Poly- and Monoamine Metabolism in *Streptomyces coelicolor*: The New Role of Glutamine Synthetase-Like Enzymes in the Survival under Environmental Stress

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Abstract

Soil bacteria from the genus *Streptomyces*, phylum Actinobacteria, feature a complex metabolism and diverse adaptations to environmental stress. These characteristics are consequences of variable nutrition availability in the soil and allow survival under changing nitrogen conditions. *Streptomyces coelicolor* is a model organism for Actinobacteria and is able to use nitrogen from a variety of sources including unusual compounds originating from the decomposition of dead plant and animal material, such as polyamines or monoamines (like ethanolamine). Assimilation of nitrogen from these sources in *S. coelicolor* remains largely unstudied. Using microbiological, biochemical and in silico approaches, it was recently possible to postulate polyamine and monoamine (ethanolamine) utilization pathways in *S. coelicolor*. Glutamine synthetase-like enzymes (GS-like) play a central role in these pathways. Extensive studies have revealed that these enzymes are able to detoxify polyamines

or monoamines and allow the survival of *S. coelicolor* in soil containing an excess of these compounds. On the other hand, at low concentrations, polyamines and monoamines can be utilized as nitrogen and carbon sources. It has been demonstrated that the first step in poly-/monoamine assimilation is catalyzed by GlnA3 (a γ -glutamylpolyamine synthetase) and GlnA4 (a γ -glutamylethanolamide synthetase), respectively. First insights into the regulation of polyamine and ethanolamine metabolism have revealed that the expression of the *glnA3* and the *glnA4* gene are controlled on the transcriptional level.

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Introduction

Streptomyces coelicolor is a filamentous soil-dwelling, non-motile, Gram-positive, obligatory aerobic bacterium with high GC-content (63–78%) and belongs to the genus *Streptomyces* from the phylum Actinobacteria [Bentley et

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al., 2002]. Streptomycetes including *S. coelicolor* feature a complex life cycle and metabolism, the ability to synthesize useful natural products, high metabolic potential as well as impressive adaptability to environmental stress [Hopwood et al., 1995].

In its natural habitat, the soil, *S. coelicolor* can live under numerous nutrient conditions. It is able to metabolize varying N/C-sources, including amino acids, peptides, amino sugars, ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and urea [Hodgson, 2000]. Members of the family Streptomycetaceae fulfil an important ecological role in the soil due to their capability to metabolize the remains of other organisms. For instance, they are able to detoxify and utilize different C- and N-sources (chitin, xylose and cellulose) as well as to release chitinases, xylanases, and cellulases for extracellular utilization of organic material [Hodgson, 2000].

In terms of genetics, physiology, and applications, *S. coelicolor* A3(2) is the best studied and described actinobacterium and, therefore, serves as a model organism for *Streptomyces* and Actinobacteria [Hopwood, 1999]. The genome of the widely used *S. coelicolor* M145 strain was fully sequenced and is remarkably large. This strain lacks two original *S. coelicolor* A3(2) plasmids SCP1 and SCP2 and contains a linear chromosome with 8,667,507 base pairs corresponding to 7,825 predicted genes [Bentley et al., 2002]. The genome of *S. coelicolor* includes multiple gene clusters for natural product synthesis, for example undecylprodigiosin, actinorhodin, calcium-dependent antibiotic, methylenomycin, perimycin [Hopwood, 2006]. Primary and secondary metabolism of *S. coelicolor* as well as morphological differentiation are controlled by interlinked regulatory networks that respond to the variety of intra- and extracellular stimuli, including signals concerning the nitrogen availability and supply [Kandikar et al., 1997]. These features allow *S. coelicolor* to be very competitive in soil and to survive under environmental stress.

Nitrogen Metabolism in *S. coelicolor*

Assimilation of Nitrogen in *S. coelicolor*

Nitrogen from the extracellular environment can be assimilated via various utilization pathways. Nitrogen control has been studied in a number of bacteria, including the actinobacterial species *Corynebacterium glutamicum*, *Mycobacterium tuberculosis*, and *S. coelicolor* [Merrick et al., 1995].

For *S. coelicolor*, common nitrogen sources in soil environment are ammonium, glutamine, glutamate, and nitrate (NO_3^-). The amino acids histidine and arginine were also shown to be preferable N-sources for *S. coelicolor* [Hodgson, 2000; Hopwood, 2006]. These compounds can diffuse through the membrane (ammonium) or are taken up directly by transporters (ammonium, nitrate/nitrite, urea, amino acids) [Thomas et al., 2000]. Ammonium is a preferred inorganic nitrogen source and can be directly incorporated into glutamine or glutamate, which are important building blocks for biomolecules. Other nitrogen sources have to be transformed first, for example cleaved like urea or deaminated/transaminated-like amino acids and are then converted to ammonium [Fischer et al., 2010] (shown in Fig. 1).

Under low N-concentrations, the intracellular ammonium is introduced into the metabolism by glutamine synthetase (GS), which catalyzes the synthesis of glutamine using ammonium and glutamate as substrates in an ATP-dependent manner. Glutamate can be produced from glutamine and 2-oxyglutarate by glutamine-2-oxyglutarate-aminotransferase (GOGAT) [Magasanik, 1982]. Under high N-concentrations, glutamate dehydrogenase (GDH) catalyzes the production of glutamate from ammonium and 2-oxyglutarate using NADPH (shown in Fig. 1). These basic reactions are also conserved in *S. coelicolor*, which possesses as a distinctive feature two GSs (GSI-GlnA and GSII-GlnII) that are able to synthesize glutamine (see section GS-Like Enzymes in *S. coelicolor*).

