050.

Siegl, T., Tokovenko, B., Myronovskyi, M., Luzhetskyy, A. (2013) Design, construction and characterisation of a synthetic promoter library for fine-tuned gene expression in actinomycetes. *Metab. Eng.*, **19**, 98-106.

Smith, K. F., Bibb, L. A., Schmitt, M. P., Oram, D. M. (2009) Regulation and activity of a zinc uptake regulator, Zur, in *Corynebacterium diphtheriae*. *J. Bacteriol*. **191**, 1595-1603.

Smith, M. J., Shoolery, J. N., Schwyn, B., Holden, I., Neilands, J. B. (1985) Rhizobactin, a structurally novel siderophore from *Rhizobium meliloti*. *J. Am. Chem. Soc.,* **107**, 1739-1743.

Spohn, M., Wohlleben, W., Stegmann, E. (2016) Elucidation of the zinc-dependent regulation in *Amycolatopsis japonicum* enabled the identification of the ethylenediamine-disuccinate ([*S*,*S*]-EDDS) genes. *Environ. Microbiol.,* **18**, 1249-1263.

Stork, M., Grijpstra, J., Bos, M. P., Torres, C. M., Devos, N., Poolman, J. T., Chazin, W. J., Tommassen, J. (2013) Zinc piracy as a mechanism of *Neisseria meningitidis* for evasion of nutritional immunity. *PLoS Pathog.*, **9**, e1003733.

Sugimoto, E. and Pizer, L. I. (1968a) The mechanism of end product inhibition of serine biosynthesis I. Purification and kinetics of phosphoglycerate dehydrogenase. *J. Biol. Chem.,* **243**, 2081-2089.

Sugimoto, E. and Pizer, L. I. (1968b) The mechanism of end product inhibition of serine biosynthesis II. Optical studies of phosphoglycerate dehydrogenase. *J. Biol. Chem.,* **243**, 2090-2098.

Tanabe, T., Funahashi, T., Miyamoto, K., Tsujibo, H., Yamamoto, S. (2011) Identification of genes, *desR* and *desA*, required for utilization of desferrioxamine B as a xenosiderophore in *Vibrio furnissii*. *Biol. Pharm. Bull.*, **34**, 570-574.

Tandy, S., Ammann, A., Schulin, R., Nowack, B. (2006) Biodegradation and speciation of residual SS-ethylenediaminedisuccinic acid (EDDS) in soil solution left after soil washing. *Environ. Pollut.* **142**, 191-199.

Teramoto, H., Inui, M. and Yukawa, H. (2012) *Corynebacterium glutamicum* Zur acts as a zinc-sensing transcriptional repressor of both zinc-inducible and zinc-repressible genes involved in zinc homeostasis. *FEBS J*., **279**, 4385-4397.

References

Tuan, L. R., D'ari, R. and Newman, E. B. (1990) The leucine regulon of *Escherichia coli* K-12: a mutation in *rblA* alters expression of L-leucine-dependent metabolic operons. *J. Bacteriol.*, **172**, 4529-4535.

Tunca, S., Barreiro, C., Sola-Landa, A., Coque, J. J. R., Martín, J. F. (2007) Transcriptional regulation of the desferrioxamine gene cluster of *Streptomyces coelicolor* is mediated by binding of DmdR1 to an iron box in the promoter of the *desA* gene. *FEBS J.*, **274**, 1110-1122.

Uguru, G. C., Stephens, K. E., Stead, J. A., Towle, J. E., Baumberg, S., McDowall, K. J. (2005) Transcriptional activation of the pathway-specific regulator of the actinorhodin biosynthetic genes in *Streptomyces coelicolor*. Mol. Microbiol, **58,** 131-150.

Umbarger, H. E., Umbarger, M. A. and Siu, P. M. (1963) Biosynthesis of serine in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol., **85***, 1431-1439.

Umezawa, H., Aoyagi, T., Ogawa, K., Iinuma, H., Naganawa, H., Hamada, M., Takeuchi, T. (1984) Histargin, a new inhibitor of carboxypeptidase B, produced by actinomycetes. *J. Antibiot*, **37**, 1088-1090.

Walshe, J. M. (1956) Penicillamine, a new oral therapy for Wilson's disease. *Am. J. Med.*, **21**, 487-495.

Wang, W., Li, X., Wang, J., Xiang, S., Feng, X., Yang, K. (2013) An engineered strong promoter for streptomycetes. *Appl. Environ. Microbiol.*, **79**, 4484-4492.

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R., Lee, S. Y., Fischbach, M. A., Müller, R., Wohlleben, W., Breitling, R., Takano, E., Medema, M. H. (2015) antiSMASH 3.0 - a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.,* **43**, W237-243.

Xu, Y., Tang, Y., Wang, N., Liu, J., Cai, X., Cai, H., Li, J., Tan, G., Liu, R., Bai, L., Zhang, L., Wu, H., Zhang, B. (2020) Transcriptional regulation of a leucine-responsive regulatory protein for directly controlling lincomycin biosynthesis in *Streptomyces lincolnensis*. *Appl. Microbiol. Biotechnol.*,, **104**, 2575-2587.

Yamanaka, K., Oikawa, H., Ogawa, H. O., Hosono, K., Shinmachi, F., Takano, H., Sakuda, S., Beppu, T., Ueda, K. (2005) Desferrioxamine E produced *by Streptomyces griseus* stimulates growth and development of *Streptomyces tanashiensis*. *Microbiol.,* **151**, 2899-2905.

Yin, X. and Zabriskie, T. M. (2004) VioC is a non-heme iron, α-ketoglutarate-dependent oxygenase that catalyzes the formation of 3S-hydroxy-l-arginine during viomycin biosynthesis. *ChemBioChem*, **5**, 1274-1277.

Zaia, A. A., Sappington, K. J., Nisapakultorn, K., Chazin, W. J., Dietrich, E. A., Ross, K. F., Herzberg, M. C. (2009) Subversion of antimicrobial calprotectin (S100A8/S100A9 complex) in the cytoplasm of TR146 epithelial cells after invasion by *Listeria monocytogenes*. *Mucosal immunology*, **2**, 43-53.

Zwicker, N., Theobald, U., Zähner, H., Fiedler, H. P. (1997) Optimization of fermentation conditions for the production of ethylene-diamine-disuccinic acid by *Amycolatopsis orientalis*. *J. Ind. Microbiol. Biotechnol.*, **19**, 280-285.

5 Appendix

5.1 Publication 1

Identification of a novel aminopolycarboxylic acid siderophore gene cluster encoding the biosynthesis of ethylendiaminesuccinc acid hydroxyarginine (EDHA)

Metallomics

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Identification of a novel aminopolycarboxylic acid siderophore gene cluster encoding the biosynthesis of ethylenediaminesuccinic acid hydroxyarginine (EDHA)†

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The mechanism of siderophore-mediated iron supply enhances fitness and survivability of microorganisms under iron limited growth conditions. One class of naturally occurring ionophores is the small aminopolycarboxylic acids (APCAs). Although they are structurally related to the most famous anthropogenic chelating agent, ethylenediaminetetraacetate (EDTA), they have been largely neglected by the scientific community. Here, we demonstrate the detection of APCA gene clusters by a computational screening of a nucleotide database. This genome mining approach enabled the discovery of a yet unknown APCA gene cluster in well-described actinobacterial strains, either known for their potential to produce valuable secondary metabolites (*Streptomyces avermitilis*) or for their pathogenic lifestyle (*Streptomyces scabies*, *Corynebacterium pseudotuberculosis*, *Corynebacterium ulcerans* and *Nocardia brasiliensis*). The herein identified gene cluster was shown to encode the biosynthesis of APCA, ethylenediaminesuccinic acid hydroxyarginine (EDHA). Detailed and comparatively performed production and transcriptional profiling of EDHA and its biosynthesis genes showed strict iron-responsive biosynthesis.

Significance to metallomics

This manuscript presents a nucleotide-based screening approach to detect genetic regions with the potential to synthesize aminopolycarboxylic acids (APCAs). This approach uses recently identified essential elements of the [*S*,*S*]-ethylenediaminedisuccinate (EDDS) biosynthetic pathway as a query sequence to discover similar genetic regions in provided nucleotide databases. Using this genome mining strategy, we have discovered a novel, previously unknown APCA gene cluster that encodes the iron-regulated synthesis of the unusual siderophore ethylenediaminesuccinic acid hydroxyarginine (EDHA).

Introduction

Many microorganisms live in habitats providing soluble metal concentrations below the intracellular concentrations required for optimal cellular growth. Such metal limitations are generally associated with eukaryotic host colonialization. As a natural anti-virulence strategy, mammals actively reduce the free metal pool by shielding it in high affinity storage and trafficking proteins. The combat between the pathogen and the host organism for metals is mainly described for iron, an essential cofactor for many basic metabolic pathways.[1–3] However, low bioavailability of iron and other metal ions is not exclusively associated with host invasion but rather a general growth limiting factor in most microbial habitats. To counteract such nutrient limitations, microorganisms have evolved small chelating compounds (ionophores) to sequester metal ions from the environment. Ionophores typically form metal complexes that serve to shuttle the metal ion across a lipid barrier and enable intracellular access for the metal.[4] Siderophores are ionophores that are specifically secreted under iron limited growth conditions

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and capture iron by high affinity binding. This leads to an increased bioavailability of iron for the producer and contributes to its fitness.[5] Some microorganisms adapted the basic siderophore-concept to sequester other poorly accessible metal ions as well. The few known examples of noniron-ionophore systems are either zinc (zincophore) or copper (chalcophore) responsive.[6–8]

For these physiological purposes, ionophores have evolved into a broad structural variety; to date, more than 500 ionophores have been described in the literature.[5,9] The majority are siderophores produced under iron-limited growth conditions. According to the chemical nature of the moieties donating the oxygen ligands for metal ion coordination, ionophores are divided into three main structural classes: catecholates, hydroxamates and carboxylates.[3,5] The donor ligand oxygen is beneficial for high affinity coordination of trivalent metal ions such as Fe(III). In contrast, softer donor atoms such as nitrogen and sulfur preferentially interact with divalent metal ions.[3]

The vast majority of known siderophores are synthesized by two secondary metabolite pathway classes. Many siderophores are polypeptides that are synthesized by nonribosomal peptide synthetases (NRPSs).[10] However, the second main pathway for siderophore biosynthesis, the so-called NRPS-independent siderophore (NIS) pathway, uses alternating dicarboxylic acid and diamine or amino alcohol building blocks.[11]

Although metal ions are essential for cellular growth, they are toxic to cells in higher concentrations. Metal ions can harm cellular functions by generating reactive oxygen species, inhibiting physiological functions of proteins by blocking important thiols, or competing with other metal ions for binding sites.[12–14] To avoid a metal overload and to guarantee an optimal cellular metal ion homeostasis, microorganisms strictly control the expression of genes encoding metal uptake and export/storage functions. The regulation of metal uptake systems, including ionophores is often mediated by transcriptional repressors. The two major metalloregulatory protein families mediating metal ion dependent gene repression in bacteria are the ferric uptake regulator (Fur) family and the iron dependent regulatory protein (IdeR), also referred to as the diphtheria toxin regulator (DtxR) family.[15,16] Fur is the major iron dependent repressor in Gram-negative and low-GC Gram-positive bacteria.[17,18] In contrast, in most high-GC Gram-positive bacteria, IdeR proteins have the function of a global iron sensor and regulate a similar spectrum of genes as Fur.[19] Iron sensing IdeR protein family members are described to fulfill their regulatory function in *Corynebacterium* spp., *Mycobacterium* spp. and *Streptomyces* spp.[20–23] Although there is no amino acid sequence similarity between Fur and IdeR proteins, they do share some similar structural features: the N-terminal domains contain the DNA-binding site with a helix-turn-helix motif, while the C-terminal domains are responsible for dimerization and metal binding. When the cytoplasmic free-iron concentration increases above a certain homeostasis level, these regulators get activated by the coordination of iron. In this ferrous ion bound form, they then bind specifically to the corresponding A/T-rich motifs in the promoter of their DNA targets. Thereby, they prevent the access of the RNA polymerase to the promoter region, which results in the repression of downstream genes.[24]

The naturally evolved concept of chelating compounds, which coordinate and thereby shield metal ions from environmental interactions, has also been adopted by humans for industrial and medical applications. Chelating agents are used in many processes to remove perturbing metal ions or to keep the metal concentration constant. A biological chelator that is in medical use in metal overload therapy is desferrioxamine B.[25] However, the vast majority of industrially applied chelating compounds such as deferasirox, deferiprone and a broad array of phosphonates and aminopolycarboxylic acids (APCAs) are of synthetic origin. Amongst them, the APCAs dominate the market. APCAs are characterized by one or more central secondary or tertiary amines and two or more carboxylic acid side chains. The most frequently used APCA is ethylenediamine-tetraacetate (EDTA), consisting of a central diaminoethane backbone and four equivalent carboxymethyl side chains. EDTA forms stable, water soluble 1 : 1 complexes with di- and trivalent metal ions. Metal ions within this complex are bound to the two tertiary amines and to a single oxygen of each of the four carboxylic groups. This leads to the formation of a highly stable hexadentate type complex.[26] A further valuable APCA that found its way into industrial applications is the EDTA-isomer [*S*,*S*]-ethylenediaminedisuccinate (EDDS), which is marketed under its trade name Envioment™.[27,28] EDDS exhibits the same metal coordination chemistry as EDTA and therefore has comparable stability constants.[26] However, in contrast to EDTA, EDDS is a naturally occurring APCA, which is readily biodegradable until complete mineralization.[29,30] This accessibility to biodegradation makes EDDS a sustainable chelating agent with a favorable environmental profile. EDDS is a naturally occurring ionophore that is synthesized by several *Amycolatopsis* strains as a response to low zinc-stress (zincophore).[8,31]

Our lab has recently discovered the EDDS biosynthesis genes (*aesA–D*) in *Amycolatopsis japonicum* and elucidated their zinc responsive gene regulation in detail.[8] Based on this knowledge, we performed an extended computational screening campaign to detect genetic regions putatively directing the synthesis of further natural APCAs, since such compounds are underrepresented in data-sets listing known ionophore structures.[5] This screening approach enabled the detection of a new APCA gene cluster within the genomes of several thoroughly investigated bacteria, including plant, animal and human pathogens. Based on the genetic information, we predicted the molecular features of the compound. Facilitated by this, we identified L-618,176 (named EDHA, ethylenediaminesuccinic acid hydroxyarginine, in the present manuscript), a previously isolated product of *Streptomyces* sp. MA5143a[32,33] as the corresponding pathway product.

In contrast to the structurally familiar EDDS, which is synthesized exclusively under zinc depleted growth conditions, we here demonstrate that EDHA is a classical, iron responsive siderophore. This was not only shown for the known EDHA producing strain *Streptomyces* sp. MA5143a but also verified by cultivating a selected pathogen, which had been identified by our initial *in silico* screening approach.

Experimental

Bacterial strains and culturing

Streptomyces sp. MA5143a was kindly provided by Dr Mike Goetz from the Natural Product Discovery Institute, Pennsylvania, USA. In liquid cultures, bacteria were grown in 500 mL baffled Erlenmeyer flasks filled with 100 mL of medium at 28 °C using an orbital shaker (220 rpm). To remove trace elements, the flasks were treated first with 0.5 M HCl, and then with 5 mM EDTA for 12 h each, before medium components and distilled water were added. To detect EDHA production, precultures were grown for 48 h in SEED medium containing dextrose (1 g L^{-1}), soluble starch (10 g L^{-1}), beef extract (3 g L^{-1}), yeast autolysate (5 g L^{-1}), peptone from casein (N-Z-Amine® B) (5 g L^{-1}), $MgSO_4 \times 7H_2O$ (0.05 g L^{-1}), KH_2PO_4 (0.18 g L^{-1}), $Na_2HPO_4 \times 2H_2O$ (0.19 g L^{-1}) and $CaCO_3$ (0.5 g L^{-1}), pH 7.0. In total, 5 mL of the 48 h old SEED culture was used to inoculate 100 mL of production medium (PM) containing corn steep powder (15 g L^{-1}), $(NH_4)_2SO_4$ (4 g L^{-1}), $CaCO_3$ (6 g L^{-1}), soluble starch (20 g L^{-1}), corn meal (1 g L^{-1}), soybean meal (5 g L^{-1}), glucose (5 g L^{-1}) and KH_2PO_4 (0.3 g L^{-1}), pH 6.7.

LC-ESI-MS and analytical instrumentation

In total, 2.5 μL of the culture supernatants was analyzed by LC-ESI-MS using a Nucleosil 100-C18 column (3 μm, 100 × 2 mm, with guard column 10 × 2 mm (Dr Maisch GmbH, Ammerbuch-Entringen, Germany)) coupled to an ESI mass spectrometer (LC/MSD Ultra Trap System XCT 6330 (Agilent Technology, Waldbronn, Germany)). Analysis was carried out at a flow rate of 0.4 mL min^{-1} with a solvent gradient of A = 0.1% formic acid in water and B = 0.06% formic acid in acetonitrile (gradient: $t_0 = t_5$ = 0% B, $t_7 = t_{10}$ = 100% B, and post time = 0% B, 40 °C, t in min). Electrospray ionization (positive ionization) in Ultra Scan mode (range 100–600 m/z) with a capillary voltage of 3.5 kV and a drying gas temperature of 350 °C was used for LCMS analysis. Data analysis was conducted with Agilent Data Analysis for 6300 Series Ion Trap LC/MS 6.1 ver. 3.4 software (Bruker-Daltonik GmbH). To generate Cu(II)–EDHA complexes, 1 mL of supernatant was incubated with 20 μL of Cu(II)SO_4 (100 mM) for 10 min, and precipitates were removed by centrifugation.

