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Review

Impact of Nitrogen-Containing Compounds on Secondary Metabolism in *Streptomyces* spp.—A Source of Metabolic Engineering Strategies

Sergii Krysenko

Valent BioSciences LLC Biorational Research Center, 1910 Innovation Way, Suite 100, Libertyville, IL 60048, USA; sergii.krysenko@valentbiosciences.com; Tel.: +1-(224)-563-7863

Abstract: Actinobacteria from the genus *Streptomyces* feature complex primary and secondary metabolism, developmental cycle, and ability to produce a variety of natural products. These soil bacteria are major producers of antibiotics and other bioactive compounds and have been extensively investigated due to the medical and industrial relevance of *Streptomyces*-derived secondary metabolites. However, the genetic toolbox for *Streptomyces* engineering as well as yield optimization strategies for the production of relevant metabolites are limited. On the one hand, the genetic potential of these organisms has not been fully utilized due to many "silent" or poorly expressed biosynthetic gene clusters, whose activation depends on environmental stimuli and nutrient availability. On the other hand, these GC-rich Gram-positive bacteria are difficult to manipulate, and traditional genetic manipulation strategies are time-consuming and have low efficiency. Recent studies of Streptomyces metabolism and genomes provided new insights into possibilities to overcome these challenges. In this review, advances and approaches for *Streptomyces* manipulations and secondary metabolite production optimization are discussed. Special focus is given to understanding the interplay between primary and secondary metabolism in Streptomyces and the supply of nitrogen-containing compounds into secondary metabolism. Existing strategies to manipulate cellular metabolism in *Streptomyces* are reviewed.

Keywords: *Streptomyces*; biotechnology; nitrogen metabolism; metabolic engineering; secondary metabolites; synthetic biology

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1. *Streptomyces* sp. as a Source of Secondary Metabolites

Streptomycetes are an excellent source of identified natural products due to their impressive ability to form a variety of interesting secondary products [1,2]. This has made these bacteria the focus of applications in industry and in research. These include applications in numerous fields in medicine, agriculture, and biotechnology. *Streptomyces*-derived natural product discoveries started in 1947 and peaked in the 1960s following a significant decline in the following decades. The development of combinatorial chemistry in combination with high-throughput screening and a rather limited funding for drug discovery contributed to this decline. Recently, new methods of genetic engineering, fermentation optimization protocols, and bioinformatics technologies including genome mining in addition to classic bioprospecting and bioassay-guided isolation reactivated the field. Different studies claim that the genus can synthesize some 150,000 more antimicrobial compounds than those currently known. These factors influenced recent constantly increasing interest in natural product discovery in the genus *Streptomyces* [3].

1.1. Streptomycetes as Biological Natural Product Producers

The secondary metabolites produced by Streptomycetes show structural diversity—the underlying chemical structure includes aminoglycosides, polyketides, non-ribosomal synthesized peptides, polypeptides, glycosides, terpenoids, lipoproteins, alkaloids, polyethers,

lantibiotics, and other compounds. Examples of secondary metabolites include antibiotics (e.g., chloramphenicol, lincomycin, neomycin, streptomycin, tetracycline), immunosuppressants (FK-506, FK-520), antimycotics/fungicides (e.g., nystatin, natamycin, amphotericin B), herbicides (phosphinothricin), anti-tumor substances (doxorubicin), anthelmintic substances (avermectin), growth promoters in ruminant animal feed (monensin), anticholesterol and coccidiostatic substances (e.g., lasalocid), and insecticides (milbemycin) [2,4].

Despite the great diversity, many secondary metabolites in *Streptomyces* are based on similar biosynthetic mechanisms. Basic units and main stages are defined by a systematic regulated production process consisting of biosynthetic steps that can be found in different production strains. More than two-thirds of all known antibiotics are synthesized by streptomycetes [5], but there are many more unknown compounds produced by *Streptomyces* [6]. In the search for new natural products, different paths have been taken including biological and chemical screening as well as genome mining [7]. These strategies allow us to reveal the entire potential of *Streptomyces* including silent biosynthetic gene clusters [8]. Streptomycetes are not only the source of many bioactive compounds but are also important biotechnological producers of such substances. Polyketides are a particularly large and diverse group of secondary metabolites produced in complex biosynthesis.

1.1.1. Secondary Metabolites as a Bacterial Survival Strategy

Secondary metabolites are not essential for primary metabolism and homeostasis in Streptomycetes, but they protect cells from environmental stress and selective pressure [9]. For instance, siderophores serve to improve the absorption of iron from the environment [10], in which biosynthesis is induced by intracellular iron deficiency and can have a growth-promoting effect on the host organism [11,12]. Another example is the colored terpenoid carotenoids [13] produced to protect against photo-oxidative damage and oxygen radicals [14]. Their biosynthesis in Streptomycetes can be induced by light but is rather inconsistent [15]. Furthermore, pigments such as melanin can protect against UV damage [16,17]. Melanin has been shown to have antibiotic and antimicrobial activities and to act as a cation chelator and antioxidant [18]. Another important group of secondary metabolites from Streptomycetes is terpenoids that can act as antibiotics, hormones, odorants, and flavorings. For example, albaflavenone, a tricyclic sesquiterpene antibiotic [19], and ectoine are effective against osmotic stress [20] and are able to prevent protein misfolding [21,22]. In *S. coelicolor* A3(2), it was shown that hopanoids are formed during the transition from substrate hyphae to aerial hyphae [23]. Other secondary metabolites such as antibiotics also represent a fitness advantage in the fight for nutrients.

1.1.2. Secondary Metabolite Production in *Streptomycetes*

Antibiotics are low-molecular-weight metabolites (M < 2000 Da) with a diverse chemical structure derived from living microorganisms, usually at low concentrations (<200 µg/mL), in stepwise biosynthesis, and can inhibit the growth of other microorganisms [24,25]. Under laboratory conditions, the production of many antibiotics can be influenced by the medium or nutrient sources available from the medium. Diverse chemical compounds acting as activators for signaling cascades that promote the production of certain antibiotics [26] may induce cryptic gene clusters that are not activated under standard conditions.

The natural role of antibiotics can be studied only in habitat-like environments where they are in response to interactions with different organisms [27]. The actual concentrations of antibiotics are difficult to estimate in nature since their production depends, among other things, on the availability of nutrients [28]. Sites of action of antibiotics include essential processes such as nucleic acid synthesis, protein biosynthesis, cell membrane and cell wall-associated enzymes, and lipid acid biosynthesis of the cell [29,30], allowing killing or growth retardation of other microorganisms.