Regulation of Nitrogen Assimilation in *S. coelicolor*

The cellular response to nitrogen limitation on the transcriptional level is controlled in *S. coelicolor* by the global nitrogen response regulator GlnR from the OmpR family of response regulators [Wray & Fisher, 1993]. It is an atypical orphan response regulator, which influences the expression of genes that are directly involved in nitrogen assimilation, e.g. the operon *amtB-glnK-glnD*, encoding the ammonium transporter AmtB, the PII signal protein GlnK, and the adenylyl transferase GlnD [Reuther & Wohlleben, 2007; Tiffert et al., 2008]. These genes are conserved across the order Actinomycetales [Fink et al., 2002; Tiffert et al., 2008]. Other GlnR target genes include *nirB* and *nasA* for nitrate/nitrite reduction, *ureA* for cleavage of urea, *glnA* and *glnII* encoding glutamine synthetases, *gdhA* encoding a glutamate dehydrogenase, *nnaR* encoding the HemD-like transcriptional regulator and seven additional genes encoding proteins of unknown function [Reuther & Wohlleben, 2007; Tiffert et

Fig. 1. Schematic illustration of nitrogen metabolism in *S. coelicolor*. GS, glutamine synthetase; GDH, glutamate dehydrogenase; GOGAT, glutamine-2-oxoglutarate-aminotransferase.

al., 2008; Wang & Zhao, 2009; Amin et al., 2012]. GlnR can be modified by phosphorylation and acetylation under *N*-variable conditions. Such post-translational modifications can change GlnR DNA-binding activity [Amin et al., 2016].

Transcriptional control of the nitrate assimilatory genes (*nnaR*, *narK*, *nasA*, *nirBD*) in *S. coelicolor* involves, in addition to GlnR, a second regulator NnaR. Under nitrogen limitation conditions, GlnR activates the expression of nitrate assimilatory genes that are enhanced by the synergistic binding of NnaR and GlnR in the presence of nitrate [Amin et al., 2012]. One more regulator GlnRII exhibits 31% amino acid sequence identity to GlnR and shows the same binding specificity as GlnR. GlnRII can also bind to the upstream regions of *glnA*, *glnII*, *amtB-glnK-glnD*, and *sco1863*. However, the deletion of *glnRII* has been shown not to result in glutamine auxotrophy, meaning that GlnRII is not a functional homologue of GlnR. Since an in vitro interaction of GlnRII with the *glnII* promoter has been demonstrated, this regulator probably has a particular role in the regulation of *glnII* [Fink et al., 2002]. GlnRII was found in a large number of screened *Streptomyces* strains, but has not been found in *Mycobacterium* or *Corynebacterium* [Reuther & Wohlleben, 2007]. The GlnR/GlnRII system may have been evolved to allow an efficient differential transcription control of both GS genes *glnA* and *glnII*. In *S. coelicolor*, GSI is predominantly responsible for primary metabolism, while GSII may be particularly involved in secondary metabolism [Hillemann et al., 1993; Reuther & Wohlleben, 2007].

Depending on the conditions, the complex regulation of nitrogen metabolism in *S. coelicolor* involves additional control by other regulators such as PhoP [Rodríguez-García et al., 2009; Sola-Landa et al., 2013], Crp [Gao et al., 2012], ArgR [Perez-Redondo et al., 2012], AfsR [Santos-Beneit et al., 2012], and AfsQ1 [Wang et al., 2013]. Under phosphate limitation, PhoP negatively controls the transcription of *glnR*, *glnA*, *glnII* as well as *amtB-glnK-glnD* [Rodríguez-García et al., 2009]. Crp positively controls *glnA*, *glnII*, and *amtB-glnK-glnD* (but not *glnR*). Also, this regulator controls the interface of primary and secondary metabolism [Gao et al., 2012]. AfsR regulates expression of *glnR* in response to an unknown nutrient stress stimulus [Santos-Beneit et al., 2012]. AfsQ1 is involved in the regulation of the carbon, nitrogen, and phosphate metabolism in the presence of glutamate [Wang et al., 2013]. Additionally, AfsR and AfsQ1 are implicated in the control of secondary metabolite production.

Regulation of nitrogen metabolism in *S. coelicolor* involves additional post-translational control. Depending on the ammonium level, ammonium uptake is post-translationally controlled by the interaction of the nitrogen sensor protein PII (GlnK) with the ammonium transporter (AmtB). Under high ammonium concentrations, GlnK is inactivated by the GlnD (adenylyltransferase)-mediated adenylation or specific proteolysis [Fink et al., 2002]. Furthermore, the activities of glutamine synthetases are controlled at the post-translational level. In response to changing nitrogen conditions, the activity of GlnA has been shown to be regulated through reversible adenylation.

Fig. 2. Schematic illustration of the post-translational regulation of nitrogen metabolism in *S. coelicolor*. GSI, glutamine synthetase I; AMP, adenosine monophosphate; UMP, uridine monophosphate; question mark, unknown regulatory protein [modified after Fink et al., 2002; Reuther & Wohlleben, 2007].