Reverse transcription (RT)-PCR analysis

RNA isolation and RT-PCR analyses were performed according to Spohn *et al.*[34] with the primer pairs listed in Table S1 (ESI†).

Sequencing, assembly and annotation of *Streptomyces* sp. MA5143a

The peqGOLD Kit (PEQLAB Biotechnology GmbH, Erlangen, Germany) was used to isolate genomic DNA of *Streptomyces* sp. MA5143a for high throughput sequencing. The quality of the DNA was assessed by gel-electrophoresis. In addition, the quantity was estimated by a fluorescence-based method using the Quant-iT PicoGreen dsDNA kit (Invitrogen, USA) and a Tecan Infinite 200 Microplate Reader (Tecan, Switzerland). For sequencing of the *Streptomyces* sp. MA5143a genome, an Illumina paired-end sequencing library (TruSeq sample preparation kit; Illumina, USA) was constructed according to the manufacturer's protocol. The *Streptomyces* sp. MA5143a draft genome sequence was established on an Illumina HiSeq system in rapid run mode (2 × 250 nt) with a pair distance of about 500 bp. Upon sequencing and processing of the obtained data, a *de novo* assembly was performed using the GS *De Novo* Assembler (version 2.8.) (Roche Diagnostics, Mannheim, Germany) with default settings. Annotation of the genome was performed by means of prokka v1.11 and the GenDB 2.0 platform.[35,36] The assembled and annotated draft sequence of *Streptomyces* sp. MA5143a was deposited in the EMBL-EBI database under Bioproject ID PRJEB20813.

Phylogenetic analysis and *in silico* DNA–DNA hybridization

The 16S rRNA gene sequence of *Streptomyces* sp. MA5143 was submitted to BLAST to identify the closest relatives.[37] For a more precise phylogenetic analysis, a phylogenetic tree including all types of strains with published genomes and 99% sequence identity of the 16S rRNA gene sequences was created. A high-resolution phylogenetic tree was obtained using 50 conserved genes that satisfy Multi Locus Sequence Analysis (MLSA) criteria: genes under purifying selection ($dN/dS < 1$), present as a single copy in all reference genomes, distributed at different loci. The 50 used genes are listed in the ESI† (Table S2). Query and reference genomes from the NCBI RefSeq database were scanned for orthologous proteins using Hidden Markov Models (HMM) from the TigrFam database and HMMER.[38–40] Codon alignments of MLSA genes were obtained by aligning amino acid sequences with MAFFT-linsi followed by back translation using pal2nal.[41,42] Alignments were then trimmed using Trimal-automated1 and concatenated into a super-matrix.[43] A maximum likelihood tree was then built using Raxml with the General Time Reversible (GTR) model, GAMMA rate distribution with invariable sites, and 1000 bootstrap replicates. The final tree was pruned to include the major clade of closely related species using the ETE toolkit.[44]

For calculating the DNA–DNA hybridization (DDH) values of *Streptomyces* sp. MA5143a and its closest relatives *in silico*, the Genome-to-Genome Distance calculator 2.0, formula 2, was used (http://ggdc.dsmz.de/).[45–47]

Results and discussion

Identification of a novel aminopolycarboxylic acid (APCA) gene cluster by implementation of a computational screening approach

In *A. japonicum*, the genes *aesA–C* are essential for the biosynthesis of the naturally occurring APCA EDDS.[8] The corresponding enzymes are predicted to catalyze the generation of the characteristic, central 1,2-diaminoethane moiety of EDDS. Although rarely found in natural compound scaffolds,[48] such central diaminoethane moieties are frequently included in industrially applied synthetic APCAs like, *e.g.*, EDTA.

To identify genetic regions that potentially direct the synthesis of natural products containing this unusual feature, we used the amino acid sequences of AesA-C as a query sequence to set up a computational search. Besides AesA-C, we included the efflux

pump AesD to our query since its gene *aesD* is part of the EDDS operon structure and is postulated to be responsible for EDDS secretion.[8]

To conduct the computational search, we applied a combinatorial BLAST and MultiGeneBlast approach and performed a homology search with our query sequence on a GenBank database provided by the MultiGeneBlast webtool.[49] This screening revealed many genetic loci containing *aesA–D* like genes in various actinobacterial genomes, including the EDDS clusters.[8] From these hits, we manually extracted a certain subset that attracted our attention owing to an additional open reading frame (ORF) 5′ upstream of the *aesA–D* genes (Fig. 2). Due to their genetic arrangement, these five genes most likely build a polycistronic unit (named *edha*-operon in this manuscript). The predicted gene product of the additional ORF exhibits similarity to VioC (Accession number: AAP92493). VioC is a biochemically well characterized oxygenase, which is involved in the synthesis of viomycin, an antibiotic with anti-tuberculosis properties.[50,51] This iron- and α-ketoglutarate-dependent oxygenase catalyzes the formation of the aproteinogenic amino acid hydroxyarginine. Such an installation of a hydroxyl group on the carbon backbone is reminiscent of the putrescine modification during the biosynthesis of alcaligin.[52] The corresponding putrescine 2-hydroxylase AlcE (Accession number: CAE35871.1), however, shows neither a sequence similarity to VioC nor to MA_5143a_00506.

A polycistronic unit combining the here described genetic features has not been reported before.

The *edha*-operon was found within the genomes of the well-known antibiotic producer *Streptomyces avermitilis* and several pathogenic actinobacterial strains like *Streptomyces scabies*, *Corynebacterium pseudotuberculosis*, *Corynebacterium ulcerans* and *Nocardia brasiliensis* (Fig. 2). *S. scabies* is a plant pathogen causing significant economic losses for the potato industry, *C. pseudotuberculosis* is an animal pathogen and a major concern to the world's sheep and goat production areas, and *C. ulcerans* and *N. brasiliensis* are human pathogens.[53,54]

The secondary metabolome of *S. avermitilis* is one of the most intensely investigated within the class of actinobacteria. Over the past few decades, more than 20 secondary metabolites were identified in this strain.[55–57] The most prominent secondary metabolite of *S. avermitilis* is the medically relevant antiparasitic compound avermectin.[56] To the best of our knowledge, although being an intensely investigated organism, neither an APCA nor the here detected genes were ever subjected to any study. This is probably due to the fact that these genes escape detection by search algorithms of routine bioinformatic tools, which are applied to identify secondary metabolite gene clusters. These tools rely on sequence similarity searches for previously identified signature genes that are highly specific for known biosynthetic pathway classes.[58] Such signature genes, however, are not yet described for the biosynthesis of small ACPAs and subsequently not integrated into the search algorithms of these genome mining tools.

The *edha*-operon combines genetic features of the EDDS biosynthetic pathway with a *vioC*-like gene. The VioC-like enzyme presumably catalyzes the formation of hydroxyarginine and

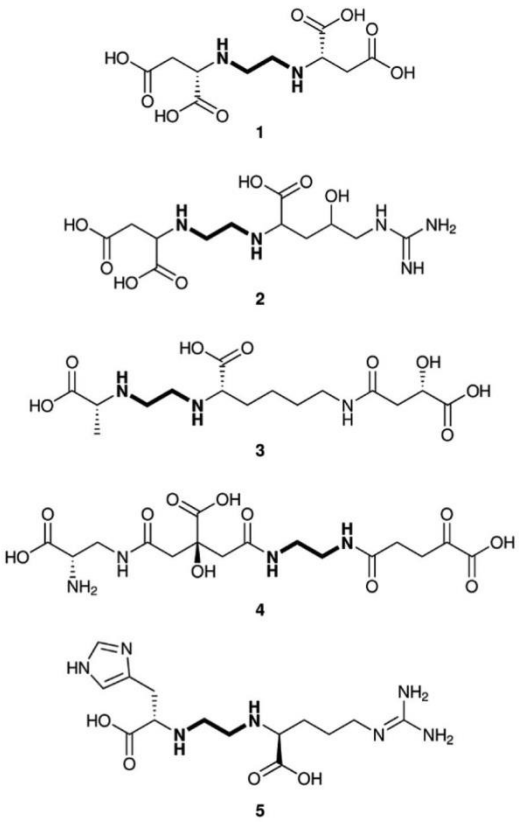

Fig. 1 Chemical structures of 1,2-diaminoethane containing natural products. (1) [*S*,*S*]-EDDS, (2) EDHA, (3) rhizobactin, (4) staphyloferrin B and (5) histargin. Central 1,2-diaminoethane moieties are highlighted in bold.

suggests a product with a similar backbone scaffold as EDDS, however expanded by a hydroxyarginine moiety. Described compounds containing a central diaminoethane scaffold are the siderophores, rhizobactin of *Sinorhizobium meliloti* DM4 and staphyloferrin B of *Staphylococcus aureus* (Fig. 1).[59,60] In addition, two further natural APCAs, which not only share an identical central diaminoethane scaffold but also a similar overall structure and size with EDDS, have been identified previously: histargin, a product of *Streptomyces roseoviridis* MF118-A5, and L-681,176, produced by *Streptomyces* sp. MA5143a (Fig. 1).[32,33,61,62] Both compounds were identified during screening programs for enzyme inhibitors. Histargin was isolated as an inhibitor of carboxypeptidase B, while L-681,176 was found during a screening for inhibitors for angiotensin converting enzymes. In both cases, the inhibitory effects arise from the capability of histargin and L-681,176 to efficiently complex zinc ions, which are essential catalytic cofactors for the respective enzymes.[61,63,64]

Strikingly, L-681,176 combines the two chemical features of a central diaminoethane moiety and a hydroxyarginine (Fig. 1). These chemical features agree with the predicted biosynthetic functions of the enzymes AesA-C and VioC-like, respectively. Consequently, we hypothesized that L-681,176 might be the pathway's end product of the here detected gene cluster.

We propose the more meaningful name ethylenediaminesuccinic acid hydroxyarginine (EDHA) for L-681,176 in accordance with the nomenclature used for natural and synthetic APCAs.

Genome sequence of *Streptomyces* sp. MA5143a

So far, *Streptomyces* sp. MA5143a was the only strain described to synthesize EDHA.[39,40] To verify the presence of the predicted EDHA gene cluster within its genome, we performed a whole genome sequencing program. The sequencing approach on the Illumina HiSeq system resulted in 7 970 359 reads, accounting for about 1.5 giga bases of total sequence information. The obtained reads were assembled by means of the GS *de novo* assembler (version 2.8) resulting in 60 scaffolds comprising 168 contigs. The genome coverage was 161-fold. The final *Streptomyces* sp. MA5143a draft genome sequence has a size of 9 838 959 bases and features a GC-content of 71.47%. Annotation of the genome within prokka[35] and the GenDB 2.0 platform[36] resulted in the identification of 8311 protein-coding sequences, 89 tRNAs and seven copies of the *rrn* operon. By conducting a MultiGene-Blast search on the genome of *Streptomyces* sp. MA5143a, we identified the genes MA_5143a_00506 to MA_5143a_00502 (Fig. 2). The predicted gene products of MA_5143a_00506 to MA_5143a_00502 exhibit similarity to VioC and the EDDS pathway enzymes AesA–D, respectively (Table 1). The detection

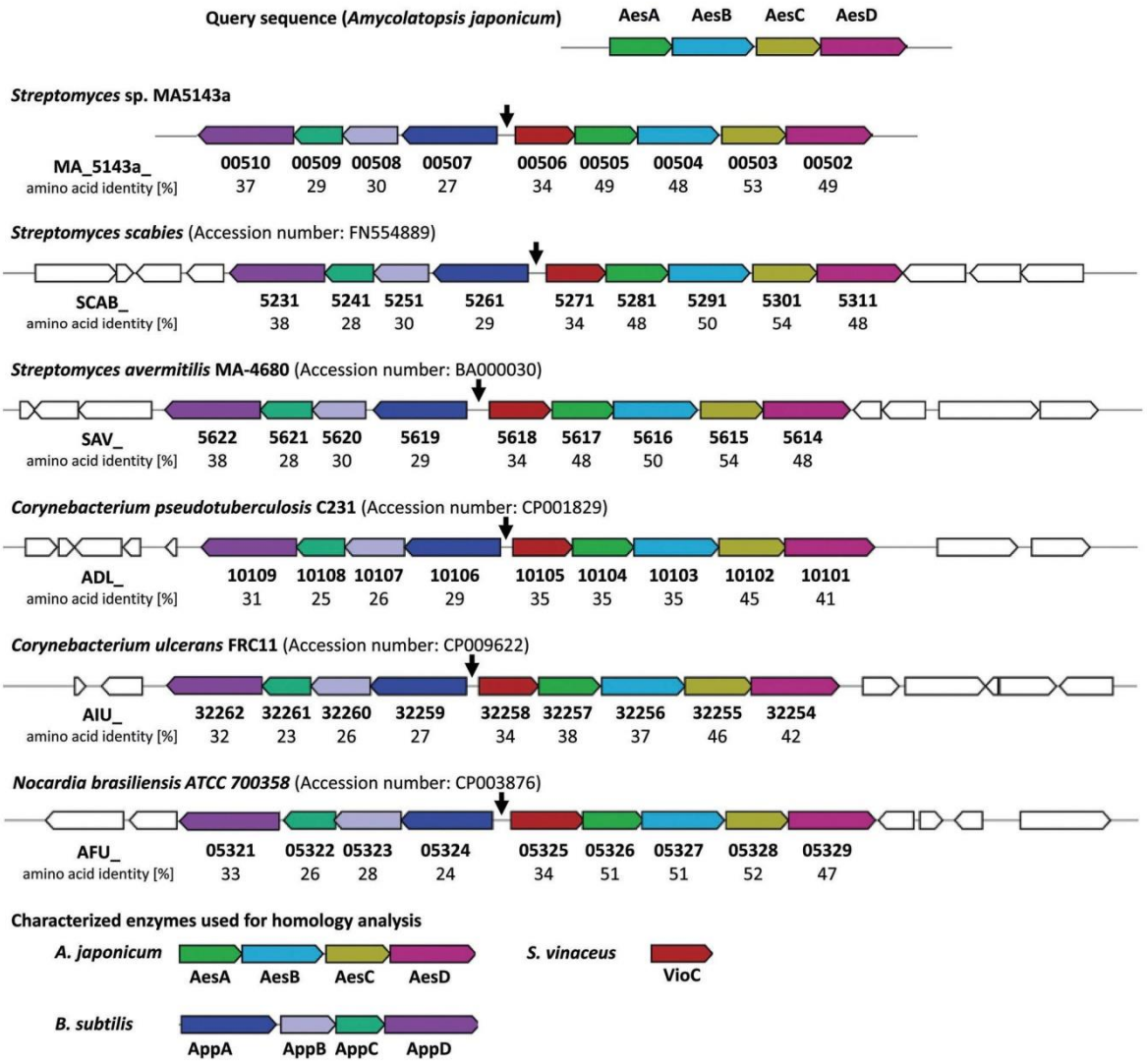

Fig. 2 Gene maps of putative EDHA gene clusters. Depiction of the identified genetic loci by the *in silico* screening approach. Amino acid sequences of AesA-D of *Amycolatopsis japonicum* were used as query. Similar genes are depicted in the same color. The Locus-Tags are shown beneath the corresponding gene. The vertical arrows indicate the location of an IdeR binding motif. Percentage of amino acid identity to AppD (P42064), AppC (P42063), AppB (P42062), and AppA (P42061) of *Bacillus subtilis*, VioC (AAP92493) of *Streptomyces vinaceus* and AesA (AIG74588), AesB (AIG74589), AesC (AIG74590) and AesD (AIG74591) of *A. japonicum* is shown beneath the corresponding gene encoding the similar gene product (See also Table 1).

Table 1 Similarity of the amino acid sequence level of identified proteins in *Streptomyces* sp. MA5143a to known enzymes with biochemically validated functions

Locus tag	Homolog enzymes	% Amino acid identity	ORF predicted function
MA_5143a_00506	VioC (accession number: AAP92493)	34	L-Arginine oxygenase
MA_5143a_00505	AesA (accession number: AIG74588)	49	Ornithine cyclodeaminase
MA_5143a_00504	AesB (accession number: AIG74589)	48	Pyridoxal-dependent decarboxylase
MA_5143a_00503	AesC (accession number: AIG74590)	53	*O*-Acetyl-L-serine sulfhydrylase
MA_5143a_00502	AesD (accession number: AIG74591)	49	Na^+-driven multidrug efflux pump
MA_5143a_00507	AppA (accession number: P42061)	27	ABC transporter substrate-binding protein
MA_5143a_00508	AppB (accession number: P42062)	30	ABC transporter permease
MA_5143a_00509	AppC (accession number: P42063)	29	ABC transporter permease
MA_5143a_00510	AppD (accession number: P42064)	37	ABC transporter ATP-binding protein

of the *edha*-operon in *Streptomyces* sp. MA5143a strongly supports our suggestion that these genes are responsible for directing the biosynthesis of EDHA.