On the other hand, experiments with sub-inhibitory antibiotic concentrations (SICs), e.g., concentrations below the minimum inhibitory concentration (MIC), were shown

to influence transcription, biofilm formation, and gene expression [31–33]. Secondary metabolites have also been described as auto-inducers of antibiotic production that can affect the biosynthesis of other antibiotics depending on the concentration of an inducer compound, which can be an antibiotic itself [34].

The biosynthesis of secondary metabolites is usually expensive to the cell, the costs of which are defined by the benefits of genes whose expression is often limited and subject to complex regulation. Diverse proteins are required for the biosynthesis of antibiotics, which are usually located in gene clusters with tightly regulated expression [35,36]. The number and organization of genes within biosynthetic gene clusters can vary greatly; mostly, these genes are required for backbone formation, modification, export, regulation, and resistance [37].

The regulation of secondary metabolism in Streptomycetes is very complex and is coordinated on different levels that are strictly coordinated with each other. The cell relies on the perception of growth-related signals that must be integrated into the complex regulatory network. The central components of the global as well as specific regulatory cascades are extra- and intracellular effector molecules. The transition from primary to secondary metabolism goes hand in hand with the morphological and physiological differentiation of Streptomycetes. On solid media, the onset of secondary metabolism is mostly associated with aerial mycelium formation involving the *bld* genes. In liquid culture, the start of secondary metabolism correlates with entry into the stationary growth phase, which is typically linked to nutrient limitation. For instance, it has been shown that low phosphate concentrations and a lack of amino acids that are sensed by the effector molecule ppGpp can act as a trigger for the production of secondary metabolites [38,39]. Furthermore, a relationship has been demonstrated between elevated cAMP (cyclic adenosine monophosphate) levels and increased production of secondary metabolites, particularly antibiotics but also other biologically active substances [40]. Another example is the γ-butyrolactones that represent a group of extracellular effector molecules in Streptomycetes [41] and other Actinomycetes [42]. They are involved in the regulation of secondary metabolism and morphological differentiation. In addition to global regulatory mechanisms, there are also biosynthetic cluster-specific regulators or regulations on systems. These are regulatory proteins that activate the transcription of genes of associated gene clusters, as well as proteins that repress the transcription of target genes. The underlying regulatory cascades can be very complex and interconnected [43].

1.2. Strategies for Discovery and Optimization of Secondary Metabolite Production in Stretpomyces

In most cases, secondary metabolite biosynthetic genes are clustered, and all genes required for synthesis, export, or resistance are located next to each other in the genome [37]. To date, only a few exceptions have been reported, e.g., gene clusters for ansamitocin [44] and pristinamycin [45].

Until recently, most of the secondary metabolites in *Streptomyces* were discovered using bioassay-guided isolation and chemical characterization of compounds of interest. Because of the biochemical complexity of biosynthetic gene clusters (BGCs), the discovery of new secondary metabolites has been challenging. This limitation has been overcome recently with the development of a genome mining approach, in which advances in DNA and RNA sequencing technologies have resulted in a rapid increase in the number of high-quality *Streptomyces* genome sequences as well as transcriptomics data. The large number of genome sequences from this genome mining provides resources for novel secondary metabolite discovery using bioinformatic analysis of *Streptomyces* genomes in silico. Interestingly, recent reports have revealed that each sequenced *Streptomyces* genome contains approximately 20–50 BGCs, a greater number than previously known.

Most BGCs are not expressed or poorly expressed in *Streptomyces* under laboratory conditions (silent BGCs) [46]. Genomes of well-studied strains such as *S. coelicolor*, *S. griseus*, and *S. avermitilis* encode more than 30 BGCs; however, only three to five secondary

metabolites were detected in these strains. For the activation of silent BGCs in *Streptomyces* strains, diverse methods have been applied, including heterologous gene expression, promoter replacement, overexpression, or repression of regulatory genes, refactoring of targeted BGCs, etc. A non-directed activation of silent BGCs has also been achieved using co-cultivation methods as well as the One Strain–Many Compounds (OSMAC) method [47–49].

1.2.1. Bioinformatics-Based Approaches for Natural Product Discovery

For the identification of secondary metabolite BGCs from data obtained with full-genome sequencing, genome-mining tools have been developed. Some of the recently proposed bioinformatics tools include ClustSCAN, NP searcher, GNP/PRISM, and antiSMASH [46]. AntiSMASH is considered to be the most widely used genome mining pipeline that features a user-friendly web interface with the possibility to predict the broad spectrum of secondary metabolite BGCs [46] (Table 1).

For the selection of genes for precursor synthesis, a genome-wide in silico reconstruction of the metabolism (genome-scale metabolic networks) has been proposed [50–52]. It is possible to simulate the growth rate, production rate, and mutation of genes. Furthermore, BGCs may be identified using low-coverage sequencing of a plasmid library with a small insert size and subsequent database comparison. These short sequences then serve as a starting point for probe design and the screening of a cosmid or BAC library [53]. Reverse genetics is another approach for *Streptomyces* engineering. It is based on the fact that secondary metabolite BGCs contain conserved core domains. Using alignments of known proteins that have similar functions in the biosynthesis of secondary metabolites, it is possible to identify previously unknown genes from genome libraries. The availability of a large number of accessible secondary metabolite biosynthesis gene cluster sequences offers a good starting point to look for specific biosynthetic gene clusters [54].

Information about secondary metabolite BGCs obtained with genome mining is essential for secondary metabolite discovery. Furthermore, it is a resource for rational design facilitation of BGCs and yields improvement in compounds of interest. In particular, polyketides (PKs) and non-ribosomal peptides (NRPs) can be redesigned because these compounds are synthesized by connected modular enzymes, which are able to recognize module-specific amino acids or CoAs. An example is a successful replacement of module 7 of AveA3 and AveA1 in the BGC of avermectin of *S. avermitilis* with MilA1 and MilA3 in the biosynthetic gene cluster of *S. hygroscopicus* that led to milbemycin production in *S. avermitilis* [55].