Fig. 3. 3D structures of GSI, GSII, and GSIII proteins. **a** The structure of GlnA from *S. coelicolor* (Swiss-Model based on 1FPY template). **b** The crystal structure of GlnII from *S. coelicolor* (PDB entry 4BAX by K. Zeth). **c** The crystal structure of GSIII from *B. fragilis* (PDB entry 3O6X by J.M. van Rooyen). Color scheme: chain (each protein chain has its own colour). Dashed arrows point out the catalytic centre of the enzyme, representing one of twelve catalytic centres between each monomer.

ylation and deadenylation by an adenylyltransferase GlnE [Fink et al., 1999], but there have been no reports of post-translational modification of the GOGAT enzyme [Harper et al., 2008]. A GlnE-mediated regulation of GSI was first shown in enterobacteria like *Escherichia coli*, where GlnE is regulated by the bicyclic regulatory cascade involving GlnD and GlnB (nitrogen regulatory protein P-II 1) [Magasanik 1982; Ninfa et al., 2001]. However, in *S. coelicolor*, the PII protein GlnK as well as the adenylyltransferase GlnD are not essential for the GlnE-dependent GSI regulation, since it also occurs in the *glnK* and *glnD* deletion mutants [Hesketh et al., 2002] (shown in Fig. 2).

Glutamine Synthetases of *S. coelicolor*

Glutamine synthetases (GS; EC 6.1.1.3) are ubiquitous, homo-oligomeric enzymes, which play a central role in nitrogen assimilation in all forms of life [Pesole et al., 1995]. Glutamine synthetases were hypothesized to be among the most ancient existing enzymes [Kumada et al., 1993] and are not restricted to a specific domain of life. Three distinct types of GS have been described. The GS type-1 (GSI) has been found in all prokaryotes, and also in mammals and plants [Merrick & Edwards, 1995]. GS type-2 (GSII) has been found in eukaryotes and like GSI also in some soil bacteria, for example *Rhizobium* sp.,

Fig. 4. 3D structures of GS-like proteins from *S. coelicolor* generated via homology modelling using Swiss-Model tool and based on the 1FPY template. **a** the structure of GlnA2; **b**: the structure of GlnA3; **c**: the structure of GlnA4. Color scheme: chain (each protein chain has its own colour). Dashed arrows point out the catalytic centre of the enzyme, representing one of twelve catalytic centres between each monomer.

Frankia sp., *Streptomyces* sp. [Behrmann et al., 1990; Kumada et al., 1993; Merrick & Edwards, 1995]. GS type-3 (GSIII) has been found in the anaerobe Gram-negative bacterium *Bacteriodes fragilis*, in cyanobacteria and some protozoans [Pesole et al., 1995].

Structurally, GSs are composed of two closed ring structures (with active sites formed between protomers). GSI are dodecamers, GSII are decamers, GSIII are dodecamers, while GSIII are much larger compared to GSI and GSII [Kumada et al., 1993; Eisenberg et al., 2000]. Each active site creates a binding pocket with distinct substrate binding sites for a nucleotide (ATP), amino acid (glutamate), ammonium ion, as well as two sites for divalent cations (Mn^{+2} or Mg^{+2}) [Eisenberg et al., 2000] (shown in Fig. 3).

In *S. coelicolor*, GlnA consists of 12 subunits and reveals high sequence similarity to other prokaryotic GSI enzymes [Wray & Fisher, 1988]. *glnA* is transcribed during all growth phases [Fink et al., 2002]. GlnII is a homodecamer and possesses high sequence similarity to eukaryotic GSII enzymes [Hillemann et al., 1993]. The *glnII* gene was detected in all *Streptomyces* species tested, but not in other actinobacteria, like *Mycobacterium* and *Corynebacterium* [Behrmann et al., 1990]. In contrast to *glnA* transcription, the expression level of the *glnII* transcript increases with the onset of mycelial differentiation [Fink et al., 2002], suggesting a role of GlnII in late growth phases.

Structural Analysis of the Glutamine Synthetase GlnII from *S. coelicolor*

The crystal structures of different types of glutamine synthetases have previously been elucidated and characterized in such bacteria as *Salmonella typhimurium* (PDB entry 1LGR (GSI); PDB entry 1FPY (GSI)), *Helicobacter pylori* (PDB entry 5ZLI (GSI)), *Bacillus subtilis* (PDB entry 3QAJ (GSI)), *Bacterioides fragilis* (PDB entry 3O6X (GSIII)) and *M. tuberculosis* (PDB entry 1HTO (GSI)). X-ray diffraction was used to determine the crystal structure of GSII (GlnII) in *S. coelicolor* with a resolution 2.55 Å (released in PDB as 4BAX). The GSII of *S. coelicolor* comprises 10 identical subunits organized in 2 rings. The biologically relevant decamer contains 10 active sites between each monomer. Similar to known GlnA structures, GlnII features a tunnel created by each active site, which contains three substrate binding sites for the ATP, glutamate and ammonium. In the middle, divalent cations of Mn^{+2} or Mg^{+2} can bind. Each ring of GlnII consists of 5 subunits that are held together through hydrogen bonding and hydrophobic interactions (shown in Fig. 3, b).

GS-Like Enzymes in *S. coelicolor*

Interestingly, an in silico analysis of *glnA*-genes across actinobacterial genomes demonstrated the existence of a hypothesized common ancestor of *glnA* and *glnII*. Apparently, the common ancestor has evolved to glutamine synthetase-encoding genes and subsequently to special-

ized *glnA*-like genes (*glnA2*, *glnA3*, *glnA4*) [Kumada et al., 1993; Hayward et al., 2009].