Directly adjacent to the EDHA biosynthesis genes (MA_5143a_00506 to MA_5143a_00502), a further operon structure was found (MA_5143a_00507 to MA_5143a_00510). This putative operon encodes proteins that exhibit similarities to the AppA–D permease systems of *B. subtilis* (Table 1). This uptake system is also present in close proximity to the EDHA biosynthesis genes within the genomes of *S. avermitilis*, *S. scabies*, *C. pseudotuberculosis*, *C. ulcerans* and *N. brasiliensis* (Fig. 2). The App uptake system is utilized by *Bacillus subtilis* to import tetra- and pentapeptides into its cytoplasm.[65] Accordingly, it is possible that the uptake system encoded by MA_5143a_00507 to MA_5143a_00510 might participate in the reassociation of ion-bound EDHA with the cell surface.

Phylogeny and *in silico* DNA–DNA hybridization of *Streptomyces* sp. MA5143a

In order to allocate *Streptomyces* sp. MA5143a within this broadly branching genus and to determine the distribution of the EDHA cluster, we performed a Multi Locus Sequence Analysis (MLSA). Therefore, all types of strains with published genomes and 99% sequence identity of the 16S rRNA gene sequences to *Streptomyces* sp. MA5143a were considered. The MLSA including 50 conserved genes (Table S1, ESI†) showed that *Streptomyces* sp. MA5143a forms a monophyletic clade with *Streptomyces torulosus* NRRL B-3889, *Streptomyces neyagawaensis* NRRL B-3092 and *Streptomyces ossamyceticus* NRRL B-3822 (Fig. S1, ESI†). These four strains belong to a major clade also containing the plant pathogen *S. scabies*, a strain that was also identified as a carrier of the EDHA gene cluster by our computational screening. However, further investigations showed that the predicted EDHA gene cluster is also present within the genomes of strains only very distantly related to *Streptomyces* sp. MA5143a (*e.g. Streptomyces avermitilis* MA-4680, *Streptomyces griseoaurantiacus* M045 and *Streptomyces clavuligerus* ATCC 27064). This shows that this genetic feature is not exclusive to the *S. scabies* clade but rather a broadly distributed feature within the analysed *Streptomyces* branch. Finally, DNA–DNA hybridization values between *Streptomyces* sp. MA5143a and its closest relatives were calculated *in silico* in addition to the MLSA. The predicted DNA–DNA hybridization values between *Streptomyces* sp. MA5143a and its relatives were as follows: for *Streptomyces neyagawaensis* strain NRRL B-3092 45.20% ± 2.55, for *Streptomyces torulosus* strain NRRL B-3889 44.30% ± 2.55 and for *Streptomyces ossamyceticus* strain NRRL B-3822 46.20% ± 2.6. These values are lower than the threshold of 70% for relatedness between different species, indicating that *Streptomyces* sp. MA5143a is a yet undescribed species.

EDHA production profiling in *Streptomyces* sp. MA5143a and *Streptomyces scabies*

The original reports describing the structurally similar chelators EDHA and EDDS were both published in the *J. Antibiot.* in 1984.[31,32] While EDDS has received attention and has been subjected to various medically, chemically, environmentally and biologically focused studies in the past few decades, no research has been reported on EDHA. Thus, neither the biosynthesis genes, nor the environmental trigger, which induces the biosynthesis and the secretion of this unusual chelating compound, were previously described.

Our initial screening approach identified several actinobacterial genera with the predicted potential to produce EDHA. To prove the capability of these strains to produce EDHA, we chose *S. scabies* as a representative strain for laboratory experiments. As a control, the known producer *Streptomyces* sp. MA5143a was cultivated in parallel. The two strains were grown for 5 days in 100 mL of medium using 500 mL Erlenmeyer flasks. The flasks had been thoroughly treated according to Zwicker *et al.*[66] to guarantee the removal of trace element pollutants. As a cultivation medium, we used a modified version of the EDHA production medium described by Huang *et al.*[32] The calculated molecular weight of EDHA is 349.343 Da with a molecular formula of $C_{12}H_{23}N_5O_7$.[32] To detect EDHA production, we analyzed the culture supernatants of *Streptomyces* sp. MA5143a and *S. scabies* by LC-ESI-MS. The culture supernatants were scanned for the presence of an ion with a mass of $[M + H]^+ = m/z$ 350 representing the uncomplexed EDHA. Both the *Streptomyces* sp. MA5143a and the *S. scabies* supernatants contained a compound that exhibits this specific mass (Fig. 3). To confirm that this mass is the ionized EDHA, we supplemented 1 mL of the culture supernatant with 20 µL of a 100 mM Cu(II)SO_4 solution in order to generate Cu(II)–EDHA complexes prior to LC-ESI-MS analysis. The expected masses of the EDHA–^{63}Cu(II)-complex and the EDHA–^{65}Cu(II)-complex are $[M + H]^+ = m/z$ 411 and $[M + H]^+ = m/z$ 413, respectively. After copper treatment of the samples, the specific masses of $[M + H]^+ = m/z$ 411 and

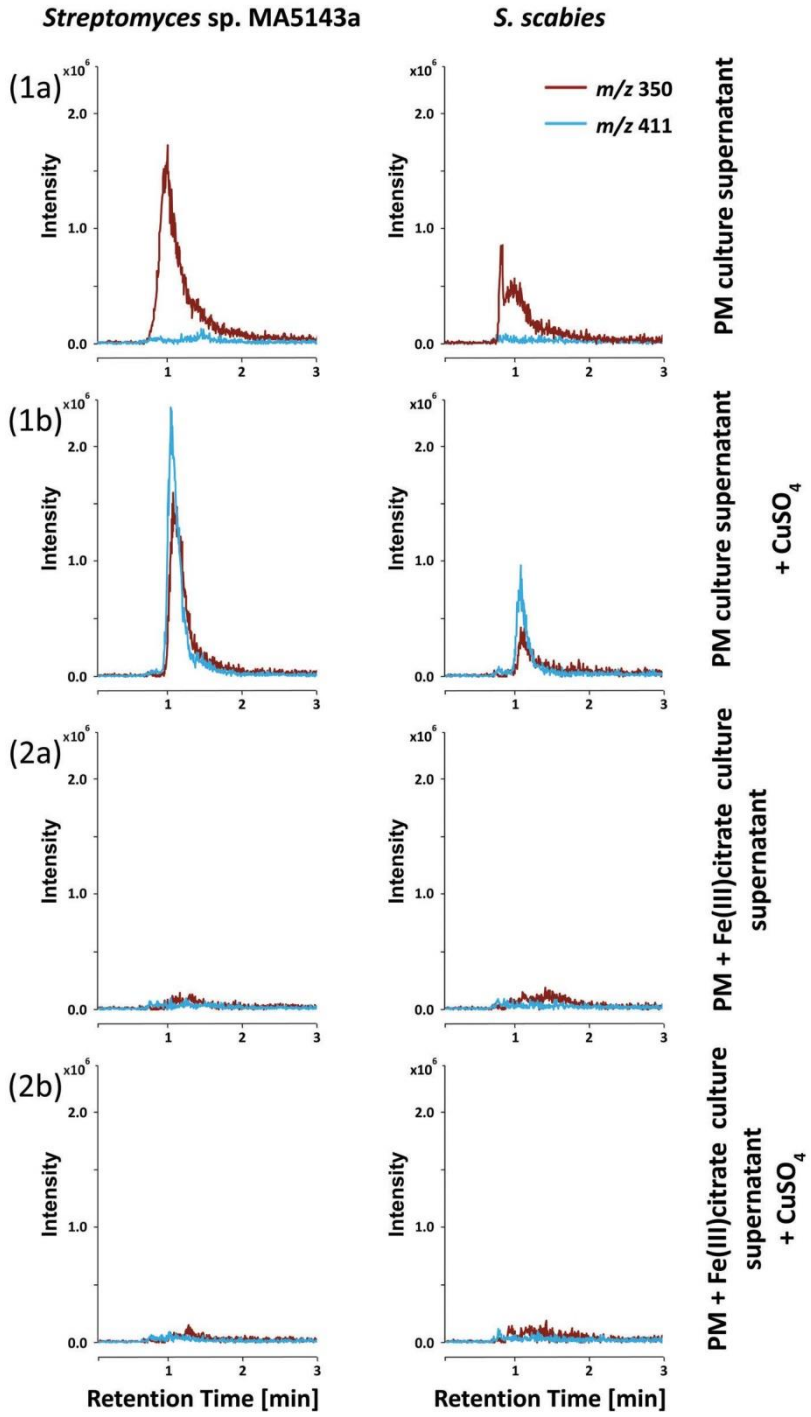

Fig. 3 LC-MS chromatograms of *Streptomyces* sp. MA5143a (left panel) and *S. scabies* (right panel) supernatants. Extracted ion chromatograms from positive mode LC-ESI-MS runs. Red lines correspond to $[M + H]^+ = m/z$ 350 of the uncomplexed EDHA; blue lines to $[M + H]^+ = m/z$ 411 of the EDHA–^{63}Cu-complex. Cultures grown without (1) or with (2) supplementation of 60 mg L^{-1} Fe(III)citrate. (a) Untreated supernatant samples and (b) after treatment with $CuSO_4$.

$[M + H]^+ = m/z$ 413 were detectable in addition to the mass of $[M + H]^+ = m/z$ 350 (Fig. 3 and 4). The relative peak heights of $[M + H]^+ = m/z$ 411 and $[M + H]^+ = m/z$ 413 reflect the natural isotope abundance of ^{63}Cu(II) and ^{65}Cu(II) (69.2% and 30.8%), respectively. The measured isotope pattern of the putative EDHA copper adduct was in accordance with the calculated isotope pattern for $C_{12}H_{22}N_5O_7Cu$ (Fig. S2, ESI†), which had been calculated using the software Universal Mass Calculator 3.7. This confirms the presence of Cu in the complex. Due to the limited mass resolution of the used ion trap, isotope peaks arising from $^{65}Cu/^{13}C_2/^{13}C^{15}N$ *etc.* could not be distinguished. Ions with the mass of $[M + H]^+ = m/z$ 411 and $[M + H]^+ = m/z$ 413 were not observed in the supernatants without supplementation of Cu(II)SO_4. This also proves that *S. scabies*, which carries the *edha*-operon in its genome, is indeed capable of producing this unusual chelating compound.

In silico prediction of metalloregulation

To date, iron responsively regulated siderophores are by far the main group of described microbial chelating compounds. In contrast, EDDS is synthesized by various *Amycolatopsis* strains as a specific response to zinc deficiency.[8] The structural similarity between EDHA and EDDS suggested that EDHA might also be a zincophore. To assess the specific low-metal ion stress, which induces the EDHA biosynthesis in *Streptomyces* sp. MA5143a, we initially conducted a computational approach. This follows the assumption that genes, which are transcriptionally regulated by protein sensing metals, exhibit a specific DNA binding motif for the corresponding regulator in their 5′ upstream regions. Therefore, we scanned the genome of *Streptomyces* sp. MA5143a for DNA binding motifs of biochemically well-characterized metalloregulatory proteins. The major bacterial regulator sensing intracellular zinc levels is the zinc uptake regulator Zur. Zur gets activated by its co-repressor zinc. In its zinc bound state, Zur represses genes encoding zinc uptake and zinc mobilization functions.[15] To detect putatively Zur regulated genes in *Streptomyces* sp. MA5143a, the published Zur binding motif TCATGAAAATCA TTTTCANNA[67] was used as the query to scan the *Streptomyces* sp. MA5143a genome by PatScanUI,[68] a tool to scan for patterns in DNA sequences. We allowed up to five mismatches and limited the search to within −200 bp of each predicted start codon. This analysis revealed Zur binding motifs upstream of an ABC uptake system exhibiting high similarity to ZnuABC of *Streptomyces coelicolor* and upstream of ribosomal proteins. These two systems were already described to be members of the Zur regulon in *S. coelicolor*.[67,69] However, no such motif was detectable upstream of MA_5143a_00506, suggesting a zinc independent production of EDHA in *Streptomyces* sp. MA5143a. Therefore, we extended the search for binding motifs of further metalloregulators. In high-GC Gram-positive bacteria, proteins of

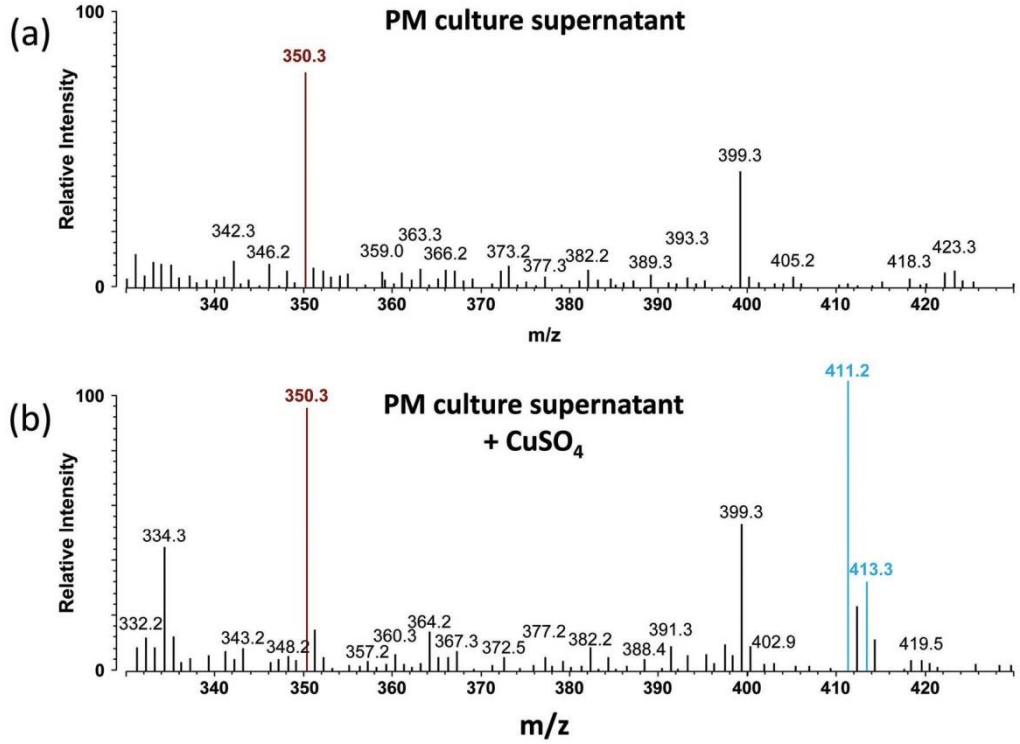

Fig. 4 Mass spectra (positive mode) measured for compounds eluting at 1.1 min from the *Streptomyces* sp. MA5143a supernatant. (a) Untreated supernatant samples and (b) after treatment with $CuSO_4$. Ions with the mass of $[M + H]^+ = m/z$ 350 representing the free EDHA are depicted in red. Ions with the mass of $[M + H]^+ = m/z$ 411 and $[M + H]^+ = m/z$ 413 representing the copper complexes of EDHA with ^{63}Cu(II) and ^{65}Cu(II) are highlighted in blue.

the IdeR protein family sense a surplus of free cytoplasmic ferrous ions and repress genes encoding iron uptake functions. The *Streptomyces* sp. MA5143a genome encodes a protein (MA_5143a_05034 = IdeR) that exhibits a nearly identical amino acid sequence (97% identity) to the iron responsive IdeR family protein DmdR1 of *S. coelicolor*.[70] To detect putatively IdeR regulated genes in *Streptomyces* sp. MA5143a, the published binding motif TWAGGTWAGSCTWACCTWA[71] was used as a reference according to the procedure described for the Zur binding motif scan. To increase the confidence level, only three mismatches were allowed. This resulted in the identification of 15 ORFs (Table S3, ESI†). These ORFs encode proteins that are annotated to be either directly involved in iron acquisition or have iron dependent catalytic activities (*e.g.* aconitase). Three predicted IdeR binding motifs are located within two discrete NIS gene clusters (upstream of MA_5143a_03356, MA_5143a_04108 and MA_5143a_04110). One of them exhibits an identical gene arrangement and high sequence similarity to the desferrioxamine gene cluster of *S. coelicolor* (Table S4, ESI†).[72] This suggests an extended metabolic repertoire of *Streptomyces* sp. MA5143a besides EDHA to respond to an environment providing insufficient metal ion bioavailability.

Our analysis also revealed the presence of a highly conserved motif upstream of the *vioC*-like gene MA_5143a_00506 (Table S3, ESI†), the first gene of the *edha*-operon. The presence of this motif in the non-coding 5′ upstream region of MA_5143a_00506 indicates that its iron-responsive transcription is under the control of IdeR. Furthermore, this also indicates the physiological function of EDHA as a siderophore for *Streptomyces* sp. MA5143a. To evaluate if this assumption is transferable to the other bacterial strains containing the EDHA biosynthesis genes, we extended our scan for IdeR binding motifs to their genomes. This revealed the presence of highly conserved IdeR binding motifs also upstream of the *vioC*-like genes in *S. scabies*, *S. avermitilis*, *C. pseudotuberculosis*, *C. ulcerans* and *N. brasiliensis* (Table S5, ESI†). Thus, analogous to *Streptomyces* sp. MA5143a, the EDHA biosynthesis in these strains is presumably also iron responsively regulated.