For the activation of silent BGCs and triggering the production of a compound of interest, various strategies have been developed. One possibility is to induce BGC expression in a native *Streptomyces* host. An example of the application of this strategy is the titer optimization of KF-506 in *S. tsukubaensis* [56]. The advantage of this strategy is that such a host is genetically tractable and genetic manipulations of the host genome are possible, including the overexpression of regulatory genes and removal of competitive pathways with gene cluster knock-out, deactivation of negative regulatory genes, and replacement of native promoters with stronger promoters. Another possibility is to clone and/or refactor BGCs and transfer them into another non-native *Streptomyces* host for heterologous expression. This is useful for the activation of silent BGCs in genetically intractable *Streptomyces* sp. [46].

1.2.2. Genetic Tools for *Streptomyces* Engineering

Classical tools for genetic manipulation of Streptomycetes include DNA overexpression, deletion, disruption, and replacement as well as the use of suicide and temperature-sensitive plasmids, which require selection and screening for single- and double-crossover recombination events (Table 1). These strategies are time-consuming and have comparably low efficiency—double-crossover mutants are rather rarely obtained in *Streptomyces*, which demonstrates inefficient DNA homologous recombination. To address these limitations,

diverse genome editing technologies were introduced. On the one hand, techniques to express BGCs in a heterologous host have been optimized: acquisition of the target SM-BGC from the native host genome (e.g., using a genomic library of cosmids, fosmids, BAC, and PAC), ligation/assembly of the BGC to the vector (sticky/blunt end ligation, Gibson cloning, recombination in different hosts), transfer of the SM-BGC-encoded vector to the heterologous host for expression (conjugation, protoplast transformation), and target secondary metabolite production with the expression of an integrative or replicative BGC vector. On the other hand, new techniques have been proposed, including the PCR-targeting system [57], Cre-loxP recombination system [58], I-SceI promoted recombination system [59], SpCas9-based genome editing [60–63], CRISPRi-mediated gene repression for single cells [64], FnCpf1-based genome editing and CRISPRi [65], base editing tools [66,67], and alternative CRISPR/Cas-based genome editing [68]. Recent discoveries on CRISPR (the clustered regularly interspaced short palindromic repeat) and CRISPR-associated protein (Cas)-based tools further improved the genetic manipulation of *Streptomyces* sp., accelerating natural product discovery, strain improvement, and genome research [46]. Application of genetics parts, such as synthetic promoters (e.g., constitutive *ermE*, *SF14P*, *kasOP*, *gapdh*, and *rpsL* promoters as well as inducible *tipA nitA* and *xylA* promoters), ribosome-binding sites (AAAGGAGG and diverse native or synthetic RBSs), terminators (e.g., Fd, TD1) and reporter genes (e.g., genes *luxAB*, *amy*, *xylE*, and *gusA* as well as eGFP, sfGFP, mRFP, and mCherry) further expanded the toolbox for *Streptomyces* engineering [46] (Table 1).

For improvement in the yield of secondary metabolites, different genetically modified *Streptomyces* hosts (also referred to as "super-hosts") were generated by removing endogenous BGCs as well as nonessential genes and genomic regions. These include optimized *Streptomyces* strains as heterologous expression hosts that were generated by removing BGCs resulting in strains that can conserve energy and SM building blocks and have a specific precursor pool. For example, engineered strains of *S. coelicolor*, *S. lividans*, *S. albus*, *S. avermitilis*, *S. chattanoogensis*, and multiple others demonstrated improved secondary metabolite production of target compounds and reduced background chemical profiles [69].

Streptomycetes possess mechanisms to control metabolic pathways, including the production of secondary metabolites, in response to external signals and nutrient availability. Antibiotic production can be induced by substrates for antibiotic-producing enzymes or by regulation of the biosynthesis, activity, and stability of these enzymes. Nitrogen-containing compounds were shown to indirectly regulate antibiotic production by affecting the primary metabolism that provides precursor molecules for secondary metabolite biosynthesis [70]. Feedback/feedforward regulation and the regulation of nutrient supply, especially in the production of antibiotics, have been demonstrated to be mechanisms that can lead to the enhancement and overproduction of secondary metabolites for industrial needs.

Table 1. A combined list of technologies for *Streptomyces* engineering.

Technology	Category	Feature	Reference
ClustSCAN	Bioinformatics	Semi-automatic annotation of modular BGCs and in silico prediction of new chemical structures	[71]
NP searcher	Bioinformatics	Automated genome mining for natural products and rapid screening for compounds with potential value	[72]
GNP/PRISM	Bioinformatics	Identification of biosynthetic gene clusters, prediction of genetically encoded non-ribosomal peptides and type I and II polyketides, and bio- and cheminformatic dereplication of known natural products	[73]
antiSMASH	Bioinformatics	Software pipeline for genome mining with a user-friendly web interface as well as prediction of the broad spectrum of BGCs	[74]
MultiMetEval	Bioinformatics	Genome-wide in silico metabolism reconstruction	[50–52]

Table 1. *Cont.*

Technology	Category	Feature	Reference
Acquisition of the target BGC	Genetic manipulation strategy	Transfer from the native host genome using a genomic library of cosmids, fosmids, BAC, and PAC	[75]
Ligation/assembly of the BGC to the vector	Genetic manipulation strategy	Sticky/blunt end ligation, Gibson cloning, and recombination in different hosts	[46]
Transfer of the BGC-encoded vector to theheterologous host for expression	Genetic manipulation strategy	Conjugation and protoplast transformation	[46]
Target secondary metabolite production by expression of the BGC vector	Genetic manipulation strategy	Expression of integrative (pSET152, pCAP01, pESAC) or replicative (pSKC2 and pUWL201) vectors	[46]
PCR-targetingsystem	Genetic manipulation strategy	Nonpolar as well as in-frame deletion of genes or gene clusters in *Streptomyces*	[57]
Cre-loxP recombination system	Genetic manipulation strategy	Can be used in combination with the PCR-targeting system or can be independently used to knock out large fragments of DNA in *Streptomyces*	[58]
I-SceI promoted recombination system	Genetic manipulation strategy	I-SceI meganuclease can recognize an 18 bp unique sequence and cause DNA double-strand breaks (DSBs), which promote double-crossover recombination events	[59]
SpCas9-based genome editing	Genetic manipulation strategy	CRISPR/Cas-based technology does not require the pre-integration of a unique enzyme recognition sequence into the genome, but uses a transcribed synthetic guide RNA to direct Cas proteins to any site on the genome. Editing plasmids: pCRISPomyces-1/2, pKCas9dO, pCRISPR-Cas9-ScaligD, and pWHU2653	[60–63]
CRISPRi-mediated gene repression for single cells	Genetic manipulation strategy	Gene repression tool based on dCas9 or ddCpf1 and the base editors (BEs) for targeted base mutagenesis based on dCas9 or Cas9n	[64]
FnCpf1-based genome editing and CRISPRi	Genetic manipulation strategy	Editing plasmids: pKCCpf1, pKCCpf1-MsmE, and pSETddCpf1	[65]
CRISPR/Cas-based base editing tools	Genetic manipulation strategy	Editing plasmids: pCRISPR-cBEST/-aBEST, and pKC-dCas9-CDA-ULstr	[66,67]
Alternative CRISPR/Cas-based genome editing	Genetic manipulation strategy	Editing plasmids: pCRISPomyces-FnCpf1, pCRISPomyces-Sth1Cas9, and pCRISPomyces-SaCas9	[68,76]
Synthetic promoters	Genetics parts	Constitutive *ermE*, SF14P, *kasOP*, *gapdh*, *rpsL* promoters as well as inducible *tipA nitA* and *xylA* promoters	[46]
Ribosome-binding sites	Genetics parts	AAAGGAGG and diverse native or synthetic RBSs	[46]
Terminators	Genetics parts	Fdand TD1	[46]
Reporter genes	Genetics parts	Genes *luxAB*, *amy*, *xylE*, and *gusA* as well as eGFP, sfGFP, mRFP, and mCherry	[46]