In the model actinobacterium, *S. coelicolor*, three *glnA*-like genes were identified in addition to *glnA* and *glnII*: *glnA2* (*sco2241*), *glnA3* (*sco6962*), and *glnA4* (*sco1613*) [Rexer et al., 2006; Reuther & Wohlleben, 2007]. Only GlnA and GlnII were shown to be functional glutamine synthetases [Krysenko et al., 2017]. Structural analysis of GS-like enzymes through homology modelling revealed that they are composed of 12 subunits in two rings with six subunits each, with an active site between each of two neighbouring subunits (shown in Fig. 3). Studies on GlnA2 (shown in Fig. 4, a) could show that it is involved in nitrogen metabolism and may be structurally more similar to GlnA (shown in Fig. 3, a) than GlnA3 and GlnA4, according to homology modelling (shown in Fig. 4, b and c). It has been shown that in *S. coelicolor* GlnA2 may play role in the post-translational regulation of the global nitrogen metabolism regulator GlnR and in the signal mediation of nitrogen excess to the adenylyl-transferase GlnE. Glutamine, synthesized by GlnA, seems to induce the expression of *glnA2*, leading to elevated levels of GlnA2 in the cell. GlnA2 binds subsequently to GlnR and GlnE and influences their activity [Nentwich, 2012]. Nevertheless, GlnA2 has been interpreted to be an important signal mediator and regulator in nitrogen metabolism under high N-concentrations; the role of GlnA2 in nitrogen metabolism has remained poorly understood.

GlnA2/3/4 of *S. coelicolor* show moderate levels of amino acid sequence identity compared to GlnA (GlnA2: 32% identity, 48% similarity; GlnA3: 31% identity, 50% similarity; GlnA4: 27% identity, 41% similarity). Since GS-like proteins are not able to synthesize glutamine from ammonium and glutamate, it was hypothesized that they may not accept ammonium as substrate, but other nitrogen containing compounds, which are typically present in soil. A phenotypic analysis of *glnA2*, *glnA3* and *glnA4* knockout mutants in the presence of different sole nitrogen sources finally revealed that GS-like enzymes can accept poly- and monoamines as substrates [Krysenko et al., 2017; 2019].

Polyamine Metabolism in Bacteria

Distribution and Role of Polyamines in Bacteria

Chemically polyamines are aliphatic polycations with a polycarbon chain and multiple amino groups. Commonly known and widely distributed natural polyamines present in all organisms are putrescine (1,4-diaminobu-

Fig. 5. Chemical formulae of common polyamines, ethanolamine, and selected amino acids.

tane), cadaverine (pentane-1,5-diamine), spermidine (N-(3-aminopropyl)-1,4-butanediamine), and spermine (N,N'-bis(3-aminopropyl)-1,4-butanediamine)) [Miller-Fleming et al., 2015] (shown in Fig. 5). There are other, rarely occurring polyamines in nature, for example thermine, thermospermine, caldopentamine, and others described in the extreme thermophile *Thermus thermophilus* [Oshima et al., 1988].

Polyamines can be found in diverse bacterial habitats. For instance, in soil, the concentration of putrescine ranges between 0.28 and 0.56 nmol/g, spermidine between 0.23 and 0.62 nmol/g, and spermine between 0.16 and 0.43 nmol/g. Increased polyamine concentrations lead to bacterial cell death [Young & Chen, 1997].

Intracellular polyamine concentrations vary between species. For example, *E. coli* contains high concentrations of putrescine (10–30 mM), while in most bacteria the intracellular content of putrescine ranges between 0.1 and 0.2 mM [Cohen, 1998]. In γ -proteobacteria, polyamine

thine decarboxylation through an ornithine decarboxylase, but it can also be synthesized from arginine via arginine decarboxylase and agmatinase [Miller-Fleming et al., 2015]. Spermidine and spermine are formed from putrescine via addition of aminopropyl groups, which are provided by decarboxylated S-adenosylmethionine (dcAdoMet, dSAM). dSAM is produced from S-adenosylmethionine by an S-adenosylmethionine decarboxylase (AdoMetDC, SpeD). The addition of aminopropyl groups is catalyzed by spermidine and spermine synthetases (SpdS/SpeE and SpmS) [Miller-Fleming et al., 2015; Kusano & Suzuki, 2015]. Cadaverine is a product of lysine decarboxylation by lysine decarboxylases (LDCs) that have been studied in *E. coli*, *Vibrio* sp., and *Lactobacillus* sp. [Tomar et al., 2013].

In *S. coelicolor*, the biosynthesis and intracellular content of polyamines have barely been investigated. It was reported that putrescine and spermidine biosynthesis may occur in the late-stationary phase in minimal liquid medium, while cadaverine may be synthesized under iron limitation [Burrell et al., 2012]. After growth in complex medium, low concentrations of putrescine, cadaverine, spermidine, and spermine (ca. 0.05–0.1 $\mu\text{mol/g}$) were detected in *S. coelicolor* JCM4357 [Hamana & Matsuzaki, 1987].