Iron responsive production and transcriptional profiling of EDHA biosynthesis

In order to confirm the predicted iron responsive production of EDHA in *Streptomyces* sp. MA5143a, the strain was grown in PM and in PM supplemented with either Fe(II)SO_4, Zn(II)SO_4, Ni(II)SO_4, Mn(II)SO_4, or Co(II)SO_4 (25 μM). In addition, a culture was grown in the presence of Fe(III)citrate (60 mg L^{-1}) to keep Fe(III) soluble in the aerobic environment and to avoid the formation of insoluble ferric ion complexes. The EDHA production profile was followed over a time range of 7 days with sampling after 2, 3, 4, 5 and 7 days of incubation. EDHA production was detectable in the presence of the divalent metal ions Zn(II), Ni(II), Mn(II) and Co(II). In contrast, there was a significant decrease in the production of EDHA in cultures supplemented with 25 μM of Fe(II)SO_4 and almost no production in the presence of Fe(III)citrate (Fig. 5). This iron-repressed biosynthesis profile of EDHA was also shown in *S. scabies* (Fig. 3). Like *Streptomyces* sp. MA5143a, *S. scabies* encodes a desferrioxamine type gene cluster.[73] The ability to produce desferrioxamine was proven in many *Streptomyces* species and is a rather general feature of this genus. The stability constants of iron–APCA complexes are quite low compared to classical siderophores of the hydroxamate and catecholate type.[3,74] This might indicate that EDHA is a second-line siderophore in these strains having a subordinate role in counteracting iron limitation. In addition to the metal ion-responsive production profile of EDHA, the transcriptional profile of the corresponding biosynthesis genes (MA_5143a_00506 to MA_5143a_00502) was analyzed in *Streptomyces* sp. MA5143a. In order to perform a qualitative reverse transcription PCR (RT-PCR), we harvested cells after 72 h of cultivation in PM and in PM supplemented with 60 mg L^{-1} Fe(III)citrate or 25 μM Zn(II)SO_4,

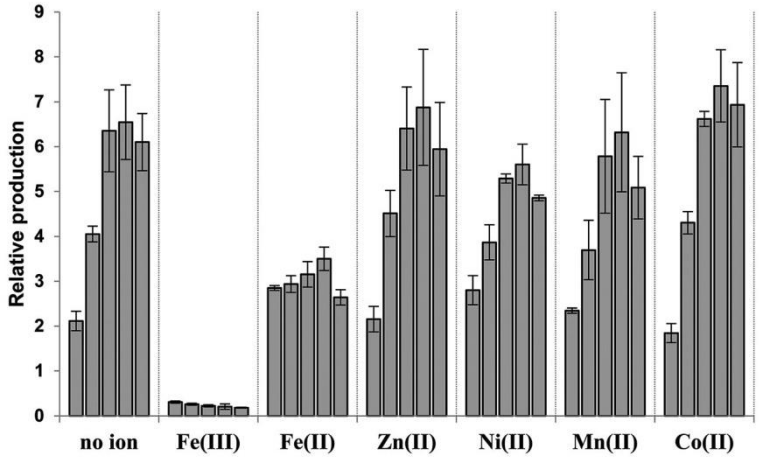

Fig. 5 Metal ion dependent EDHA production in *Streptomyces* sp. MA5143a. EDHA production was followed over five days of cultivation by LC-ESI-MS. *Streptomyces* sp. MA5143a was grown in PM without the addition of any ion, in the presence of 25 μM $FeSO_4$, $ZnSO_4$, $NiSO_4$, $MnSO_4$ or $CoSO_4$ and 60 mg L^{-1} Fe(III)citrate. The samples were taken after 2, 3, 4, 5 and 7 days of incubation. Experiments were performed in triplicate (n = 3).

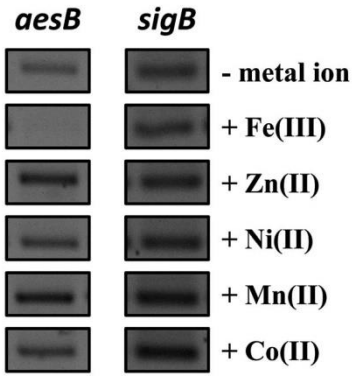

Fig. 6 Trace metal-dependent transcriptional pattern of *aesB* (MA_5143a_00504). The gene names are indicated at the top of the gels. *Streptomyces* sp. MA5143a was grown in PM without the addition of any ion, in the presence of Fe(III)citrate (60 mg L^{-1}), $ZnSO_4$, $NiSO_4$, $MnSO_4$ or $CoSO_4$ (25 μM). The samples were taken after 72 h of incubation to isolate RNA. *sigB* was used as a housekeeping gene to normalize the cDNA.

Ni(II)SO_4, Mn(II)SO_4 or Co(II)SO_4 and prepared cDNA samples from isolated RNA. The presence of MA_5143a_00506 to MA_5143a_00502 transcripts was monitored with specific primer pairs (Table S2, ESI†) and *sigB* was used as a housekeeping gene to normalize the cDNA levels. For reasons of simplicity, we only illustrate *aesB* (MA_5143a_00504) while the transcriptional profiles of the other genes are depicted in the ESI.† The RT-PCR experiments showed that all five analyzed genes (MA_5143a_00506 to MA_5143a_00502) were expressed in the presence of Zn(II), Ni(II), Mn(II) and Co(II) (Fig. 6 and Fig. S3, ESI†). However, there was no transcript detectable in RNA samples taken from iron replete conditions. This strict iron-repressed transcriptional pattern of the EDHA operon is coincident with the observed EDHA production pattern and is further evidence that these genes indeed encode EDHA synthesis.

The two naturally occurring APCAs, EDHA and EDDS, were identified during screening programs for enzyme inhibitors where their inhibitory effects arise from their capability to efficiently complex zinc ions.[31,32] Our finding that EDHA is an iron-responsively produced compound and its suggested biological role as a siderophore is fundamentally different to the zinc regulated biosynthesis of EDDS.[8] Although isolated by its capability to complex zinc and shown to be zinc responsively produced, EDDS exhibits preferred binding towards the metal ions copper and iron *in vitro*.[74] The specificity of ionophores in biological systems is therefore rather reflected by metal responsive biosynthesis and specific reassociation of the once formed metal–ionophore complex than by the simple ability to form a complex.

Conclusions

Naturally occurring aminopolycarboxylic acids (APCAs) are an underrepresented class of microbial chelating compounds. In this work, we developed and implemented a nucleotide-based screening approach to detect and to prioritize genetic regions with potential APCA biosynthesis function. Thereby, we identified the genes directing the synthesis of the unusual zwitterionic siderophore ethylenediaminesuccinic acid hydroxyarginine (EDHA) in a panel of well-known and phylogenetically distinct actinomycetes. The EDHA biosynthesis genes were detected, *e.g.*, in the genome of *Streptomyces avermitilis*, an organism known for its potential to produce valuable secondary metabolites, and in several actinomycetes characterized by a pathogenic lifestyle (*Streptomyces scabies* (plant), *Corynebacterium pseudotuberculosis* (animal), *Corynebacterium ulcerans* and *Nocardia brasiliensis* (human)). To investigate the physiological function of EDHA, we analyzed its production profile and the transcriptional pattern of its biosynthesis genes with respect to various low-metal stress conditions. These comparative studies show an iron-responsive EDHA biosynthesis, which strongly indicates a role of EDHA in iron acquisition.

The here presented workflow provides an easy and generally applicable strategy to detect genetic regions directing the synthesis of APCAs in nucleotide databases and can therefore help to extend our knowledge on the chemical diversity and physiological relevance of this class of natural APCAs.

Conflicts of interest

There are no conflicts to declare.

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References

1 U. E. Schaible and S. H. Kaufmann, Iron and microbial infection, *Nat. Rev. Microbiol.*, 2004, **2**, 946–953.
2 B. J. Cherayil, The role of iron in the immune response to bacterial infection, *Immunol. Res.*, 2011, **50**, 1–9.
3 M. Miethke and M. A. Marahiel, Siderophore-based iron acquisition and pathogen control, *Microbiol. Mol. Biol. Rev.*, 2007, **71**, 413–451.
4 M. E. Helsel and K. J. Franz, Pharmacological activity of metal binding agents that alter copper bioavailability, *Dalton Trans.*, 2015, **44**, 8760–8770.
5 R. C. Hider and X. L. Kong, Chemistry and biology of siderophores, *Nat. Prod. Rep.*, 2010, **27**, 637–657.
6 H. J. Kim, D. W. Graham, A. A. DiSpirito, M. A. Alterman, N. Galeva, C. K. Larive, D. Asunskis and P. M. Sherwood, Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria, *Science*, 2004, **305**, 1612–1615.
7 D. Kallifidas, B. Pascoe, G. A. Owen, C. M. Strain-Damerell, H. J. Hong and M. S. Paget, The zinc-responsive regulator

Zur controls expression of the coelibactin gene cluster in *Streptomyces coelicolor*, *J. Bacteriol.*, 2010, **192**, 608–611.

8 M. Spohn, W. Wohlleben and E. Stegmann, Elucidation of the zinc-dependent regulation in *Amycolatopsis japonicum* enabled the identification of the ethylenediamine-disuccinate ([*S*,*S*]-EDDS) genes, *Environ. Microbiol.*, 2016, **18**, 1249–1263.

9 M. Miethke, Molecular strategies of microbial iron assimilation: from high-affinity complexes to cofactor assembly systems, *Metallomics*, 2013, **5**, 15–28.

10 J. H. Crosa and C. T. Walsh, Genetics and assembly line enzymology of siderophore biosynthesis in bacteria, *Microbiol. Mol. Biol. Rev.*, 2002, **66**, 223–249.

11 G. L. Challis, A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases, *ChemBioChem*, 2005, **6**, 601–611.

12 M. Kasahara and Y. Anraku, Succinate- and NADH oxidase systems of *Escherichia coli* membrane vesicles. Mechanism of selective inhibition of the systems by zinc ions, *J. Biochem.*, 1974, **76**, 967–976.

13 A. Aagaard and P. Brzezinski, Zinc ions inhibit oxidation of cytochrome *C* oxidase by oxygen, *FEBS Lett.*, 2001, **494**, 157–160.

14 S. J. Dixon and B. R. Stockwell, The role of iron and reactive oxygen species in cell death, *Nat. Chem. Biol.*, 2014, **10**, 9–17.

15 M. F. Fillat, The FUR (ferric uptake regulator) superfamily: diversity and versatility of key transcriptional regulators, *Arch. Biochem. Biophys.*, 2014, **546**, 41–52.

16 A. T. Merchant and G. A. Spatafora, A role for the DtxR family of metalloregulators in Gram-positive pathogenesis, *Mol. Oral Microbiol.*, 2014, **29**, 1–10.

17 K. Hantke, Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant, *Mol. Gen. Genet.*, 1981, **182**, 288–292.

18 A. Bagg and J. B. Neilands, Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*, *Biochemistry*, 1987, **26**, 5471–5477.

19 K. Hantke, Iron and metal regulation in bacteria, *Curr. Opin. Microbiol.*, 2001, **4**, 172–177.

20 E. Pohl, R. K. Holmes and W. G. J. Hol, Crystal structure of the iron-dependent regulator (IdeR) from *Mycobacterium tuberculosis* shows both metal binding sites fully occupied, *J. Mol. Biol.*, 1999, **285**, 1145–1156.

21 E. Pohl, R. K. Holmes and W. G. J. Hol, Crystal structure of a cobalt-activated diphtheria toxin repressor-DNA complex reveals a metal-binding SH3-like domain, *J. Mol. Biol.*, 1999, **292**, 653–667.

22 K. Günter-Seeboth and T. Schupp, Cloning and sequence-analysis of the *Corynebacterium diphtheriae dtxR* homolog from *Streptomyces lividans* and *S. pilosus* encoding a putative iron repressor protein, *Gene*, 1995, **166**, 117–119.

23 S. Tunca, C. Barreiro, A. Sola-Landa, J. J. R. Coque and J. F. Martin, Transcriptional regulation of the desferrioxamine gene cluster of *Streptomyces coelicolor* is mediated by binding of DmdR1 to an iron box in the promoter of the *desA* gene, *FEBS J.*, 2007, **274**, 1110–1122.

24 J. W. Lee and J. D. Helmann, Functional specialization within the Fur family of metalloregulators, *Biometals*, 2007, **20**, 485–499.

25 N. Mobarra, M. Shanaki, H. Ehteram, H. Nasiri, M. Sahmani, M. Saeidi, M. Goudarzi, H. Pourkarim and M. Azad, A Review on Iron Chelators in Treatment of Iron Overload Syndromes, *Int. J. Hematol. Oncol. Stem. Cell. Res.*, 2016, **10**, 239–247.

26 L. Chen, T. Liu and C. Ma, Metal complexation and biodegradation of EDTA and S,S-EDDS: a density functional theory study, *J. Phys. Chem. A*, 2010, **114**, 443–454.

27 G. M. St. George and D. A. Wilson, *US Pat.*, 3158635, 1964.

28 J. A. Neal and N. J. Rose, Stereospecific ligands and their complexes. I. A cobalt(III) complex of ethylenediaminedisuccinic acid, *Inorg. Chem.*, 1968, **7**, 2405–2412.

29 D. Schowanek, T. C. Feijtel, C. M. Perkins, F. A. Hartman, T. W. Federle and R. J. Larson, Biodegradation of [*S*,*S*], [*R*,*R*] and mixed stereoisomers of ethylene diamine disuccinic acid (EDDS), a transition metal chelator, *Chemosphere*, 1997, **34**, 2375–2391.

30 R. Takahashi, N. Fujimoto, M. Suzuki and T. Endo, Biodegradabilities of ethylenediamine-*N*,*N*′-disuccinic acid (EDDS) and other chelating agents, *Biosci., Biotechnol., Biochem.*, 1997, **61**, 1957–1959.

31 T. Nishikiori, A. Okuyama, H. Naganawa, T. Takita, M. Hamada, T. Takeuchi, T. Aoyagi and H. Umezawa, Production by actinomycetes of (*S*,*S*)-*N*,*N*′-ethylenediamine-disuccinic acid, an inhibitor of phospholipase C, *J. Antibiot.*, 1984, **37**, 426–427.

32 L. Huang, G. Rowin, J. Dunn, R. Sykes, R. Dobna, B. A. Mayles, D. M. Gross and R. W. Burg, Discovery, purification and characterization of the angiotensin converting enzyme-inhibitor, L-681,176, produced by *Streptomyces* sp. MA 5143a, *J. Antibiot.*, 1984, **37**, 462–465.

33 O. D. Hensens and J. M. Liesch, Structure elucidation of angiotensin converting enzyme inhibitor L-681,176 from *Streptomyces* sp. MA 5143a, *J. Antibiot.*, 1984, **37**, 466–468.

34 M. Spohn, N. Kirchner, A. Kulik, A. Jochim, F. Wolf, P. Münzer, O. Borst, H. Gross, W. Wohlleben and E. Stegmann, Overproduction of ristomycin A by activation of a silent gene cluster in *Amycolatopsis japonicum* MG417-CF17, *Antimicrob. Agents Chemother.*, 2014, **58**, 6185–6196.

35 T. Seemann, Prokka: rapid prokaryotic genome annotation, *Bioinformatics*, 2014, **30**, 2068–2069.

36 F. Meyer, A. Goesmann, A. C. McHardy, D. Bartels, T. Bekel, J. Clausen, J. Kalinowski, B. Linke, O. Rupp, R. Giegerich and A. Puhler, GenDB–an open source genome annotation system for prokaryote genomes, *Nucleic Acids Res.*, 2003, **31**, 2187–2195.

37 S. F. Altschul, W. Gish, W. Miller, E. W. Myers and D. J. Lipman, Basic local alignment search tool, *J. Mol. Biol.*, 1990, **215**, 403–410.

38 N. A. O'Leary, M. W. Wright, J. R. Brister, S. Ciufo, D. Haddad, R. McVeigh, B. Rajput, B. Robbertse, B. Smith-White, D. Ako-Adjei, A. Astashyn, A. Badretdin, Y. Bao, O. Blinkova, V. Brover, V. Chetvernin, J. Choi, E. Cox, O. Ermolaeva, C. M. Farrell, T. Goldfarb, T. Gupta, D. Haft, E. Hatcher, W. Hlavina, V. S. Joardar, V. K. Kodali, W. Li, D. Maglott, P. Masterson, K. M. McGarvey, M. R. Murphy,

K. O'Neill, S. Pujar, S. H. Rangwala, D. Rausch, L. D. Riddick, C. Schoch, A. Shkeda, S. S. Storz, H. Sun, F. Thibaud-Nissen, I. Tolstoy, R. E. Tully, A. R. Vatsan, C. Wallin, D. Webb, W. Wu, M. J. Landrum, A. Kimchi, T. Tatusova, M. DiCuccio, P. Kitts, T. D. Murphy and K. D. Pruitt, Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation, *Nucleic Acids Res.*, 2016, **44**, D733–D745.