2. Importance of Nitrogen for Secondary Metabolism in *Streptomyces*

2.1. Nitrogen as a Key Element for Cellular Metabolism

Nitrogen is one of the essential elements in living systems along with carbon, hydrogen, and oxygen. It makes up to 14% of the cellular dry weight. It is a key element for the

biosynthesis of nucleotides and amino acids—building blocks of DNA, RNA, and proteins. Soil, a natural environment for *Streptomyces*, is characterized by comparatively low nitrogen availability (<0.1 mM) and, at the same time, variable nitrogen amounts [77].

The main source of nitrogen in the soil is plant matter, but it is rather low because the major component of plant cell walls (~50%) is the unbranched polysaccharide cellulose [78]. Nitrogen has a major influence on morphological and physiological differentiation in bacteria [79]. A limitation of nitrogen is considered a signal to initiate aerial mycelium formation and sporulation in Streptomycetes [80]. Furthermore, the production of many secondary metabolites is subject to nitrogen repression in Streptomycetes [81]. For instance, the formation of actinorhodin in *S. coelicolor* has been demonstrated to be inhibited by high ammonium concentrations [82].

Limited nitrogen availability in the soil is reflected in Streptomycetes in the control of primary nitrogen metabolism and the supply of nitrogen into secondary metabolism. For instance, feedback inhibition in amino acid biosynthetic pathways allows for the control of nitrogen availability, ensuring amino acid uptake from soil [78].

In addition to being important as a nutrient in primary metabolism, nitrogen also plays a key role in the morphological and physiological differentiation of *Streptomyces*. These two processes generally are temporally coupled and share genetic control elements. A signal to trigger morphological differentiation is nutrient limitation [83]. For example, it has been demonstrated that nitrogen deficiency and initiation of aerial mycelium and sporulation are coupled [80].

The formation of many secondary metabolites in Streptomycetes depends on nitrogen availability—the presence of nitrogen-containing compounds influences secondary metabolite synthesis [81]. For example, the formation of actinorhodin in *S. coelicolor* [82] and the production of pristinamycin in *S. pristinaespiralis* is inhibited at high ammonium concentrations [84].

2.1.1. Nitrogen Assimilation in *Streptomyces*

The preferred source of nitrogen for *Streptomyces* is ammonium [85]. However, in soil, nitrogen is predominantly available from nitrogen-containing compounds like proteins, amino acids, monoamines, and polyamines that are released from dead animal and plant matter [86]. Other nitrogen-containing compounds in soil include nitrate, nitrite, and urea, as well as high-molecular-weight nitrogen-containing compounds such as polymers or nucleic acids [87]. High-molecular-weight compounds are first broken down by exoenzymes into smaller molecules like glutamate and ammonium, which are then taken up from the extracellular environment and metabolized in the cell [86] (Figure 1).

Due to naturally low levels of nitrogen in the soil habitat, nitrogen assimilation in Streptomycetes occurs mainly via glutamine synthetase (GS) and glutamine-2-oxoglutarate-aminotransferase (GOGAT) [85]. GS allows nitrogen assimilation under conditions of nitrogen deficiency (<0.1 mM) because GS has a high substrate affinity (e.g., in *E. coli*: KM = 0.2 mM for ammonium). GS provides condensation of ammonium and glutamate; it catalyzes the synthesis of glutamine under ATP consumption [87]. Glutamate can be regenerated through the conversion of glutamine to alpha-ketoglutarate, which is then converted into two glutamate molecules via transamination. Furthermore, in many bacteria, glutamate is generated from glutamine and 2-oxyglutarate by glutamine-2-oxoglutarate-aminotransferase (GOGAT). Glutamine synthetase fulfills two important tasks: on the one hand ammonium assimilation and, on the other hand, the synthesis of the nitrogen donor glutamine. In addition to GS-catalyzed ATP-dependent nitrogen assimilation, many bacteria including *Streptomyces* sp. possess an alternative glutamine biosynthetic pathway. At high ammonium concentrations (>1 mM), glutamate can be formed directly from ammonium and alpha-ketoglutarate via the glutamate dehydrogenase (GDH) enzyme. This enzyme has a low affinity for substrate ammonium [85,87]. Functional glutamine synthetases have been described to occur in three forms: GSI, GSII, and GSIII. Many Streptomycetes possess multiple GS and GS-like enzymes. For instance, *S. coelicolor* contains

two functional glutamine synthetases, GlnA (GSII) and GlnII (GSII), as well as three GS-like enzymes, GlnA2, GlnA3, and GlnA4, which can catalyze glutamylation reactions [86] (Figure 1).