In addition to de novo biosynthesis, the uptake of external polyamines can provide polyamines for bacterial cells. Since polyamines are positively charged and hydrophilic molecules at physiological pH, they cannot pass cellular membranes by diffusion. Hence, an active transport system is required for polyamine uptake. Moreover, polyamine uptake conserves energy, since the biosynthesis of S-adenosylmethionine (AdoMet) requires ATP [Kusano & Suzuki, 2015]. Transport of polyamines has been investigated predominantly in pathogenic bacteria, such as *Proteus mirabilis*, *Vibrio cholerae*, *Streptococcus pneumoniae*, and *Aggregatibacter actinomycetemcomitans*, as well as in *E. coli*, which possesses several polyamine transporters [Kusano & Suzuki, 2015]. However, the polyamine uptake in *S. coelicolor* is largely uninvestigated. Some candidate genes that may encode proteins for polyamine uptake were defined in an RNAseq analysis (see section Further Identification of Polyamine-Associated Genes in *S. coelicolor*).

Polyamine Assimilation in Bacteria

Polyamine assimilation is required to control the intracellular polyamine pool, to detoxify polyamines under excess and to utilize them as C/N source under deficiency conditions. This process has been studied extensively in the Gram-negative bacteria *E. coli* and *P. aeruginosa* POA1.

It was reported that these bacteria can efficiently utilize polyamines as carbon and nitrogen sources [Kurihara et al., 2005; Yao et al., 2011; Kusano & Suzuki, 2015]. In *P. aeruginosa* and *E. coli*, putrescine can be catabolized to succinate via the aminotransferase pathway [Schneider & Reitzer, 2012] or the γ -glutamylase pathway [Kurihara et al., 2008; Yao et al., 2011; Schneider & Reitzer, 2012; Krysenko et al., 2017] (shown in Fig. 6, in black). Further polyamine utilization pathways have been described in bacteria, such as the direct oxidation pathway for putrescine in *P. aeruginosa* (shown in Fig. 6, in orange), the spermine/spermidine dehydrogenase pathway for spermine in *P. aeruginosa* (shown in Fig. 6, in orange), the acetylation pathway for spermidine in *E. coli* and *B. subtilis* (shown in Fig. 6, in blue) [Forouhar et al., 2005; Yao et al., 2011; Foster et al., 2013; Campilongo et al., 2014]. The different pathways and enzymes involved are shown in Figure 6.

Regulation of Polyamine Assimilation Genes in Bacteria

Since polyamines are involved in multiple bacterial functions, but can lead to cell death in excess, strict regulation of intercellular polyamine concentration at the level of biosynthesis, degradation, uptake, and efflux is required. Regulation of the polyamine-associated genes, such as those from the aminotransferase pathway and the gamma-glutamylase pathway, have been extensively studied in *E. coli*. It has been shown that in *E. coli* the gene regulation involves the alternative sigma factor σ^S , the alternative sigma factor for nitrogen-controlled genes σ^{54} , the nitrogen regulatory protein C (NtrC) and the nitrogen assimilation control protein (Nac). The genes of the gamma-glutamylase pathway in *E. coli* can be regulated by the repressor PuuR, which controls the expression of *puu* genes in *puuAP* and *puuDRCBE* operons containing polyamine utilization genes [Partridge et al., 2006; Kusano & Suzuki, 2015].

Regulation of the polyamine-associated genes was only recently studied also in *S. coelicolor* (see section Regulation of Polyamine Assimilation Genes in *S. coelicolor*) revealing the participation of components similar to those known from Gram-negative bacteria.

Ethanolamine Metabolism in Bacteria

Distribution and Role of Ethanolamine in Bacteria

The monoamine ethanolamine is a primary amine and primary alcohol and belongs to the class of aliphatic amino alcohols (shown in Fig. 5). Ethanolamine is a nitrog-

Fig. 7. Combined model of ethanolamine utilization pathways within prokaryotes [modified after Krysenko et al., 2019]. EUTP, *eut*-pathway; GGP, gamma-glutamylation pathway; AMP, amination pathway; BUE, biosynthetic utilization of ethanolamine. Dashed arrow represents diffusion. Pathways in black: *E. coli* and *S. typhimurium*, blue: *M. tuberculosis*, orange: *C. salexigens*, red: *S. coelicolor*.

enous base in phospholipids, and together with glycerol, fatty acid esters, and phosphoric acid, it is a building block of biomembranes in animals. It is a frequently occurring compound in nature as a component of cell membranes in form of phosphatidylethanolamine, which is the second most-abundant head group for phospholipids. Ethanolamine is an abundant compound in the human intestinal tract as well as in processed food [Kofoid et al., 1999; Anderson & Kendall, 2016].

Ethanolamine has been demonstrated to be a source of carbon and nitrogen for gut-associated predominantly Gram-negative bacteria, such as *Escherichia* and *Salmonella* [Tsoy et al., 2009]. Ethanolamine utilization is a virulence determinant and contributes to a competitive advantage for intestinal pathogens [Kaval & Garsin 2018].

Ethanolamine, its incorporation into the cell membrane and its biological role, were also studied in Gram-positive bacteria such as *Mycobacterium* sp. [Nandedkar, 1974], *Corynebacterium* sp., *Listeria* sp., *Enterococcus* sp. and *Clostridium* sp. [Tsoy et al., 2009; Garsin, 2010], and recently in *S. coelicolor* [Krysenko et al., 2019].