39 D. H. Haft, J. D. Selengut and O. White, The TIGRFAMs database of protein families, *Nucleic Acids Res.*, 2003, **31**, 371–373.

40 R. D. Finn, J. Clements and S. R. Eddy, HMMER web server: interactive sequence similarity searching, *Nucleic Acids Res.*, 2011, **39**, W29–W37.

41 K. Katoh and D. M. Standley, MAFFT multiple sequence alignment software version 7: improvements in performance and usability, *Mol. Biol. Evol.*, 2013, **30**, 772–780.

42 M. Suyama, D. Torrents and P. Bork, PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments, *Nucleic Acids Res.*, 2006, **34**, W609–W612.

43 S. Capella-Gutierrez, J. M. Silla-Martinez and T. Gabaldon, trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses, *Bioinformatics*, 2009, **25**, 1972–1973.

44 J. Huerta-Cepas, J. Dopazo and T. Gabaldon, ETE: a python Environment for Tree Exploration, *BMC Bioinf.*, 2010, **11**, 24.

45 A. F. Auch, H. P. Klenk and M. Goker, Standard operating procedure for calculating genome-to-genome distances based on high-scoring segment pairs, *Stand. Genomic Sci.*, 2010, **2**, 142–148.

46 A. F. Auch, M. von Jan, H. P. Klenk and M. Goker, Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison, *Stand. Genomic Sci.*, 2010, **2**, 117–134.

47 J. P. Meier-Kolthoff, A. F. Auch, H. P. Klenk and M. Goker, Genome sequence-based species delimitation with confidence intervals and improved distance functions, *BMC Bioinf.*, 2013, **14**, 60.

48 M. Bucheli-Witschel and T. Egli, Environmental fate and microbial degradation of aminopolycarboxylic acids, *FEMS Microbiol. Rev.*, 2001, **25**, 69–106.

49 M. H. Medema, E. Takano and R. Breitling, Detecting sequence homology at the gene cluster level with MultiGeneBlast, *Mol. Biol. Evol.*, 2013, **30**, 1218–1223.

50 M. G. Thomas, Y. A. Chan and S. G. Ozanick, Deciphering tuberactinomycin biosynthesis: Isolation, sequencing, and annotation of the viomycin biosynthetic gene cluster, *Antimicrob. Agents Chemother.*, 2003, **47**, 2823–2830.

51 X. H. Yin and T. M. Zabriskie, VioC is a non-heme iron, alpha-ketoglutarate-dependent oxygenase that catalyzes the formation of 3*S*-hydroxy-L-arginine during viomycin biosynthesis, *ChemBioChem*, 2004, **5**, 1274–1277.

52 B. Li, T. Lowe-Power, S. Kurihara, S. Gonzales, J. Naidoo, J. B. MacMillan, C. Allen and A. J. Michael, Functional Identification of Putrescine C- and N-Hydroxylases, *ACS Chem. Biol.*, 2016, **11**, 2782–2789.

53 D. R. D. Bignell, R. F. Seipke, J. C. Huguet-Tapia, A. H. Chambers, R. J. Parry and R. Loria, *Streptomyces scabies* 87-22 contains a coronafacic acid-like biosynthetic cluster that contributes to plant-microbe interactions, *Mol. Plant-Microbe Interact.*, 2010, **23**, 161–175.

54 F. A. Dorella, A. Gala-Garcia, A. C. Pinto, B. Sarrouh, C. A. Antunes, D. Ribeiro, F. F. Aburjaile, K. K. Fiaux, L. C. Guimaraes, N. Seyffert, R. A. El-Aouar, R. Silva, S. S. Hassan, T. L. Castro, W. S. Marques, R. Ramos, A. Carneiro, P. de Sa, A. Miyoshi, V. Azevedo and A. Silva, Progression of OMICS methodologies for understanding the pathogenicity of *Corynebacterium pseudotuberculosis*: the Brazilian experience, *Comput. Struct. Biotechnol. J.*, 2013, **6**, e201303013.

55 S. Omura, H. Ikeda, J. Ishikawa, A. Hanamoto, C. Takahashi, M. Shinose, Y. Takahashi, H. Horikawa, H. Nakazawa, T. Osonoe, H. Kikuchi, T. Shiba, Y. Sakaki and M. Hattori, Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 12215–12220.

56 H. Ikeda, J. Ishikawa, A. Hanamoto, M. Shinose, H. Kikuchi, T. Shiba, Y. Sakaki, M. Hattori and S. Omura, Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*, *Nat. Biotechnol.*, 2003, **21**, 526–531.

57 H. Ikeda, K. Shin-ya and S. Omura, Genome mining of the Streptomyces avermitilis genome and development of genome-minimized hosts for heterologous expression of biosynthetic gene clusters, *J. Ind. Microbiol. Biotechnol.*, 2014, **41**, 233–250.

58 M. H. Medema and M. A. Fischbach, Computational approaches to natural product discovery, *Nat. Chem. Biol.*, 2015, **11**, 639–648.

59 M. J. Smith, J. N. Shoolery, B. Schwyn, I. Holden and J. B. Neilands, Rhizobactin, a structurally novel siderophore from *Rhizobium meliloti*, *J. Am. Chem. Soc.*, 1985, **107**, 1739–1743.

60 H. Drechsel, S. Freund, G. Nicholson, H. Haag, O. Jung, H. Zahner and G. Jung, Purification and Chemical Characterization of Staphyloferrin-B, a Hydrophilic Siderophore from Staphylococci, *Biometals*, 1993, **6**, 185–192.

61 H. Umezawa, T. Aoyagi, K. Ogawa, H. Iinuma, H. Naganawa, M. Hamada and T. Takeuchi, Histargin, a new inhibitor of carboxypeptidase B, produced by Actinomycetes, *J. Antibiot.*, 1984, **37**, 1088–1090.

62 K. Ogawa, H. Naganawa, H. Iinuma, T. Aoyagi and H. Umezawa, The structure of histargin, *J. Antibiot.*, 1984, **37**, 984–987.

63 E. Hough, L. K. Hansen, B. Birknes, K. Jynge, S. Hansen, A. Hordvik, C. Little, E. Dodson and Z. Derewenda, High-resolution (1.5 A) crystal structure of phospholipase C from *Bacillus cereus*, *Nature*, 1989, **338**, 357–360.

64 P. Bünning and J. F. Riordan, The functional-role of zinc in angiotensin converting enzyme - implications for the enzyme mechanism, *J. Inorg. Biochem.*, 1985, **24**, 183–198.

65 A. Koide and J. A. Hoch, Identification of a second oligopeptide transport system in *Bacillus subtilis* and determination of its role in sporulation, *Mol. Microbiol.*, 1994, **13**, 417–426.

66 N. Zwicker, U. Theobald, H. Zähner and H. P. Fiedler, Optimization of fermentation conditions for the production of ethylene-diamine-disuccinic acid by *Amycolatopsis orientalis*, *J. Ind. Microbiol. Biotechnol.*, 1997, **19**, 280–285.

67 G. A. Owen, B. Pascoe, D. Kallifidas and M. S. Paget, Zinc-responsive regulation of alternative ribosomal protein genes in *Streptomyces coelicolor* involves Zur and σR, *J. Bacteriol.*, 2007, **189**, 4078–4086.

68 M. Dsouza, N. Larsen and R. Overbeek, Searching for patterns in genomic data, *Trends Genet.*, 1997, **13**, 497–498.

69 J. H. Shin, S. Y. Oh, S. J. Kim and J. H. Roe, The zinc-responsive regulator Zur controls a zinc uptake system and some ribosomal proteins in *Streptomyces coelicolor* A3(2), *J. Bacteriol.*, 2007, **189**, 4070–4077.

70 F. J. Flores and J. F. Martin, Iron-regulatory proteins DmdR1 and DmdR2 of *Streptomyces coelicolor* form two different DNA-protein complexes with iron boxes, *Biochem. J.*, 2004, **380**, 497–503.

71 B. Gold, G. M. Rodriguez, S. A. E. Marras, M. Pentecost and I. Smith, The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages, *Mol. Microbiol.*, 2001, **42**, 851–865.

72 F. Barona-Gomez, U. Wong and A. E. Giannakopulos, P. J. Derrick and G. L. Challis, Identification of a cluster of genes that directs desferrioxamine biosynthesis in *Streptomyces coelicolor* M145, *J. Am. Chem. Soc.*, 2004, **126**, 16282–16283.

73 R. F. Seipke, L. Song, J. Bicz, P. Laskaris, A. M. Yaxley, G. L. Challis and R. Loria, The plant pathogen *Streptomyces scabies* 87-22 has a functional pyochelin biosynthetic pathway that is regulated by TetR- and AfsR-family proteins, *Microbiology*, 2011, **157**, 2681–2693.

74 D. Kołodyńska, in *Expanding issues in desalination*, ed. R. Y. Ning, InTech, Rijeka, Croatia, 3rd edn, 2011, ch. 17, pp. 339–370, DOI: 10.5772/826.

5.2 Publication 2

Metabolic engineering of *Amycolatopsis japonicum* for optimized production of [*S,S*]-EDDS, a biodegradable chelator

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Metabolic engineering of *Amycolatopsis japonicum* for optimized production of [*S,S*]-EDDS, a biodegradable chelator

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ABSTRACT

The actinomycete *Amycolatopsis japonicum* is the producer of the chelating compound [*S,S*]-ethylenediamine-disuccinc acid (EDDS). [*S,S*]-EDDS is an isomer of ethylenediamine-tetraacetic acid (EDTA), an economically important chelating compound that suffers from an extremely poor degradability. Frequent use of the persistent EDTA in various industrial and domestic applications has caused an accumulation of EDTA in soil as well as in aqueous environments. As a consequence, EDTA is the highest concentrated anthropogenic compound present in water reservoirs. The [*S,S*]-form of EDDS has chelating properties similar to EDTA, however, in contrast to EDTA it is readily biodegradable. In order to compete with the cost-effective chemical synthesis of EDTA, we aimed to optimize the biotechnological production of [*S,S*]-EDDS in *A. japonicum* by using metabolic engineering approaches. Firstly, we integrated several copies of the [*S,S*]-EDDS biosynthetic genes into the chromosome of *A. japonicum* and replaced the native zinc responsive promoter with the strong synthetic constitutive promoter *SP44**. Secondly, we increased the supply of *O*-phospho-serine, the direct precursor of [*S,S*]-EDDS. The combination of these approaches together with the optimized fermentation process led to a significant improvement in [*S,S*]-EDDS up to 9.8 g/L with a production rate of 4.3 mg/h/g DCW.

1. Introduction

[*S,S*]-Ethylenediamine-disuccinic acid (EDDS), produced by the actinomycete *Amycolatopsis japonicum* MG417-CF17, is an aminopolycarboxylic acid discovered during a screening for phospholipase C inhibitors (Nishikiori et al., 1984). It exhibited a positive reaction by complexing zinc ions, which are essential cofactors of this enzyme (Hough et al., 1989). In principle, [*S,S*]-EDDS forms hexadentate complexes with bivalent metal ions (Chen et al., 2009). It is an isomer of the well-known synthetic complexing agent ethylenediamine-tetraacetic acid (EDTA) (Fig. 1). EDTA is used as a chelator in a wide range of applications with a volume of > 100,000 tons per year, e.g. in the textile, pulp and paper industry, in agriculture or in cosmetics, and it is especially an important ingredient in industrial or household cleaners. It enhances the cleaning effect and prevents the precipitation of metals, which can cause encrustation. However, EDTA is not retained or degraded by conventional wastewater treatment and is therefore released in high amounts into the aquatic environment where it is hardly degradable. The environmental persistence of EDTA depends in particular on the stability of the complex formed with metals, with copper and iron being the most stable ones. In laboratory experiments, in which EDTA was applied to different types of soil, only 14% degradation was observed after 20 days (Wen et al., 2009). In plant experiments with high EDTA concentrations, however, no EDTA degradation at all occurred after 40 days (Meers et al., 2005).

The metal-binding properties of [*S,S*]-EDDS are similar to those of

Abbreviations: ethylenediamine-disuccinic acid, EDDS; ethylenediamine-tetraacetic acid, EDTA; EDDS biosynthetic genes, *aesA-D*; overproducer, OP; zinc uptake regulator, Zur; zinc regulated producer, ZRP; β-glucuronidase, GusA

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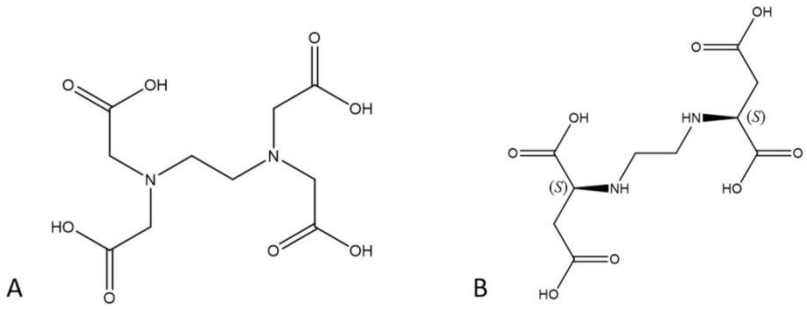

Fig. 1. Structure of the synthetic chelator EDTA (A) and the biodegradable chelator [*S,S*]-EDDS (B).

EDTA, with the benefit that its high biodegradability significantly reduces the environmental risk compared to EDTA. It is noteworthy that only the [*S,S*]-isomer of EDDS is readily biodegradable, but not the [*R,R*]-isomer and the meso-forms. Following an initial lag period of 7-11 days, [*S,S*]-EDDS is completely degraded in different types of soil with a half-life range of 3.8–7.5 days (Meers et al., 2005; Tandy et al., 2006).

The advantages of [*S,S*]-EDDS have already been demonstrated for several applications: In agriculture, for example, [*S,S*]-EDDS can be applied as fertilizer, since plants treated with [*S,S*]-EDDS-iron complexes took up as much iron as plants treated with EDTA-iron complexes. Thus [*S,S*]-EDDS-iron complexes were as effective as EDTA-iron complexes in preventing chlorosis and improving soybean growth (López-Rayo et al., 2019). Additionally, [*S,S*]-EDDS was more effective than EDTA in pot experiments in increasing the concentration of Cu and Zn in corn and beans. Compared to EDTA [*S,S*]-EDDS increased soluble Cu and Zn concentrations by a factor of about 192 and 8, respectively, especially in the first days after treatment (Luo et al., 2005). Furthermore, the uptake of [*S,S*]-EDDS mobilized metal ions by plants is beneficial for the purification of contaminated soils (Hauser et al., 2005; Meers et al., 2008). The mobilized heavy metals are taken up by the plants, which can subsequently be harvested and incinerated to ensure safe disposal of the heavy metals.

Another potential application for [*S,S*]-EDDS could be the substitution of EDTA in pharmaceuticals. EDTA has synergistic effects on many antibiotics and increases their uptake and/or their efficacy (Buckley et al., 2013; Farca et al., 1997; Lambert et al., 2004; Wooley et al., 1984, 2004). A gentamicin/EDTA lock therapy is even able to eradicate a gentamicin-resistant *Staphylococcus aureus* in catheter-associated biofilms (Chauhan et al., 2012).

The chemical synthesis for [*S,S*]-EDDS has been developed in the 1960s. The first established EDDS process was based on maleic acid and ethylenediamine (Barbier et al., 1963), leading to the formation of a racemic mixture of [*S,S*]-, [*R,R*]- and [*meso*]-EDDS from which [*S,S*]-EDDS had to be laboriously separated. The most conventional chemical route for the production of [*S,S*]-EDDS today is the alkylation of ethylenedibromide with L-aspartic acid (US Patent 3158635), a stereospecific reaction forming exclusively the [*S,S*]-EDDS isomer. However, the substrate ethylenedibromide is toxic and carcinogenic.

Advantageously, *A. japonicum* MG417-CF17 produces exclusively the [*S,S*]-isomer of EDDS. A characteristic of [*S,S*]-EDDS production in *A. japonicum*, that interferes with biotechnological [*S,S*]-EDDS production, is its strict repression by traces of zinc (< 2 µmol/L) (Spohn et al., 2016; Zwicker et al., 1997), a concentration occurring ubiquitously in the steel/glass fermenters as well as in the standard media components. Our recent identification of the [*S,S*]-EDDS biosynthesis genes *aesA-D* and the gene encoding the global zinc uptake regulator Zur in *A. japonicum* enabled the construction of the first zinc deregulated [*S,S*]-EDDS production strain *A. japonicum* Δ*zur* (Spohn et al., 2016; Stegmann et al., 2016). This mutant, which is able to produce [*S,S*]-EDDS even in the presence of high zinc concentrations, presented the first step towards the biotechnological production of [*S,S*]-EDDS. However, *A. japonicum* Δ*zur* has the disadvantage of slow growth and lack of spore formation, phenotypes which resulted presumable from the deletion of *zur*. Drastic effects caused by the *zur* deletion were also described for *S. coelicolor* where the primary metabolism, the ribosomal activity and spore formation were affected (Shin et al., 2007; Kallifidas et al., 2010). In order to optimize the [*S,S*]-EDDS production and to overcome the zinc regulation of the EDDS biosynthesis without interfering with the zinc response of the strain, we applied metabolic engineering approaches including modification of the transcription of the biosynthetic genes, the exchange of the zinc regulated promoter by a constitutive one, and the optimization of the precursor supply.