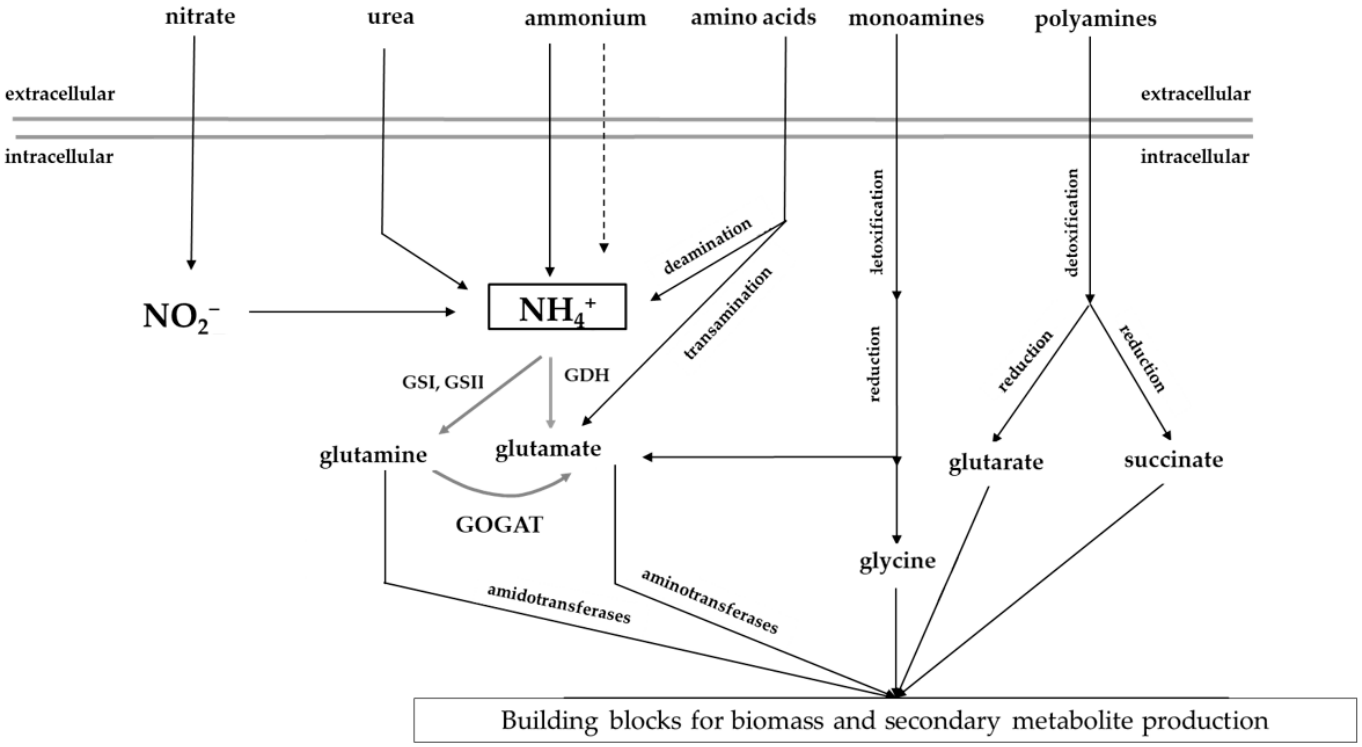

Figure 1. Schematic illustration of primary nitrogen metabolism in *S. coelicolor*. GS, glutamine synthetase; GDH, glutamate dehydrogenase; GOGAT, glutamine-2-oxoglutarate-aminotransferase (modified from [88]).

2.1.2. Regulation of Primary Nitrogen Metabolism in *Streptomyces*

GS is regulated at different levels. In *Streptomyces*, GSI expression can be detected throughout all growth phases, while significant GSII expression is detected only during the stationary phase. This suggests that the corresponding enzymes are active during these growth phases [89]. While GSI can be modified post-translationally, a modification of GSII by adenylation has not been observed so far. The enzyme activity of GSI in *S. coelicolor* is analogous to *E. coli* and is regulated post-translationally via reversible covalent modification of the adenyltransferase GlnE [89].

The regulation of GSs is linked to the nitrogen content in cells, which is a protective mechanism to maintain the intracellular glutamate pool. In contrast to GlnE in *E. coli*, where GlnD and the two PII proteins GlnK and GlnB modify GlnE depending on nitrogen availability, GlnE in *S. coelicolor* is not regulated through the interaction with the PII protein GlnK but is indirectly regulated via adenyltransferase GlnD, depending on the nitrogen concentration [90]. At low nitrogen concentrations, GlnK is adenylated at the conserved Tyr-51 residue, while an increase in nitrogen concentration leads to the deadenylation of GlnK [90]. Adenyltransferase GlnD is responsible for the modification of GlnK in *S. coelicolor* [90], although such adenylation is unusual for prokaryote PII modification and has been observed only in *C. glutamicum* [91]. Further interactions between GlnK and other components of nitrogen metabolism and resulting involvement in their regulation are very likely [92]. The deletion of *glnK* in *S. coelicolor* has been demonstrated to lead to a loss of antibiotic production [93]. However, the mechanism of action of GlnK on antibiotic production in *S. coelicolor* is still unclear (Figure 2).

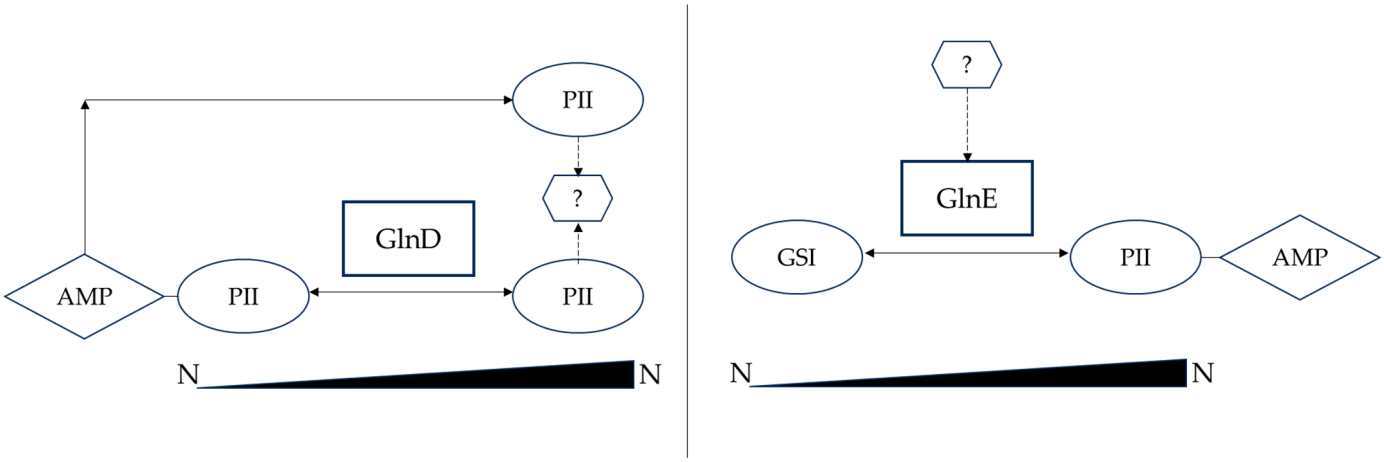

Figure 2. Scheme of the post-translational regulation of primary nitrogen metabolism in *S. coelicolor*. AMP, adenosine monophosphate; GSI, glutamine synthetase I; UMP, uridine monophosphate; question mark, unknown regulatory protein (modified after [89–92]).