Ethanolamine Biosynthesis and Uptake in Bacteria

The biosynthesis of ethanolamine can occur from serine. This process has been described in plants, where ethanolamine is biosynthesized from serine by decarboxylation, a process which involves a phosphatidylserine decarboxylase. As an endogenous alkalamine, ethanolamine is a principal precursor of phosphoglycerides, which are important elements in the structure of choline in biological membranes [Bingham et al., 2001]. However, in comparison to plants and mammals the biosynthesis of ethanolamine from serine has less been studied in bacteria. Similar to plants, yeast and bacteria also possess the enzyme phosphatidylserine decarboxylase for decarboxylation, but they do not synthesize ethanolamine directly as a precursor of phosphatidylserine. Instead, phosphatidylserine is predominantly synthesized in a reaction catalyzed by a phosphatidylserine synthase. The intracellularly produced phosphatidylserine can be decarboxylated into phosphatidylethanolamine, which subsequently can be cleaved by phosphodiesterases into glycerol and ethanolamine, for example during cell lysis [Cronan, 2003].

Extracellular ethanolamine can also pass into the cell through carrier-mediated transport or diffusion [Penrod et al., 2004]. For instance, in *E. coli* and *S. typhimurium*, ethanolamine is transported into the cell by the transport protein EutH, which is similar to permeases [Stojiljkovic et al., 1995]. Most Actinobacteria and Proteobacteria can take up ethanolamine with a transporter encoded by the *eat* gene, which is a functional, non-homologous equivalent to *eutH* from *S. typhimurium* [Tsoy et al., 2009; Garsin, 2010].

Ethanolamine Assimilation in Bacteria

Ethanolamine utilization as a source of carbon and nitrogen has been reported in *Salmonella*, *Escherichia*, *Erwinia*, *Flavobacterium*, *Klebsiella*, *Pseudomonas*, *Achromobacter*, *Vibrio*, *Escherichia* as well as in *Enterococcus*, *Arthrobacter*, *Mycobacterium*, *Corynebacterium*, *Clostridium* [Del Papa & Perego et al., 2008; Tsoy et al., 2009; Garsin, 2010], and in *S. coelicolor* [Krysenko et al., 2019].

Ethanolamine utilization in *E. coli* and *S. typhimurium* occurs in a bacterial microcompartment called the metabolosome [Kofoed et al., 1999]. In *S. typhimurium* and *E. coli*, the ethanolamine catabolic pathway includes 17 proteins encoded by genes from the ethanolamine utilization operon *eut* [Blackwell et al., 1976; Stojiljkovic et al., 1995; Kofoed et al., 1999]. The metabolosome is required to retain acetaldehyde, the intermediate of ethanolamine utilization. It prevents the loss of this volatile C-source and protects the cell from the toxic effects of acetaldehyde [Penrod & Roth, 2006]. After ethanolamine enters the cell via the ethanolamine transporter EutH or by diffusion, it reaches the metabolosome and is subsequently broken down via ethanolamine ammonia lyase (EutBC) into acetaldehyde and ammonia. The ammonia serves as a supply of reduced nitrogen, while acetaldehyde is converted into acetyl-CoA, which is used in the TCA cycle, glyoxylate cycle, and lipid biosynthesis [Garsin, 2010; Kaval & Garsin, 2018] (shown in Fig. 7, in black).

Besides canonical ethanolamine utilization pathways that involve the metabolosome, some alternative pathways have been described. Reductive amination of glyoxylate to glycine by glycine dehydrogenase has been reported in *M. tuberculosis* [Goldman & Wagner, 1962]. The conversion of ethanolamine to glycine via a pathway with the intermediates glycoaldehyde and glyoxalate was reported in *Mycobacterium* sp. 607 [Nandedkar, 1974]. However, it was proposed that in *Mycobacterium* sp. ethanolamine cannot be directly utilized as a C- or N-source. It can be

transformed into phosphatidylethanolamine (a process also described as biosynthetic utilization of ethanolamine) [Nandedkar, 1975; Shukla & Turner, 1980] (shown in Fig. 7, in blue).

It was also shown that diverse organisms ranging from the Actinobacteria to the Proteobacteria may possess the genetic capability for ethanolamine metabolism, which does not require *eut* genes. An ethanolamine glutamylation pathway was proposed for a γ -proteobacterium *Chromohalobacter salexigens* based on the EFI-GNT web tool input. In *C. salexigens*, ethanolamine may be glutamylated by a member of the glutamine synthase family (PF00120; ethanolamine γ -glutamylase) and subsequently reduced to L-glutamate and glycine, which are further assimilated as an N-source [Gerlt, 2016] (shown in Fig. 7, in orange).

Regulation of Ethanolamine Assimilation Genes in Bacteria

Strict control of the ethanolamine utilization genes is required in order to regulate the intracellular ethanolamine concentration and to avoid its toxic effects.

Two regulatory mechanisms of ethanolamine utilization genes were studied in *E. coli* and *S. typhimurium* (the EutR system) as well as in *Enterococcus faecalis* (the EutV-EutW system). In *S. typhimurium*, an EutR regulator, which belongs to the AraC family of transcriptional regulators, positively regulates the transcription of the *eut* operon [Roof & Roth, 1992; Garsin, 2010]. In contrast, *E. faecalis* lacks the *eutR* gene, and the ethanolamine utilization is regulated by a two-component system composed of EutV (response regulator) and EutW (sensor histidine kinase) [Del Papa & Perego, 2008].