2. Material and methods

2.1. Strains, plasmids and oligonucleotides

All strains used in this study are listed in Table S1, plasmids and oligonucleotides are listed in Table S2 and Table S4.

2.2. Media and culture conditions

Escherichia coli was grown in Luria Broth (LB) medium at 37 °C with the respective antibiotics (Table S4) to maintain plasmids. *A. japonicum* strains were cultivated and manipulated as described in Stegmann et al. (2001) and Spohn et al. (2014). For measurement of the [*S,S*]-EDDS production the strains were cultured in 100 ml volume in EDTA-treated flasks according to Zwicker et al. (1997). For production, strains were grown for 120 h in SM medium (25 g/L glycerol, 1.2 g/L $MgSO_4$ x 7 H_2O, 60 mg/L ferric(III)citrate, 8 g/L KH_2PO_4, 12 g/L Na_2HPO_4 x 2 H_2O and 11.3 g sodium glutamate monohydrate). To suppress [*S,S*]-EDDS production in the wild type and to check for zinc deregulated [*S,S*]-EDDS production in recombinant strains, the SM medium was supplemented with zinc sulphate to a final concentration of 6 µM. For the seed-culture the strains were grown in a complex culture medium consisting of 20 g/L glycerol and 20 g/L soy flour at pH 7.5. Seeding cultures were supplemented with the respective antibiotics (Table S3) to maintain the integrative plasmids. After 48 h of cultivation, 5 ml of seed culture were used to inoculate 100 ml of the main culture. All liquid cultures of *A. japonicum* were grown on a rotary shaker (120 rpm) at 27 °C.

2.3. Construction of the overexpression plasmid pMS_aesA-D

For the construction of the overexpression plasmid pMS_*aesA-D* the plasmid pSET-*aesA-D* (Spohn et al., 2016), which already contained the [*S,S*]-EDDS biosynthesis genes *aesA-D,* was cut with the restriction enzymes VspI and HindIII. The 6698 bp fragment containing the genes *aesA-D* was purified using a gel extraction kit (QIAGEN, Hilden,

Germany) and ligated with the plasmid pIJ10257 (cut with NdeI and HindIII) resulting in the recombinant plasmid pMS_*aesA-D*. The plasmid pSET-*aesA-D* was transferred into *A. japonicum* WT via conjugation where it integrated via the ΦC31 *att* site into the chromosome, resulting in the strain *A. japonicum* ZRP1. The plasmid pMS_*aesA-D* was transferred to *A. japonicum* ZRP1 via *E. coli* conjugation, where it integrated via the ΦBT1 *att* site into the chromosome resulting in the strain *A. japonicum* ZRP2.

2.4. Construction of promoter probe plasmids

For the construction of the promoter probe plasmids the *SP44** promoter (Bai et al., 2015), the *actII-orf4*p promoter and the *ermE*p* promoter were amplified via PCR with the primer pairs given in Table S4. For the *SP44** promoter the plasmid pUC57_*SP44** (see 2.5) was used as template DNA, for the *ermE*p* promoter the plasmid pRM4 (Menges et al., 2007) and for the *actII-orf4*p promoter genomic DNA of *Streptomyces coelicolor* A3(2) (for more details to the promoters see below). Amplified sequences were integrated into the plasmid pGUS directly upstream of *gusA* using KpnI and SpeI restriction sites. Plasmids were sequenced to check their correctness and were transferred into *A. japonicum* wildtype (WT) via conjugation. The strength of a promoter corresponds to the activity of the enzyme GusA. GusA is a β-glucuronidase, which converts *p*-nitrophenyl-β-D-glucuronide into glucuronic acid and p-nitrophenol. After the dissociation of p-nitrophenol, the yellow colour of the *p*-nitrophenolate product was measured to determine enzyme activity/gene expression. For this assay the strains were grown in SM medium with 6 μM $ZnSO_4$ for 72 h, the cells were harvested and washed twice with SM medium. Biomass was adjusted to 100 mg/ml with SM-medium and glycerol. 600 μl of this cell stock were centrifuged, resuspended in 0.5 ml lysis buffer (0.05 M phosphate buffer, 5 mM DTT, 0.1% Triton X-100, 1 mg/ml lysozyme, pH 7.0) and incubated for 90 min at 37 °C. After incubation, cell lysates were centrifuged and 100 μl of the supernatants were mixed with 100 μl of reaction buffer (0.05 M phosphate buffer, 5 mM DTT, 0.1% Triton X-100, 0.2 M p-nitrophenyl-β-D-glucuronide), incubated for 45 min at 37 °C and then OD was measured at 405 nm in a Tecan infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Miller Units were calculated as 1000 x OD_{405} of sample - OD_{405} of blank)/(incubation time) (Spohn et al., 2016).

2.5. Synthesis of the constitutive promoter SP44*

For increasing the expression of the [*S*,*S*]-EDDS biosynthesis genes *aesA-D* the synthetic promoter *SP44** (Bai et al., 2015) was used. This promoter was synthesised together with a ribosome binding site (SR41) and the RiboJ insulator as described in Bai et al. (2015) by the company BioCat GmBH (Heidelberg, Germany) and cloned into the vector pUC57_*SP44**. For further cloning experiments restriction sites were added to the original sequence. The complete synthesised sequence including the restriction sites PspXI-KpnI-VspI and NdeI (underlined sequences) was as follows $^{5'}$ACTCGAGGTACCATTAATTGTTCACATTC GAACCGTCTCTGCTTTGACAACATGCTGTGCGGTGTTGTAAAGTCTGG TGTAAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGA CGAAACAGCCTCTACAAATAATTTTGTTTAATCTAAGTAAGGAGTAGG CTGACATATG$^{3'}$.

2.6. Construction of the promoter exchange plasmid pGUSA21 SP44*

For the construction of the promoter exchange plasmid pGUSA21 *SP44** the genomic regions upstream and downstream of the Zur binding motif between the genes *aesA* and *aesE* were amplified with the primers PromEx-US-FP/PromEx-US-RP, PromEx-DS-FP/PromEx-DS-RP (Table S3) using genomic DNA of *A. japonicum* as template. These about 1500 bp large regions were inserted into the plasmid pGUSA21 via the primer attached restriction sites EcoRI, SphI, HindIII and XbaI. Subsequently, the constitutive promoter *SP44** was inserted into the newly constructed plasmid in between the upstream and downstream regions, resulting in the plasmid pGUSA21 *SP44**. For cloning the promoter, the sequence was amplified from pUC57_*SP44** (see above) via the primers PromEx-SP44-FP and PromEx-SP44-RP (Table S4) and inserted via the restriction sites EcoRI and HindIII. Sequencing of the fragments confirmed their correctness and subsequently the plasmid was transferred into *A. japonicum* via *E. coli* conjugation. After integration via single cross over, a second cross over was provoked by applying an adapted stress protocol, described by Puk et al. (2002) with a temperature shift to 37 °C for 12 h, followed by protoplast formation to disintegrate and loose the plasmid. The generation of protoplast ensures formation of single cells and the loss of the cell wall enhances the cell stress and thereby the probability of crossing over events. To generate protoplasts, cells were treated as described in Stegmann et al. (2001). For selection, blue-white screening was carried out and the loss of GusA activity (visible as white-coloured colonies) indicated the excision of the plasmid. For the screening, protoplasts were plated on agar plates containing the GusA substrate 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-Gluc) (86 mg/L) to regenerate. GusA-negative colonies were selected and checked by PCR. In the resulting mutant *A. japonicum* SP44*, the Zur DNA-binding motif including most of the intergenic region upstream of *aesA* was exchanged for the synthetic promoter *SP44** (Fig. 4).

2.7. Construction of the plasmid pSE_aesA-D

To increase the transcription of the *aesA-D* in *A. japonicum* the genes were expressed under the control of the constitutive promoter *SP44**. For the construction of the plasmid pSE_*aesA-D*, the integrative plasmid pIJ10257 was used as backbone, and the *ermE*p* promoter was exchanged for the *SP44** promoter. Firstly, the constitutive promoter *SP44** was synthesised and cut from the plasmid pUC57_*SP44** with the restriction enzymes NdeI and KpnI. The *SP44** promoter was then cloned into the plasmid pIJ10257 via KpnI and NdeI, replacing the *ermE*p* promoter and resulting in the plasmid pSE01. Subsequently, the [*S*,*S*]-EDDS biosynthesis genes *aesA-D* of *A. japonicum* were amplified with the primers *aesA*-FP and *aesD*-RP (Table S4) using pMS_*aesA-D* as template. The amplified genes were cloned into pSE01 via their primer attached restriction sites. The correctness of the plasmid pSE_*aesA-D* was checked by sequencing. The plasmid was transferred into *A. japonicum SP44** via conjugation, generating the overexpression strain *A. japonicum* OP1.

2.8. Construction of A. japonicum ΔserC

Upstream (1445 bp) and downstream (1278 bp) regions of *serC* (AJAP_RS02725) were amplified via PCR with the primer pairs serC-US-FP/serC-US-RP and serC-DS-FP/serC-DS-RP (Table S4). The fragments were purified from the agarose gel and cloned into the plasmid pGUSA21 via the primer-attached restriction sites. The plasmid was sequenced to confirm the correctness of the inserts. The resulting plasmid pGUS Δ*serC* was transferred into *A. japonicum* WT via conjugation. After its integration into the chromosome via homologous recombination, a stress protocol was applied (as described in 2.6) to disintegrate and loose the plasmid. Blue white-screening was carried out to select for loss of the plasmid and GusA-negative colonies were checked by PCR to obtain *A. japonicum ΔserC*.

2.9. Construction of the plasmid pRM4 serAserC

To construct the overexpression plasmid pRM4 *serAserC* the *serA* (AJAP_RS08210) and *serC* (AJAP_RS02725) genes of *A. japonicum* were amplified via PCR with the primer pairs serA-FP/serA-RP and serC-FP/serC-RP (Table S4). The amplified fragment of *serA* as well as the plasmid pRM4 were cut with the restriction enzymes NdeI and HindIII,

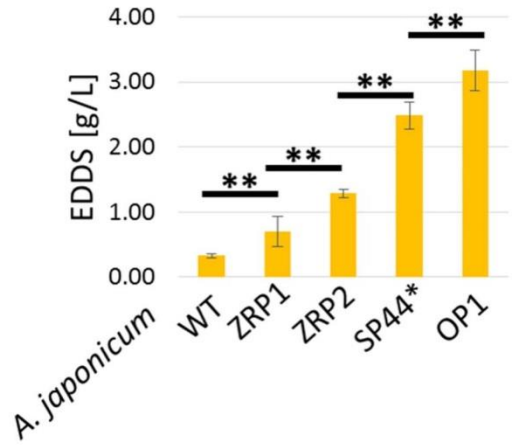

Fig. 2. [*S,S*]-EDDS production titers of different *A. japonicum* strains grown in zinc depleted medium after five days of cultivation. The [*S,S*]-EDDS production was stepwise increased by adding one copy (*A. japonicum* ZRP1) or two copies (*A. japonicum* ZRP2) of the *aesA-D* genes into the genome of *A. japonicum* WT. In *A. japonicum* SP44* the native *aesA* promoter of *A. japonicum* WT was exchanged for a synthetic one. To generate *A. japonicum* OP1, an additional copy of the *aesA-D* genes under control of *SP44** was introduced into *A. japonicum* SP44*. Statistically significant differences are marked with ** (**: $p \leq 0.01$, Man-Whitney *U*-test, n = 6).

purified and used for ligation. Correct plasmids containing *serA* as well as amplified fragment of *serC* were cut with MunI and HindIII, purified and used for ligation. The resulting plasmid pRM4 *serAserC* was sequenced to check the correctness and were later on transferred to *A. japonicum* OP1, generating the overexpression strain *A. japonicum* OP2.

2.10. Detection of [S,S]-EDDS biosynthesis using HPLC-DAD

Samples for [*S,S*]-EDDS measurement were treated as described by Spohn et al. (2016). [*S,S*]-EDDS analyses were carried out on a HP1090M liquid 490 chromatograph equipped with a thermostated autosampler, a diode-array detector and a HP Kayak 491 XM 600 ChemStation (Agilent Technologies, Santa Clara, USA). 10 µl sample were injected onto a Hypersil ODS column (125 × 4 mm, 3 µm) fitted with a guard column (10 × 4 mm, 3 µm; Stagroma AG, Reinach, Swiss) and analysed by isocratic elution with solvent A – acetonitrile (96:4, v/v) at a flow rate of 1 ml/min. Solvent A 493 consisted of 20 mM Sorensen's phosphate buffer (pH 7.2) with 5 mM tetrabutyl-ammoniumhydrogensulfate. UV detection was performed at 253 nm. For data analysis Chemstation LC3D software Rev. A.08.03 was used. [*S,S*]-EDDS in solution (Sigma Aldrich, St. Louis, USA) was used as a standard.

2.11. Statistics

The data from production tests were analysed for statistics, using the Mann-Whitney test to calculate *p* values (n = 6). On each figure p values lower than 0.01 are indicated by asteriks **, respectively. *p* values higher than 0.05 are indicated with ns (not significant). All statistics were performed using Past 3 (Hammer et al., 2001).

2.12. Growth of OP2 strain in pilot scale bioreactor

A preculture was prepared by inoculation of 4 mL of glycerol spore stock into 400 mL 2 L Erlenmayer flask with of complex medium containing 20 g/L glycerol (Carlo Erba, Val de Reuil, France), 20 g/L soy flour (Sojaprotein, Bečej, Serbia) with initial pH of 7.5. 200 µg/L of hygromycin and 100 µg/L of apramycin was used for plasmid maintenance. Growth of pre-culture was performed on a rotatory shaker at 220 rpm, 5 cm throw at 30 °C for 48 h. Batch cultivations were performed in 10 L fermentation system (Infors, Bottmingen, Swiss) with 6 L starting volume. This fermentation system has 7 L working volume. inoculated with 300 mL of preculture (5% seed inoculum). Production medium contained 80 g/L glycerol, 4 g/L soluble starch (Sigma Aldrich, St. Louis, USA), 10 g/L soy flour (Sojaprotein, Bečej, Serbia), 10 g/L peptone (Sigma Aldrich, St. Louis, USA), 10 g/L yeast extract (VWR, Radnor, USA), 10 g/L NaCl (Gram-mol, Zagreb, Croatia) and 4 g/L $CaCO_3$ (Solvay, Brussels, Belgium). Temperature was maintained at 30 °C, pH was controlled at 7.0 ± 1.0 with addition of $NaOH/H_2SO_4$. Dissolved oxygen was kept above 30% using 0.5 vol per liquid volume per minute (vvm) and stirring rate controlled by DO content. Fermentations samples were taken two times daily and OD_{600}, NH_4^+, sulphate, phosphate, dry cell weight (DCW) and [*S,S*]-EDDS concentrations were measured. OD_{600} was measured using a spectrophotometer (Tecan Infinite M200). NH_4^+ and phosphate concentrations were measured using commercially available colorimetric assays according to the manufactory's instructions; Ammonium test (Merck, Darmstadt, Germany) and Phosphate test (Merck), respectively. For DCW measurements 1 mL of whole broth was analysed on an automated moisture analyser PMX50/NH (Radwag Balances and Scales, Radom, Poland). Commercially available strips Quantofix Sulphate (Macherey-Nagel, Dueren, Germany) were used to measure sulphate concentration, according to the manufacturer's instructions.

3. Results

3.1. Increased [S,S]-EDDS production by introducing additional copies of the biosynthetic genes aesA-D into A. japonicum

The *aesA-D* genes in *A. japonicum* encode the stepwise biosynthesis of [*S,S*]-EDDS (Fig. S1), with *aesD* encoding the exporter of [*S,S*]-EDDS (Spohn et al., 2016). In order to optimize the production, we firstly introduced additional copies of the [*S,S*]-EDDS biosynthesis genes *aesA-D* into the genome of *A. japonicum* wild type (WT). For this, the operon was cloned under the control of the native promoter in the plasmid pSET152 (Bierman et al., 1992) (pSET-*aesA-D*), which integrates via the ΦC31 *att* site. The plasmid was introduced into *A. japonicum* WT via conjugation, resulting in the recombinant strain *A. japonicum* ZRP1 (zinc regulated producer). The strain was cultivated under zinc depletion conditions in [*S,S*]-EDDS production medium SM and its [*S,S*]-EDDS productivity was determined by HPLC. The analyses proved that *A. japonicum* ZRP1 produced significantly more [*S,S*]-EDDS (0.7 ± 0.2 g/L) than *A. japonicum* WT (0.3 g/L) (Fig. 2; for yield see Fig. S2).

To investigate the impact of further *aesA-D* copies on the [*S,S*]-EDDS production a second plasmid (pMS_*aesA-D*) was integrated via the ΦBT1 *att* site into *A. japonicum* ZRP1. This plasmid also contained the [*S,S*]-EDDS genes *aesA-D* under the control of the native promoter (*A. japonicum* ZRP2). The introduction of the second plasmid led to a four times higher [*S,S*]-EDDS production (1.3 ± 0.1 g/L in *A. japonicum* ZRP2) than in *A. japonicum* WT (Fig. 3).