A central regulator of nitrogen metabolism in *Streptomyces* is GlnR [94]. It regulates the transcription of the GSI encoding gene *glnA* and the GSII encoding gene *glnII* as well as *gdhA* (encodes the Gdh), *nirB* (encodes the nitrite reductase), *ureA* (encodes the urease), and the *amt* operon *amtB-glnK-glnD* (encodes the ammonium transporter AmtB, the PII signaling protein GlnK, and the adenyltransferase GlnD). It has been shown that under nitrogen deficiency conditions, GlnR activates the expression of *glnA*, *glnII*, *nirB*, and the *amt* operon and represses the expression of *gdhA* and *ureA* [95]. Thus, GlnR regulates essential steps of nitrogen assimilation at lower nitrogen availability through the activation of the transport of ammonium into the cell, nitrite reduction to ammonium, and synthesis of glutamine. In the case of nitrogen deficiency, GlnR represses the splitting of urea into ammonium and CO_2 and the synthesis of glutamate via glutamate dehydrogenase. The DNA-binding affinity of GlnR is modulated by post-translational modifications in response to changing N-conditions in order to elicit a proper transcriptional response [96]. An acetylation version of GlnR has been demonstrated to bind to the promoter region of *glnA2*, which encodes a gamma-glutamylpolyamine synthetase required for polyamine utilization in *S. coelicolor* [97].

Another transcriptional regulator, GlnRII has been identified as a GlnR homologue in *S. coelicolor*. It was shown, that GlnRII, similar to GlnR, also recognizes the promoter regions *glnA* and *amtB* and binds to the promoter region of *glnII* [89]. Both GlnR and GlnRII belong to the OmpR-like transcription factors, which are characterized by a C-terminal winged-helix-turned-helix motif [89,98]. They show sequence similarities at the protein level but are not functionally homologous since deletion of *glnRII* did not result in the glutamine auxotrophs phenotype [92].

2.1.3. Regulation of Secondary Metabolism in *Streptomyces*

In addition to GlnR, complex nitrogen metabolism in Streptomycetes involves control by diverse transcriptional regulators, such as Crp, ArgR, PhoP, AfsR, DasR, and AfsQ1 as well as the response regulator MtrA (master transcriptional regulator A) [70] (Table 2). Crp regulates the interplay between primary and secondary metabolism, activating the genes *glnA*, *glnII*, and *amtB-glnK-glnD*. AfsR controls the expression of *glnR* in response to nutrient-stress stimuli. PhoP represses the transcription of the *amtB-glnK-glnD* operon as well as the genes *glnA*, *glnII*, and *glnR* under conditions of phosphate limitation. AfsQ1 is required for the regulation of carbon, nitrogen, and phosphate metabolism in the presence of glutamate [86]. The overexpression of the global regulator Crp in *S. coelicolor* leads to the overproduction of actinorhodin, undecylprodigiosin, and calcium-dependent antibiotics [99]. This is in contrast to the direct activating effect of Crp on antibiotic production in

S. coelicolor [99]. Crp overexpression has been shown to enhance the titers of the secondary metabolite tacrolimus (FK-506) in *S. tsukubaensis* [100] (Table 2).

Table 2. List of regulatory proteins involved in primary and secondary metabolism regulation in *Stretpomyces* [70].

Regulator Name	Function	Reference
GlnR	Central regulator of nitrogen metabolism regulating *glnA*, *glnII*, *gdhA*, *nirB*, *ureA*, and *amtB-glnK-glnD*	[87]
GlnRII	A GlnR homologue that recognizes *glnA*, *amtB*, and *glnII*	[82]
Crp	Regulates the interplay of primary and secondary metabolism, activating *glnA*, *glnII*, and *amtB-glnK-glnD*	[99]
ArgR	Controls the expression of *glnR* in response to nutrient-stress stimuli	[101]
PhoP	Represses the *amtB-glnK-glnD* operon and *glnA*, *glnII*, and *glnR* under conditions of phosphate limitation	[102]
AfsR	Controls expression of *glnR* in response to unknown nutrient stress stimulus	[103]
DasR	Links nutrient stress to antibiotic production	[104]
AfsQ1	Required for regulation of carbon, nitrogen, and phosphate metabolism in the presence of glutamate	[105]
MtrA	Activates antibiotic biosynthetic gene clusters	[70]

2.2. Influence of Nitrogen-Containing Compounds on Antibiotic Production—Interplay between Primary and Secondary Metabolism

Antibiotic production in bacteria is largely affected by available nutrient sources. To produce secondary metabolites, precursors come mainly from primary metabolism [70]. Ammonium, nitrate, amino acids (e.g., glutamate, glutamine, lysine, serine) and polyamines (putrescine, spermidine, spermine, cadaverine) have an impact on secondary metabolism and are required for it (Figure 3).

Examples are lysine for the biosynthesis of FK-506 [100] and asparagine and malonyl CoA for the biosynthesis of lysolipin [106]. Certain nutrients can increase, decrease, or even stop the production of secondary metabolites. Secondary metabolite production also depends on the regulation network and the type of the product. For instance, the regulation of the synthesis of classic antibiotics differs from the regulation of lantibiotics production. Classic antibiotics require sophisticated cellular machinery with specialized enzymes, such as non-ribosomal peptide synthetases (NRPS) for the synthesis of non-ribosomal peptide antibiotics (NRP), or polyketide synthases (PKS) for polyketides (PK) [107,108]. Synthesis of these antibiotics depends on the activity of PKS and NRPS enzymes. However, lantibiotics synthesis depends on the activity and specificity of ribosomes and modifying enzymes. Thus, NRPs occur in the stationary phase of the growth synthesized from precursors such as amino acids, fatty acids, and α–hydroxy acids [109]. On the one hand, some enzymes involved in antibiotic synthesis, e.g., phosphatases, usually only become active when the strain's growth is slowed down under nutrient deficiency, e.g., phosphate depletion [110]. On the other hand, lantibiotic production typically coexists with the growth of a producer strain, e.g., in the case of epidermin, gallidermin, and nisin [111,112], but not in the case of mersacidin, which is produced only after active growth [113]. A typical lantibiotic biosynthesis reflects NAI-107 production—its amount in the late growth phase is several times greater than at the beginning of cultivation [114,115].