It has been shown that the regulation of the *eut* operon may involve also other regulators. In *S. typhimurium*, the expression of the *eut* operon may be influenced by CsrA, the global regulator of invasion genes. The expression of the *eut* operon in *E. faecalis* may be affected by Fsr, the global transcriptional regulator of serine protease- and gelatinase-encoding genes [Fox et al., 2009; Garsin, 2010].

Polyamine and Ethanolamine Metabolism in *S. coelicolor*

Polyamine Utilization in S. coelicolor

In recent studies, a polyamine utilization pathway in *S. coelicolor* was postulated. It has been shown that this actinobacterium possesses a gamma-glutamylolation path-

way for polyamine utilization, which has similarities to that known in *E. coli* and *P. aeruginosa* [Krysenko et al., 2017]. The initial step of the polyamine utilization is catalyzed by the GlnA3 enzyme, which is able to glutamylate the polyamines putrescine, cadaverine, spermidine, and spermine [Krysenko et al., 2017].

Based on transcriptional studies and in silico analysis, it was possible to postulate a pathway for the subsequent steps: The glutamylated products are transformed by the predicted gamma-glutamylpolyamine oxidoreductase (SCO5671) in the second step. The SCO5671 enzyme is a close ortholog of the gamma-glutamylpolyamine oxidoreductases PuuB from *E. coli* and PauB1-B4 from *P. aeruginosa*. In the second step of the polyamine utilization pathway, SCO1281 and SCO6051 are involved. The third step of the utilization pathway is catalysed by the predicted dehydrogenases (SCO5666 and SCO5657), which are predicted homologs of the (gamma-glutamyl-) gamma-aminobutyraldehyde dehydrogenases PuuC and PatD from *E. coli*. The fourth step of the pathway requires predicted hydrolases (SCO5666, SCO5657 and SCO6961) and results in the production of aminovalerate or GABA. Afterwards, the GABA aminotransferase (SCO5676) that is a predicted homolog of GabT from *E. coli*, catalyses the production of glutarate semialdehyde or succinate semialdehyde in the fifth step. In the last step of the polyamine utilization pathway, SCO5679, which is a predicted homolog of the succinic semialdehyde dehydrogenase GabD from *E. coli*, is involved, terminating the pathway by releasing succinate or glutarate and feed the tricarboxylic acid (TCA) cycle (shown in Fig. 6, see GGP). Moreover, the expression of *sco5679* appeared to be induced by cadaverine, which was confirmed by RNAseq, supporting the hypothesis that SCO5679 is involved in the last step of polyamine utilization in *S. coelicolor* (see section Further Identification of Polyamine-Associated Genes in *S. coelicolor*).

There is a possibility of an alternative polyamine utilization pathway in *S. coelicolor*, since a predicted amidotransferase (SCO5655) was identified. This enzyme is an orthologue of the putrescine aminotransferase (PatA) from *E. coli* (shown in Fig. 6, see AMTP). Transcriptional analysis revealed that the expression of *sco5655*, *sco6960*, *sco6961* was enhanced in presence of polyamines [Krysenko et al., 2017] and *sco5655* was reported to be induced by a diamide [Kallifidas et al., 2010] and not by arginine [Perez-Redondo et al., 2012]. However, further analysis of SCO5655 as well as other potential aminotransferase homologs of PatA (SCO1223, SCO1284, SCO6769) are required in order to determine

the functionality of the PatA-dependent pathway (shown in Fig. 6, see AMTP).

Regulation of Polyamine Assimilation Genes in S. coelicolor

The recently postulated gamma-glutamylation polyamine utilization pathway in *S. coelicolor* (see above) is likely to be regulated by SCO5656 (EpuRII). *sco5656* is localized close to the genes that encode predicted enzymes of the polyamine utilization pathway and is annotated as putative regulator. A possible role of EpuRII in the regulation of polyamine metabolism is supported by the observation that the expression of the *epuRII* gene was enhanced in the presence of putrescine, cadaverine, and spermidine [Krysenko et al., 2017]. Moreover, preliminary EMSA analysis of potential regulatory targets of EpuRII revealed hints towards a complex regulation of several polyamine-associated genes, including *glnA3*, *sco5676* (encoding a putative homolog of the 4-amino-butyrate aminotransferase GabT of *E. coli* K12), and *sco5977* (encoding a putative polyamine antiporter) [Krysenko, 2018]. Further candidate genes that may encode regulators of polyamine metabolism were defined by RNAseq analysis (see section Further Identification of Polyamine-Associated Genes in *S. coelicolor*).

Further Identification of Polyamine-Associated Genes in S. coelicolor

In order to identify further genes, which may encode enzymes required to metabolize polyamines or glutamylated polyamines (shown in Fig. 6), respectively, an in silico analysis was performed. Subsequently, the association of these genes to the polyamine metabolism was investigated using RT-PCR and RNAseq analysis. Expression of genes in the presence of polyamines (putrescine, cadaverine, spermidine, spermine) as the only nitrogen source was compared with their expression in the presence of ammonium. RNAseq analysis revealed significantly ($p_{\text{adj}} \leq 0.01$, A-value: 6) induced expression of the amino acid transporter-encoding gene *sco2724* by cadaverine, of the ammonium transporter AmtB-encoding gene *sco5583* by putrescine and of the transmembrane efflux protein *sco3915* by spermine. Some genes (*sco3435* and *sco5877*), whose gene products were annotated as transcriptional regulators, showed elevated expression levels in the presence of polyamines: the expression of *sco3435* appeared to be induced by spermine, while the expression of *sco5877* by putrescine.