3.2. Glucuronidase activity testing of heterologous promoters

Even though production was increased in *A. japonicum* ZRP1 and *A. japonicum* ZRP2, the synthesis of [*S,S*]-EDDS was still suppressed in the presence of zinc, as all copies of the biosynthesis genes were still under the control of the native zinc regulated promoter. Basically, there are two possibilities to circumvent this zinc regulation: either the deletion of the zinc regulator gene *zur* or the replacement of the Zur-regulated promoter of the [*S,S*]-EDDS biosynthetic genes with a strong promoter. Previous studies revealed that the deletion of *zur* allowed [*S,S*]-EDDS to be produced in the presence of zinc. However, this mutation caused

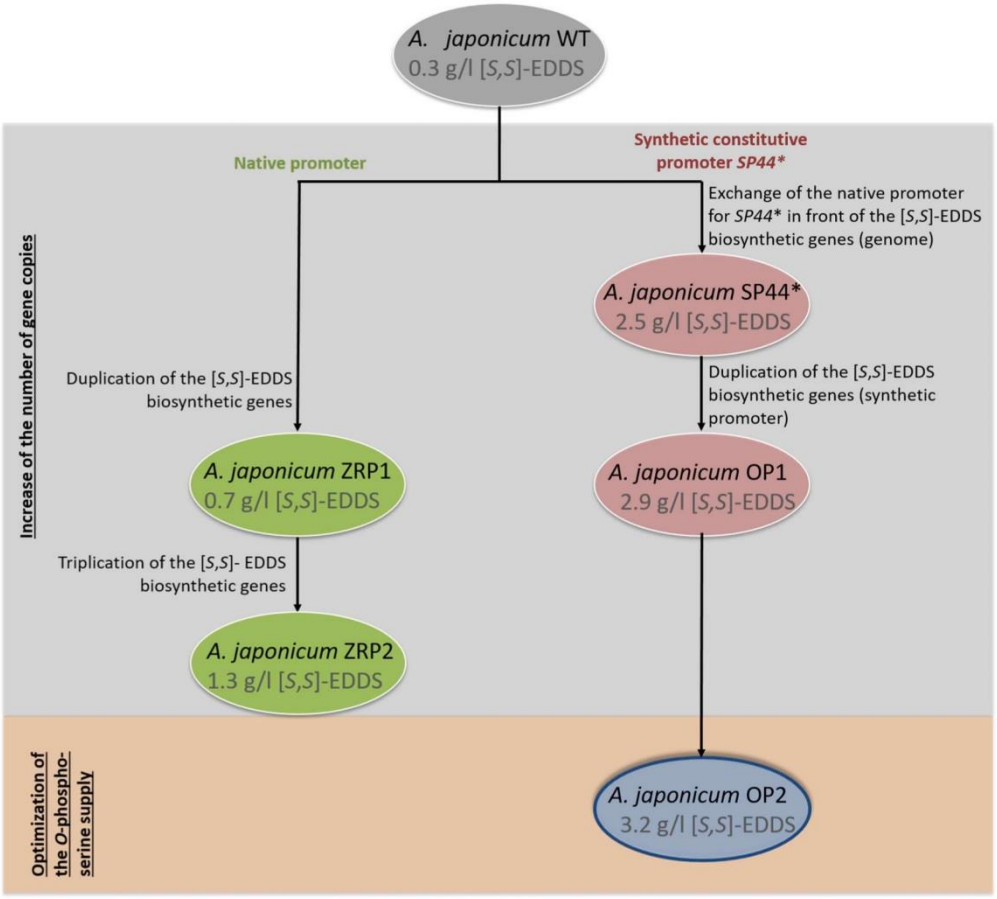

Fig. 3. Genetic engineering steps to optimize the [*S,S*]-EDDS production rates in *A. japonicum.* Average [*S,S*]-EDDS production of the strains in zinc depleted SM medium is given.
WT: wild type, ZRP: additional copy of the [*S,S*]-EDDS biosynthetic genes *aesA-D* under the control of the native, zinc regulated promoter, OP: the [*S,S*]-EDDS biosynthetic genes under the control of the synthetic promoter *SP44**.

undesired side effects like slow growth. Hence, optimization steps were implemented, targeting only the [*S,S*]-EDDS biosynthesis genes rather than the Zur regulon. The replacement of the native promoter by a strong constitutive promoter could on the one hand increase the transcription level and on the other hand abolish zinc dependence without directly affecting the primary metabolism. Therefore, an optimal constitutive promoter was considered. A widely used constitutive promoter in actinomycetes is *ermE*p* (Bibb et al., 1994; Schmitt-John and Engels, 1992; Siegl et al., 2013). Another strong constitutive promoter is a synthetic derivative of the *kasO* promoter, *SP44** (Wang et al., 2013; Bai et al., 2015). KasO is a regulator of the SARP family and regulates the expression of the polyketide synthase type I gene cluster for coelimycin of *S. coelicolor*. *SP44** showed a 20-fold higher activity than the *ermE*p* promoter (Bai et al., 2015) when tested in *Streptomyces venezuelae*. To investigate its suitability for *A. japonicum*, we determined its activity and compared it to the activity of the *ermE*p* as well as to the native promoter of the actinorhodin biosynthesis of *S. coelicolor*, *actII-orf4*p, by using the glucuronidase activity assay adapted for *A. japonicum* (Spohn et al., 2016). The *SP44**, *ermE*p* and *actII-orf4*p promoter were cloned in front of the glucuronidase reporter gene *gusA* into the vector pGUS (Myronovskyi et al., 2011) and the resulting plasmids pGUS *SP44**, pGUS *ermE*p*, pGUS *actII-orf4*p, were integrated in *A. japonicum* WT. *A. japonicum* pGUS P*aesA* (Spohn et al., 2016), containing the native promoter of the [*S,S*]-EDDS biosynthesis and the strain *A. japonicum* pGUS (Spohn et al., 2016), containing the unaltered promoterless pGUS plasmid, served as controls. After cultivation of the strains for 72 h in a zinc containing medium, the cells were harvested and a cell wet weight of 100 mg/ml was adjusted for each sample. Following disruption of the cells, the glucuronidase activities were measured in Miller units per milligram biomass using a spectrophotometric assay (Table 1).

In the presence of zinc the native EDDS promoter was not active at all, whereas the *actII-orf4*p promoter of *S. coelicolor* showed the weakest activity. In comparison, the glucuronidase gene was highly expressed under the control of the *ermE*p* promoter. The use of *SP44** even led to a four-fold increased glucuronidase activity than *ermE*p*. These results display the same tendency as the results obtained by Bai et al. (2015) who demonstrated that the synthetic promoter *SP44** is much stronger than the *ermE*p* promoter in *S. venezuelae* ISP5230. That means that the *SP44** promoter is not only suitable for increasing secondary metabolite production in *Streptomyces spp*., but also in other actinomycetes like *A. japonicum*.

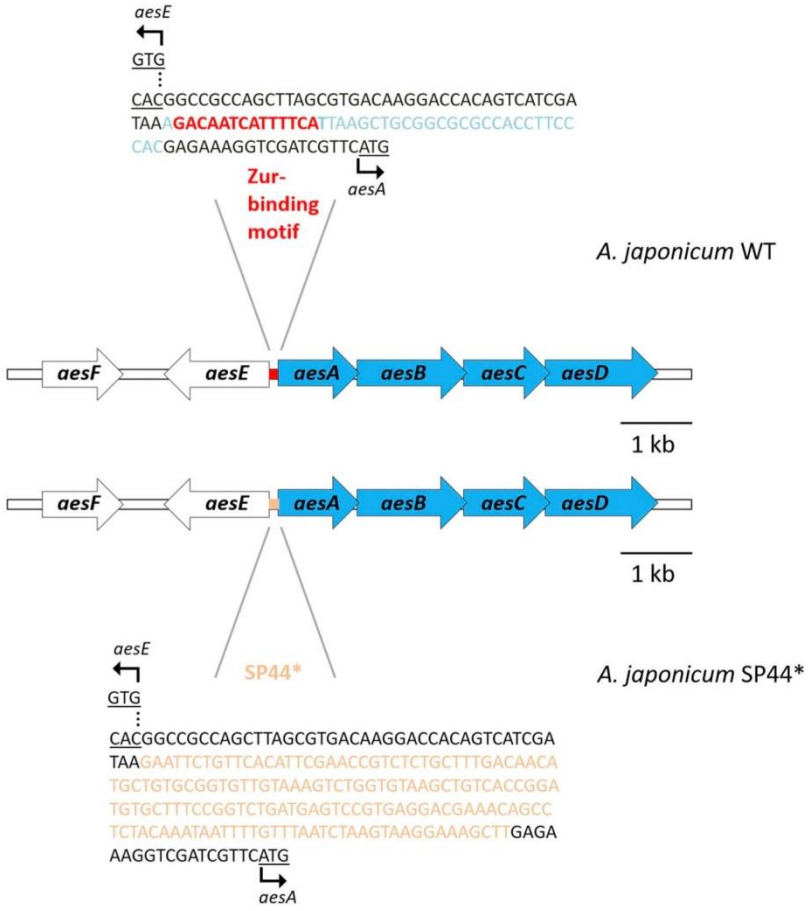

Fig. 4. Native and synthetic promoter regions used for expression of *aesA-D*.
Intergenic region upstream of *aesA* in *A. japonicum* wild type (WT) (A) and *A. japonicum* SP44* (B). The Zur-binding box is shown in red, exchanged promoter regions are shown in yellow and blue. Start codons of *aesA* and *aesE* are underlined. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Determination of the promoter activity using different promoters fused with the glucuronidase reporter gene.

Promoter fusion strains *A. japonicum* pGUS	Average glucuronidase activity [Miller units]
- (neg. control)	3 ± 2.4
PaesA	2 ± 0.4
*actII-orf4*p	380 ± 107.0
*ermE*p*	1797 ± 197.4
*SP44**	7630 ± 324.8

± standard deviation is given. n = 3.

3.3. Construction of a zinc deregulated strain by exchanging the aesA-D promoter region

According to the glucuronidase activity assay, the *SP44** promoter was by far the strongest promoter in *A. japonicum*. Therefore, in order to increase the expression of the *aesA-D* genes we exchanged the native P*aesA* for the *SP44** promoter in the genome of *A. japonicum* WT. For this purpose, the plasmid pGUSA21 *SP44** was used (see 2.6). In the resulting mutant *A. japonicum* SP44* the Zur DNA-binding motif including most of the intergenic region upstream of *aesA* was exchanged for the synthetic promoter *SP44** (Fig. 4). We left the 18 base pair sequence upstream of *aesA* unchanged in *A. japonicum* SP44* to ensure the presence of the native ribosomal binding site.

HPLC analyses of the supernatant of the *A. japonicum* strains cultivated under zinc depleted conditions revealed that *A. japonicum* SP44* produced eight times more [*S,S*]-EDDS than *A. japonicum* WT and almost twice as much as *A. japonicum* ZRP2 containing three copies of the [*S,S*]-EDDS biosynthesis genes under the control of the native promoter (Fig. 2).

3.4. Overexpression of the aesA-D genes in A. japonicum

In order to combine the positive effects obtained by introducing additional copies of the *aesA-D* genes and by exchanging the native promoter by the *SP44** promoter, we introduced an integrating plasmid containing *aesA-D* (pSE_*aesA-D*) under the control of the *SP44** promoter into *A. japonicum* SP44*. In zinc depleted SM medium the resulting strain *A. japonicum* OP1 produced about 3.2 ± 0.3 g/L [*S,S*]-EDDS, thus significantly more than *A. japonicum* SP44* and ten times more [*S,S*]-EDDS than *A. japonicum* WT (Fig. 2).

3.5. Optimization of the precursor supply

The advancement of *A. japonicum* OP1 was based on the increased

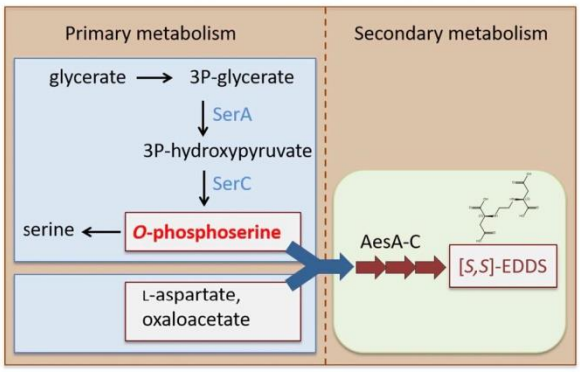

Fig. 5. Scheme of the precursor supply for [*S,S*]-EDDS biosynthesis.
SerA (phosphoglycerate dehydrogenase) and SerC (phosphoserine transaminase) catalyse essential steps for providing the [*S,S*]-EDDS precursor *O*-phosphoserine.

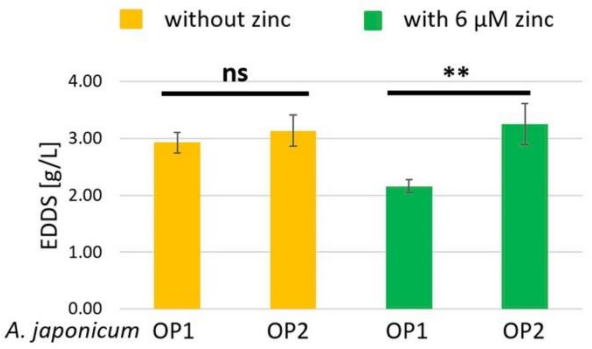

Fig. 6. [*S,S*]-EDDS production titers of the *A. japonicum* strains OP1 and OP2. Cells were grown for five days in either zinc depleted or zinc supplemented (6 µmol/L) SM-medium.
A. japonicum OP1 contains additional copies of the [*S,S*]-EDDS genes (*aesA-D*) under the control of the constitutive promoter *SP44**.
A. japonicum OP2 contains in addition to *A. japonicum* OP1 an additional copy of the *O*-phosphoserine biosynthesis genes *serA* and *serC* under control of the *ermEp**. Statistically significant differences are marked with ** (**: $p \leq 0.01$, ns: not significant, Man-Whitney *U*-test, n = 6).

transcription rates of the [*S,S*]-EDDS biosynthesis genes by introducing multiple copies and a strong constitutive promoter. However, the production rates of secondary metabolites are often limited by the provision of the building blocks. Therefore, in the next step, we addressed the precursor supply of [*S,S*]-EDDS to overcome this potential bottleneck in product formation.

Spohn et al. (2016) postulated that *A. japonicum* synthesizes [*S,S*]-EDDS from aspartic acid, oxaloacetic acid and *O*-phosphoserine. Feeding experiments with labelled amino acids performed with *A. japonicum* confirmed the incorporation of aspartic acid in [*S,S*]-EDDS (Cebulla, 1995). A corresponding feeding experiment with *O*-phosphoserine was not possible, since *A. japonicum* was not able to take up *O*-phosphoserine (data not shown). In bacteria, *O*-phosphoserine is an intermediate of the serine biosynthesis (Fig. 5). Key enzymes for this pathway are the phosphoglycerate dehydrogenase SerA (Sugimoto and Pizer 1968a, 1968b; Schuller et al., 1995) and the phosphoserine transaminase SerC (Pizer, 1963; Umbarger et al., 1963). To verify the hypothesis that *O*-phosphoserine is a [*S,S*]-EDDS precursor, we deleted *serC* in *A. japonicum*. The mutant *A. japonicum* Δ*serC* showed a retarded growth compared to *A. japonicum* WT. This phenotype could be reversed by feeding serine to the mutant, suggesting that the effects on growth were caused by the elimination of the main serine biosynthetic pathway and the necessity of the mutant to synthesize serine by using other amino acids (e.g. glycine) as precursors. In addition to growth, the deletion of *serC* also effected [*S,S*]-EDDS production. Under producing conditions, no [*S,S*]-EDDS could be detected in the culture of *A. japonicum* Δ*serC*, confirming that *O*-phosphoserine is indeed a precursor of [*S,S*]-EDDS. For further optimization of the [*S,S*]-EDDS production we therefore focused on increasing the supply of *O*-phosphoserine by overexpressing the respective biosynthesis genes under the control of a strong promoter.

The genes *serA* (AJAP RS_08210) and *serC* (AJAP RS_02725) of *A. japonicum* were amplified and cloned into pRM4 (pRM4 *serAserC*). The integration of pRM4 *serAserC* into the chromosome of *A. japonicum* OP1 resulted in *A. japonicum* OP2 containing an additional copy of its *serA* and *serC* genes under control of the *ermEp** promoter. Finally, both [*S,S*]-EDDS overproducer strains *A. japonicum* OP1 and *A. japonicum* OP2 were tested in zinc supplemented [6 µmol/L] and zinc depleted medium. The [*S,S*]-EDDS production assay revealed that under zinc depleted conditions *A. japonicum* OP2 did not produce more [*S,S*]-EDDS than *A. japonicum* OP1 (Fig. 6), indicating that *O*-phosphoserine is not limiting [*S,S*]-EDDS production. Surprisingly, *A. japonicum* OP1 showed a zinc dependent reduction in [*S,S*]-EDDS production (2.9 ± 0.2 without zinc and 2.2 ± 0.1 g/L [*S,S*]-EDDS with zinc), although the zinc regulated promoter region upstream of the *aesA-D* genes was replaced by the constitutive promoter *SP44** in this strain. However, this reduction was not observed in *A. japonicum* OP2, which produced as much [*S,S*]-EDDS in the presence (3.3 ± 0.4 g/L [*S,S*]-EDDS) as in the absence of zinc (3.1 ± 0.3 g/L [*S,S*]-EDDS) (Fig. 6).