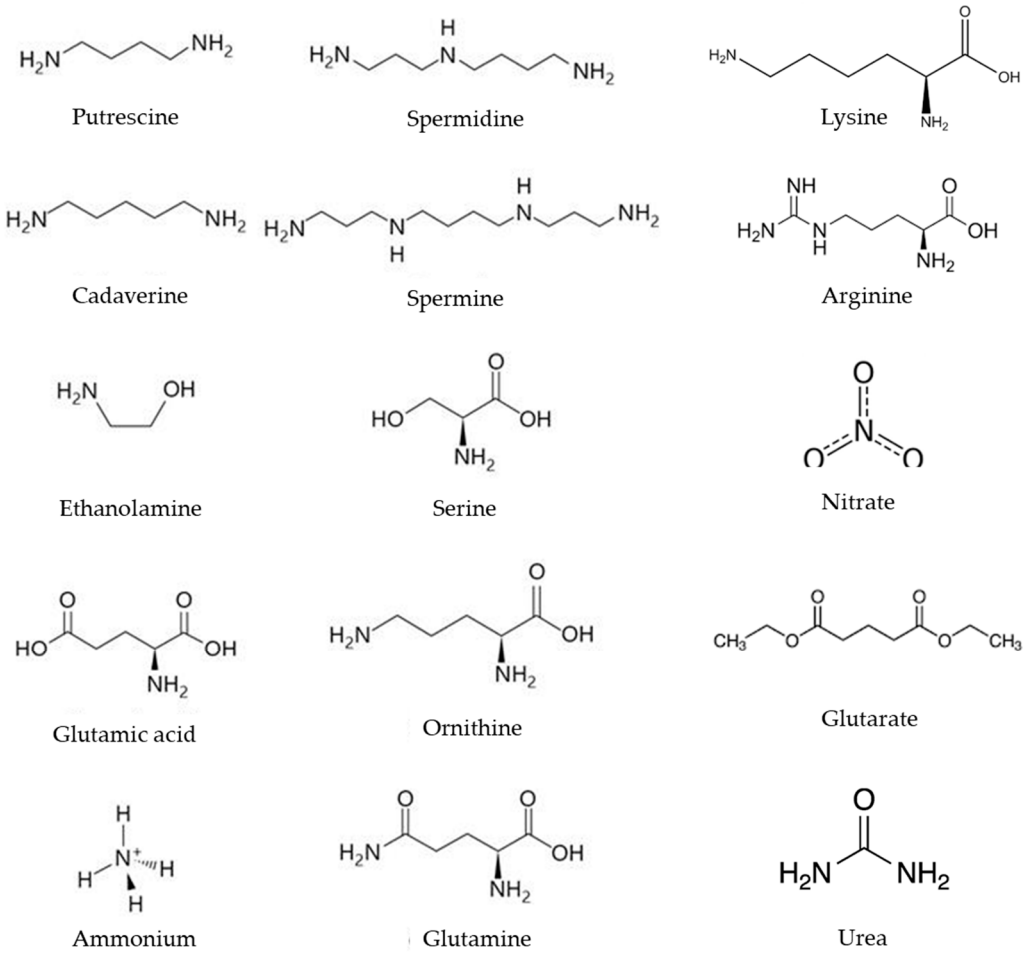

Figure 3. Chemical formulae of common nitrogen-containing compounds used by cellular metabolism in *Streptomyces* sp.

2.2.1. Influence of Ammonium on Antibiotic Production in *Streptomyces*

Nitrogen is a basic nutrient for biosynthesis of antibiotics and lantibiotics, and the regulation of nitrogen metabolism has been shown to influence antibiotic production, both positively and negatively. It has been shown that the biosynthesis of many antibiotics can be suppressed by ammonium excess [116–118]. Nitrogen repression of secondary metabolism has been observed in connection with the inhibition, reduction, or delay in the production of diverse compounds in the presence of nitrogen-containing molecules [81]. Repression of secondary metabolism has been demonstrated for high concentrations of ammonium (10–120 mM) present in the medium. The addition of yeast extract and soy peptone for an improvement in nisin production has been demonstrated to be effective only with slow feeding during fermentation [119]. Negative effects of nitrogen have also been reported for nisin production [120]. For some *Streptomyces*, amino acid degradation pathways were demonstrated to be inhibited by ammonium [121].

In contrast to amino acids, the use of inorganic nitrogen sources can also lead to an increase in some secondary metabolite production. For instance, the positive influence of high ammonium concentration (60–200 mM ammonium) was observed for the production of lantibiotics epidermin, Pep5, and gallidermin in a complex medium [111] as well as neomycin [122] and streptonigrin [123]. In contrast, a greater excess of ammonium (up to 300 mM ammonium) in a defined amino acid-containing medium did not change gallidermin production. The highest production of epidermin was found in the medium with 40 mM ammonium or 80 mM nitrate [111]. The production of another lantibiotic,

NAI-107, under ammonium excess and ammonium limitation distinguishes this group of antibiotics from classic antibiotics [114,115] (Table 3).

2.2.2. Influence of Nitrate on Antibiotic Production in *Streptomyces*

Nitrogen-containing compounds have been shown to positively influence antibiotic production, e.g., nitrate [124–126]. A nitrate-stimulating effect has been described at the transcriptional level for both the preparation of antibiotics at a preliminary stage and for the genes encoding enzymes involved in production [126]. Efficient nitrate concentrations were reported to range from 12.5 mmol/L to 40 mmol/L, positively influencing the production of antibiotics such as azalomycin, erythromycin, lividomycin, lincomycin, and rifamycin [124–126] (Table 3). It has been demonstrated that the transcription of the GS-encoding gene *glnA* is activated with the addition of nitrate. The transcription of *glnA* is determined by the global transcription regulator GlnR, which plays a central role in nitrogen metabolism in *Streptomyces* [124–126] (Table 3).