Furthermore, this transcriptional analysis indicated that the expression of *sco5679* is induced by cadaverine. This finding strengthened the hypothesis that *sco5679* is involved in polyamine metabolism by encoding an aldehyde dehydrogenase responsible for the last step of polyamine utilization in *S. coelicolor*.

Ethanolamine Utilization in *S. coelicolor*

Studies on GS-like enzymes from *S. coelicolor* revealed that GlnA3 is involved in polyamine metabolism, and GlnA2 may play a regulatory role in the nitrogen metabolism (see above). In silico analysis of GlnA4 showed low amino acid sequence similarity with known proteins involved in nitrogen metabolism but predicted structural features that differ from GlnA and other GS-like proteins. A phenotypic analysis of the *glnA4* mutant revealed growth defects in the presence of ethanolamine. Ethanolamine is glutamylated by the gamma-glutamylethanolamide synthetase GlnA4 in the first step of the utilization pathway [Krysenko et al., 2019]. Further steps of the ethanolamine utilization pathway were postulated based on in silico and transcriptional analysis. The pathway may require a predicted gamma-glutamylethanolamine dehydrogenase (SCO1611), a predicted gamma-glutamylaldehyde dehydrogenase (SCO1612) and a predicted gamma-glutamylglycine amidohydrolase (SCO1615) in further reactions. Interestingly, the RNAseq analysis revealed that the *sco1615* gene was induced by putrescine. The pathway may end in the production of glycine and glutamate [Krysenko, 2018; Krysenko et al., 2019] (shown in Fig. 7, in red; Table 1).

Regulation of Ethanolamine Assimilation Genes in *S. coelicolor*

In contrast to *S. typhimurium* and *E. faecalis*, until recently, nothing was known about the regulation of ethanolamine utilization in Actinobacteria and in *S. coelicolor*. In the genome of *S. coelicolor*, a gene *sco1614* (*epuRI*) can be found, annotated as gene for a putative repressor and localized close to the gene *glnA4* (*sco1613*). It was therefore assumed that genes encoding predicted enzymes for the ethanolamine utilization pathway may be regulated by EpuRI (SCO1614). EMSA analysis indeed revealed that *glnA4* as well as the promoter sequence of *epuRI* are potential targets of EpuRI [Krysenko, 2018]. The genes located downstream of *glnA4* that encode predicted enzymes of the ethanolamine utilization pathway (see above) are organized in one putative operon. Thus, these genes are most likely transcribed together with *glnA4* and regulated by EpuRI. Its possible role as a nega-

tive transcriptional regulator of the ethanolamine utilization associated genes was shown in a transcriptional analysis. The deletion of *epuRI* allowed *glnA4* expression in the presence of ammonium that does not occur in the parental strain [Krysenko et al., 2019].

Conclusion

Streptomycetes have evolved a large number of metabolic properties required for the survival under diverse environmental conditions found in soil. This is also reflected by the large genome of most streptomycetes. One of these special properties is their ability to detoxify polyamines and ethanolamine and to utilize them as C/N-sources. The key mechanism of polyamine/ethanolamine assimilation is the glutamylation of the substrate. GlnA3 and GlnA4, which are required for this reaction, are evolutionary derived from a universal GS enzyme. Instead of glutamylation of ammonium, GlnA3 and GlnA4 are able to glutamylate polyamines and ethanolamine in an analogous reaction. For subsequent conversions that are required for the integration of polyamines and ethanolamine into primary metabolism, candidate proteins were identified using in silico as well as transcriptional analysis. This enabled the first postulation of polyamine/ethanolamine utilization pathways in *S. coelicolor*. The role of these proteins in polyamine/ethanolamine metabolism will be demonstrated in biochemical analyses in the near future.

Enzymes that are able to glutamylate mono- and polyamines have been described in a small number of Gram-negative bacteria, such as *E. coli* (PuuA) [Kurihara et al., 2008], *P. aeruginosa* (PauA7) [Yao et al., 2011], and *C. salexigens* (PF00120 family member) [Gerlt, 2016]. These enzymes share similarities to GlnA3 and GlnA4 from *S. coelicolor*. Furthermore, in silico analysis showed that orthologs of GlnA3 and GlnA4 can be found in Gram-negative and Gram-positive bacteria that live and colonize specific habitats. For example, across Actinobacteria (24 genera) orthologs of GlnA3 are present in about 29% of representative species and GlnA4 in 54% of species.

The presence of GS-like enzymes GlnA3 and GlnA4 are a special feature of streptomycetes. Only *Mycobacterium* sp., which has a very similar nitrogen metabolism, shares this feature. While streptomycetes have to cope with polyamines and ethanolamine in their main habitat soil, pathogenic mycobacteria are exposed to elevated polyamine concentrations during the infection process in

macrophages. In this case, GlnA3 enables the pathogens to survive in their host. Thus, the inhibition of GlnA3 should prevent survival of *Mycobacterium* in macrophages and offers a novel approach to combat mycobacterial infections.

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Conflict of Interest Statement

All authors declare no conflict of interest to declare.

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Author Contributions

S.K. wrote the manuscript. S.K. and T.B. performed the RNA-seq analysis. W.W. corrected the manuscript and provided helpful feedback of each draft of the paper. A.M. and A.B. provided helpful feedback.

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