The introduction of a constitutively expressed copy of the *O*-phosphoserine biosynthesis genes *serA* and *serC* into *A. japonicum* OP1 prevented the zinc dependent reduction of [*S,S*]-EDDS. These data indicated that not only [*S,S*]-EDDS production, but also the formation of its precursor *O*-phosphoserine is affected by zinc. However, unlike to the biosynthesis genes of [*S,S*]-EDDS, no Zur-binding box could be identified in the upstream regions of *serA* and *serC*, suggesting the presence of an additional (zinc responsive) regulator controlling the transcription of those genes.

3.6. Scale-up of the EDDS overproducing strain OP2

The next step towards biotechnological production is the optimization of the fermentation process. Several attempts have been made to optimize the bioprocess for the production of [*S,S*]-EDDS in *A. japonicum*. Previously, production titers of about 20 g/L [*S,S*]-EDDS were achieved by fed-batch fermentations of *A. japonicum* WT under low zinc conditions (Zwicker et al., 1997) and of the zinc deregulated mutant *A. japonicum* Δzur in a zinc-containing environment (Fig. S3). However, since the fermentation time was 900 h, these conditions were not transferable to an industrial process.

In order to demonstrate the potential of the used metabolic engineering approaches for an industrial production of [*S,S*]-EDDS we cultivated the best performing strain, *A. japonicum* OP2, in a pH-controlled batch bioprocess in a 10 L bioreactor with 7 L working volume. Using nutrient-rich and low-cost industrial media (2.12), the titers of [*S,S*]-EDDS reached up to 9.8 g/L in 135 h fermentation time, with a calculated [*S,S*]-EDDS production rate of 4.3 mg/h/g DCW (Fig. 7).

The new strain *A. japonicum* OP2 was dramatically superior to the strains *A. japonicum* WT and *A. japonicum* Δzur in [*S,S*]-EDDS production. Volumetric productivity was highest in the first 70 h of bioprocess and reached 80 mg/L/h, compared to 20 mg/L/h in bioprocess of *A. japonicum* Δzur (Fig. S1) in defined medium. These results demonstrated that *A. japonicum* OP2 has excellent potential for further development of an economically viable industrial bioprocess. In the next step, volumetric productivity of the *A. japonicum* OP2 strain should be further increased through optimization of biomass growth (ie. by feeding of carbon and rapidly available nitrogen sources) during the bioprocess.

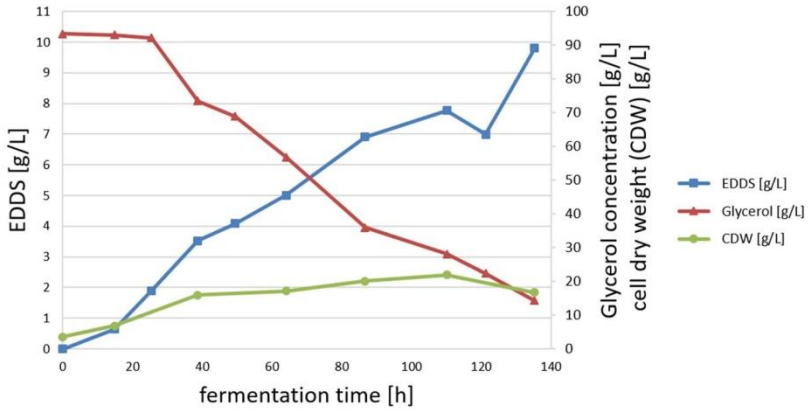

Fig. 7. [*S*,*S*]-EDDS production, glycerol concentration in the medium and dry cell weight (DCW) of the *A. japonicum* strain OP2. Representative batch bioprocess was done in 10 L bioreactor in industrial complex medium is shown.

4. Conclusion

[*S*,*S*]-EDDS is the more environment-friendly substitute for the industrially widely applied EDTA. With the successful engineering and optimizing of the [*S*,*S*]-EDDS producer *A. japonicum*, we generated the overproduction strain *A. japonicum* OP2, which produces up to 9.8 g/L [*S*,*S*]-EDDS, even in the presence of zinc. *A. japonicum* OP2 is a highly efficient [*S*,*S*]-EDDS producer which fulfills all requirements for an economic biotechnological production, necessary to substitute [*S*,*S*]-EDDS for EDTA.

Surprisingly, deregulation and overexpression of the biosynthesis genes *aesA-D* and optimization of the precursor supply was not enough to construct this high yielding strain. Engineering the *O*-phosphoserine production in *A. japonicum* OP1 revealed a so far unknown effect of zinc on the biosynthesis of the amino acid and [*S*,*S*]-EDDS precursor. In addition to that, this zinc effect is seemingly not caused by the major zinc sensing regulatory protein Zur. This demonstrates how much primary metabolism influences the output of secondary metabolite production and stresses the importance of better knowledge of regulatory networks. In particular, more research is needed to investigate how lack of micronutrients e.g. zinc, changes primary and thus secondary metabolic fluxes either by regulation or simply by essential, but non-functional metalloproteins.

Author Contributions

Simone Edenhart: Performance of the experiments, Writing-Original draft preparation; Marius Denneler: Performance of the promoter probe experiments; Marius Spohn: Construction of recombinant strains; Eva Doskocil: Construction of mutants; Martin Kavšček, Tadeja Amon, Gregor Kosec, Jernej Smole: Batch-Fermentation of the optimized strains OP1 and OP2, Original draft preparation; Bettina Bardl, Michael Biermann, Martin Roth: Fed batch fermentation, Original draft preparation; Wolfgang Wohlleben: Writing-Reviewing and Editing; Evi Stegmann: Supervision, Conceptualization, Writing- Reviewing and Editing

Declaration of competing interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2020.04.003.

References

Bai, C., Zhang, Y., Zhao, X., Hu, Y., Xiang, S., Miao, J., Lou, C., Zhang, L., 2015. Exploiting a precise design of universal synthetic modular regulatory elements to unlock the microbial natural products in *Streptomyces*. Proc. Natl. Acad. Sci. Unit. States Am. 112, 12181–12186. https://doi.org/10.1073/pnas.1511027112.

Barbier, M., Bogdanovsky, D., Vetter, W., Lederer, E., 1963. Synthese und Eigenschaften eines Analogen des Lycomarasmins und der Aspergillomarasmine. Liebigs Ann. Chem. 668, 132–138.

Bibb, M.J., White, J., Ward, J.M., Janssen, G.R., 1994. The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. Mol. Microbiol. 14, 533–545. https://doi.org/10.1111/j.1365-2958.1994.tb02187.x.

Bierman, M., Logan, R., O'brien, K., Seno, E.T., Rao, R.N., Schoner, B.E., 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene 116, 43–49. https://doi.org/10.1016/0378-1119(92)90627-2.

Buckley, L.M., McEwan, N.A., Nuttall, T., 2013. Tris–EDTA significantly enhances antibiotic efficacy against multidrug-resistant *Pseudomonas aeruginosa in vitro*. Vet. Dermatol. 24https://doi.org/10.1111/vde.12071. 519-e122.

Cebulla, I., 1995. Gewinnung komplexbildender Substanzen mittels *Amycolatopsis orientalis*. University of Tübingen, Doctoral Thesis.

Chauhan, A., Lebeaux, D., Ghigo, J.M., Beloin, C., 2012. Full and broad-spectrum *in vivo* eradication of catheter-associated biofilms using gentamicin-EDTA antibiotic lock therapy. Antimicrob. Agents Chemother. 56, 6310–6318. https://doi.org/10.1128/AAC.01606-12.

Chen, L., Liu, T., Ma, C.A., 2009. Metal complexation and biodegradation of EDTA and *S,S*-EDDS: a density functional theory study. J. Phys. Chem. 114, 443–454. https://doi.org/10.1021/jp904296m.

Farca, A.M., Nebbia, P., Robino, P., Re, G., 1997. Effects of the combination antibiotic-EDTA-Tris in the treatment of chronic bovine endometritis caused by antimicrobial-resistant bacteria. Pharmacol. Res. 36, 35–39. https://doi.org/10.1006/phrs.1997.0199.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 9.

Hauser, L., Tandy, S., Schulin, R., Nowack, B., 2005. Column extraction of heavy metals from soils using the biodegradable chelating agent EDDS. Environ. Sci. Technol. 39, 6819–6824. https://doi.org/10.1021/es050143r.

Hough, E., Hansen, L.K., Birknes, B., Jynge, K., Hansen, S., Hordvik, A., Little, C., Dodsen, E., Derewenda, Z., 1989. High-resolution (1.5 Å) crystal structure of phospholipase C from *Bacillus cereus*. Nature 338, 357. https://doi.org/10.1038/338357a0.

Kallifidas, D., Pascoe, B., Owen, G.A., Strain-Damerell, C.M., Hong, H.J., Paget, M.S., 2010. The zinc-responsive regulator Zur controls expression of the coelibactin gene cluster in *Streptomyces coelicolor*. J. Bacteriol. 192, 608–611. https://doi.org/10.1128/JB.01022-09.

Lambert, R.J.W., Hanlon, G.W., Denyer, S.P., 2004. The synergistic effect of EDTA/antimicrobial combinations on *Pseudomonas aeruginosa*. J. Appl. Microbiol. 96, 244–253. https://doi.org/10.1046/j.1365-2672.2004.02135.x.

López-Rayo, S., Sanchis-Pérez, I., Ferreira, C.M., Lucena, J.J., 2019. [*S,S*]-EDDS/Fe: a new chelate for the environmentally sustainable correction of iron chlorosis in calcareous soil. Sci. Total Environ. 647, 1508–1517. https://doi.org/10.1016/j.scitotenv.2018.08.021.

Luo, C., Shen, Z., Li, X., 2005. Enhanced phytoextraction of Cu, Pb, Zn and Cd with EDTA and EDDS. Chemosphere 59, 1–11. https://doi.org/10.1016/j.chemosphere.2004.09.100.

Meers, E., Ruttens, A., Hopgood, M.J., Samson, D., Tack, F.M.G., 2005. Comparison of EDTA and EDDS as potential soil amendments for enhanced phytoextraction of heavy metals. Chemosphere 58, 1011–1022. https://doi.org/10.1016/j.chemosphere.2004.09.047.

Meers, E., Tack, F.M.G., Verloo, M.G., 2008. Degradability of ethylenediaminedisuccinic acid (EDDS) in metal contaminated soils: implications for its use soil remediation. Chemosphere 70, 358–363. https://doi.org/10.1016/j.chemosphere.2007.07.044.

Menges, R., Muth, G., Wohlleben, W., Stegmann, E., 2007. The ABC transporter Tba of *Amycolatopsis balhimycina* is required for efficient export of the glycopeptide antibiotic balhimycin. Appl. Microbiol. Biotechnol. 77, 125–134. https://doi.org/10.1007/s00253-007-1139-x.

Myronovskyi, M., Welle, E., Fedorenko, V., Luzhetskyy, A., 2011. β-Glucuronidase as a sensitive and versatile reporter in actinomycetes. Appl. Environ. Microbiol. 77, 5370–5383. https://doi.org/10.1128/AEM.00434-11.

Nishikiori, T., Okuyama, A., Naganawa, H., Takita, T., Hamada, M., Takeuchi, T., Aoyagi, T., Umezawa, H., 1984. Production by actinomycetes of (*S,S*)-*N,N*-ethylenediaminedisuccinic acid, an inhibitor of phospholipase c. J. Antibiot. 37, 426–427. https://doi.org/10.7164/antibiotics.37.426.

Pizer, L.I., 1963. The pathway and control of serine biosynthesis in *Escherichia coli*. J. Biol. Chem. 238, 3934–3944.

Puk, O., Huber, P., Bischoff, D., Recktenwald, J., Jung, G., Süßmuth, R.D., Pée, K.-H., Wohlleben, W., Pelzer, S., 2002. Glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM5908: function of a halogenase and a haloperoxidase/perhydrolase. Chem. Biol. 9, 225–235. https://doi.org/10.1016/S1074-5521(02)00101-1.

Schmitt-John, T., Engels, J.W., 1992. Promoter constructions for efficient secretion expression in *Streptomyces lividans*. Appl. Microbiol. Biotechnol. 36, 493–498. https://doi.org/10.1007/BF00170190.

Schuller, D.J., Grant, G.A., Banaszak, L.J., 1995. The allosteric ligand site in the V max-type cooperative enzyme phosphoglycerate dehydrogenase. Nat. Struct. Biol. 2, 69. https://doi.org/10.1038/nsb0195-69.

Shin, J.H., Oh, S.Y., Kim, S.J., Roe, J.H., 2007. The zinc-responsive regulator Zur controls a zinc uptake system and some ribosomal proteins in *Streptomyces coelicolor* A3 (2). J. Bacteriol. 189, 4070–4077. https://doi.org/10.1128/JB.01851-06.

Siegl, T., Tokovenko, B., Myronovskyi, M., Luzhetskyy, A., 2013. Design, construction and characterisation of a synthetic promoter library for fine-tuned gene expression in actinomycetes. Metab. Eng. 19, 98–106. https://doi.org/10.1016/j.ymben.2013.07.006.

Spohn, M., Kirchner, N., Kulik, A., Jochim, A., Wolf, F., Muenzer, P., Borst, O., Gross, H., Wohlleben, W., Stegmann, E., 2014. Overproduction of ristomycin A by activation of a silent gene cluster in *Amycolatopsis japonicum* MG417-CF17. Antimicrob. Agents Chemother. 58, 6185–6196. https://doi.org/10.1128/AAC.03512-14.

Spohn, M., Wohlleben, W., Stegmann, E., 2016. Elucidation of the zinc-dependent regulation in *Amycolatopsis japonicum* enabled the identification of the ethylenediamine-disuccinate ([*S,S*]-EDDS) genes. Environ. Microbiol. 18, 1249–1263. https://doi.org/10.1111/1462-2920.13159.

Stegmann, E., Pelzer, S., Wilken, K., Wohlleben, W., 2001. Development of three different gene cloning systems for genetic investigation of the new species *Amycolatopsis japonicum* MG417-CF17, the ethylenediaminedisuccinic acid producer. J. Biotechnol. 92, 195–204. https://doi.org/10.1016/S0168-1656(01)00360-1.

Stegmann, E., Wohlleben, W., Spohn, M., Weber, T., 2016. [*S,S*]-EDDS Biosynthesis Genes and Proteins and Method of Biosynthesis of [*S,S*]-EDDS. WO2015/03275, PCT/EP2014/068612.

Sugimoto, E., Pizer, L.I., 1968a. The mechanism of end product inhibition of serine biosynthesis I. Purification and kinetics of phosphoglycerate dehydrogenase. J. Biol. Chem. 243, 2081–2089.

Sugimoto, E., Pizer, L.I., 1968b. The mechanism of end product inhibition of serine biosynthesis II. Optical studies of phosphoglycerate dehydrogenase. J. Biol. Chem. 243, 2090–2098.

Tandy, S., Ammann, A., Schulin, R., Nowack, B., 2006. Biodegradation and speciation of residual *S,S*-ethylenediaminedisuccinic acid (EDDS) in soil solution left after soil washing. Environ. Pollut. 142, 191–199. https://doi.org/10.1016/j.envpol.2005.10.013.

Umbarger, H.E., Umbarger, M.A., Siu, P.M., 1963. Biosynthesis of serine in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. 85, 1431–1439.

Wang, W., Li, X., Wang, J., Xiang, S., Feng, X., Yang, K., 2013. An engineered strong promoter for streptomycetes. Appl. Environ. Microbiol. 79, 4484–4492. https://doi.org/10.1128/AEM.00985-13.

Wen, J., Stacey, S.P., McLaughlin, M.J., Kirby, J.K., 2009. Biodegradation of rhamnolipid, EDTA and citric acid in cadmium and zinc contaminated soils. Soil Biol. Biochem. 41, 2214–2221. https://doi.org/10.1016/j.soilbio.2009.08.006.

Wooley, R.E., Jones, M.S., Shotts Jr., E.B., 1984. Uptake of antibiotics in gram-negative bacteria exposed to EDTA-Tris. Vet. Microbiol. 10, 57–70. https://doi.org/10.1016/0378-1135(84)90056-7.

Wooley, R.E., Ritchie, B.W., Burnley, V.V., 2004. *In vitro* effect of a buffered chelating agent and neomycin or oxytetracycline on bacteria associated with diseases of fish. Dis. Aquat. Org. 59, 263–267. https://doi.org/10.3354/dao059263.

Zwicker, N., Theobald, U., Zähner, H., Fiedler, H.P., 1997. Optimization of fermentation conditions for the production of ethylene-diamine-disuccinic acid by *Amycolatopsis orientalis*. J. Ind. Microbiol. Biotechnol. 19, 280–285. https://doi.org/10.1038/sj.jim.2900458.