The global regulator of nitrogen metabolism GlnR has been demonstrated to play an important role in influencing the production of antibiotics. For example, in *A. mediterranean* GlnR is necessary for nitrate-stimulating effect on rifamycin production. In comparison, GlnR inhibits rifamycin biosynthesis in the absence of nitrate [127]. In *S. erythraea*, GlnR has been shown to inhibit erythromycin production in a complex TSB medium as well as in glutamine-containing Evans medium, while the transcription of genes encoding erythromycin polyketide synthases in the wild-type compared with Δ*glnR* were down-regulated [128]. In *S. carnosus*, nitrate is completely reduced to nitrite in the first step and then reduced to ammonium in the second step. In *S. carnosus*, nitrite reduction can be inhibited by nitrate [129].

2.2.3. Influence of Amino Acids on Antibiotic Production in *Streptomyces*

Amino acids serve as building blocks of peptides, proteins, and lipids (as components of phospholipids in cellular membranes), and they form precursors for diverse metabolites. It has been shown that when absorbed into the cell, amino acids are metabolized as precursors rather than directly incorporated into antibiotics and do not necessarily increase production [130]. On the other hand, it has been demonstrated that the effect of amino acid addition on secondary metabolite production is mainly based on its supply as a carbon source [131]. The amino acids leucine, isoleucine, lysine, and valine serve as precursors for commercially important polyketides.

While microorganisms and plants can synthesize all amino acids themselves, mammals have evolved the ability to synthesize about half of the 20 proteinogenic amino acids. Proteinogenic amino acids are built from common precursors that originate from the citrate cycle or catabolic carbohydrate metabolism. They are assigned into five families: glutamate, aspartate, pyruvate, serine, and the aromatic family. De novo, glutamate becomes glutamine, proline, and arginine; aspartate forms asparagine, lysine, methionine, and threonine; pyruvate forms alanine, valine, isoleucine, and leucine; and serine forms glycine and cysteine. Phosphoenolpyruvate is a precursor for synthesis of the aromatic amino acids phenylalanine, tryptophan, and tyrosine. Amino acids can be degraded through deamination, transamination reactions, or by oxidative, hydrolytic, or eliminating deamination reactions. Of the 20 proteinogenic amino acids, seven different degradation products (alpha-ketoglutarate, succinyl-CoA, fumarate, oxaloacetate, pyruvate, acetyl-CoA, and acetoacetate) are produced, providing intermediates or precursors (acetyl-CoA and pyruvate) for the citrate cycle, amino acid degradation pathways, or secondary metabolism [131].

Most amino acid biosynthetic and utilization genes are only expressed at a low constitutive level. In *Streptomycetes*, the amino acid biosynthetic pathways are often regulated through inhibition, presumably due to the natural soil habitat of the Streptomycetes lacking nitrogen and most amino acids [78]. For example, amino acid biosynthetic pathways involving feedback inhibition are the synthesis of arginine and aromatic amino acids [132].

The amino acid lysine is a non-proteinogenic amino acid, a common building block of secondary metabolites, and a frequently used precursor and media additive in biotechnology. A gene that codes for a lysine cyclodeaminase is therefore localized in the associated biosynthetic gene clusters, such as in the cluster of the streptogramin pristinamycin (PipA) [133] or the lipopeptide antibiotic friulimicin (Pip). It had already been shown for all producers that the overexpression of the respective lysine cyclodeaminase genes resulted in an increase in the desired secondary metabolites. A positive correlation between increased lysine availability and increased secondary metabolite production was shown in cephamycin biosynthesis in *S. clavuligerus* [134] and tacrolimus (SK-506) biosynthesis in *S. tsukubaensis* [56] (Table 3).

The aspartate kinase enzyme catalyzing the first reaction of lysine biosynthesis was shown to be subject to strict regulation via inhibition by the end products lysine and threonine. The combined overexpression of the feedback-deregulated aspartate kinase Ask_{Cg^*} and the dihydropicolinate synthase $DapA_{St}$, showed a strong enhancement in the intracellular lysine pool in *S. tsukubaensis* and consequently increased the yield of FK-506 by approximately 73% compared with the wild type. Furthermore, FK506 production was enhanced by heterologous overexpression of the mutated version of lysine cyclodeaminase Pip_{Af} [56] (Table 3).

2.2.4. Influence of Polyamines on Antibiotic Production in *Streptomyces*

Polyamines are aliphatic polycations with multiple amino groups and polycarbon chains. Common widely distributed natural polyamines are putrescine, cadaverine, spermidine, and spermine [135,136]. Polyamines can be observed in diverse bacterial habitats, e.g., in soil, the concentration of polyamine 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 [137]. In *S. coelicolor* putrescine, spermidine and diaminopropan biosynthesis has been detected in the late-stationary phase, while cadaverine synthesis occurred only under iron limitation [138]. In the strain *S. coelicolor* JCM4357 grown in a complex medium, the intracellular concentration of polyamines was reported to be as follows: putrescine 0.127, cadaverine 0.103, spermidine 0.040, and spermine 0.024 µmol/g. The biosynthesis of polyamines occurs from amino acid precursors arginine, ornithine, and methionine as well as lysine [139] (Figure 4). Increased polyamine concentrations lead to bacterial cell death. For example, 200 mM of exogenous putrescine was reported to be toxic for *S. coelicolor* [140]. Under such high conditions of polyamines, the detoxification and utilization of elevated levels of intracellular polyamines are needed to escape cell death. In *Streptomyces*, a gamma-glutamylation pathway for polyamine utilization has been characterized [97]. Involving gamma-glutamylpolyamine synthetases GlnA2 and GlnA3 as well as multiple catalytic steps downstream, polyamines can be efficiently converted into glutarate and succinate supplying the TCA cycle [97] (Figure 4).

Polyamines have been demonstrated to play important roles in bacterial, plant, and mammal cells including cellular growth, developmental processes, and environmental stress responses. Furthermore, polyamines are essential nutrients for intracellular pathogens, and polyamine metabolism is considered to be a new target for pharmaceutical drug development [141]. Interestingly, in plants, they have been considered a new kind of plant biostimulant. In plants, polyamines serve as precursor molecules for secondary metabolite synthesis, namely, for several groups of alkaloids (pyrrolizidine, tropane, and quinolizidine alkaloids) and phenolamides [142]. Also, in fungi and bacteria, polyamines can modulate the biosynthesis of secondary metabolites. For instance, spermidine has been demonstrated to stimulate the biosynthesis of benzylpenicillin in *Penicillum chrysogenum*, causing increases in transcript levels of penicillin biosynthetic genes [143]. Putrescine has been shown to induce secondary metabolism in *Nocardia lactamdurans* [144].

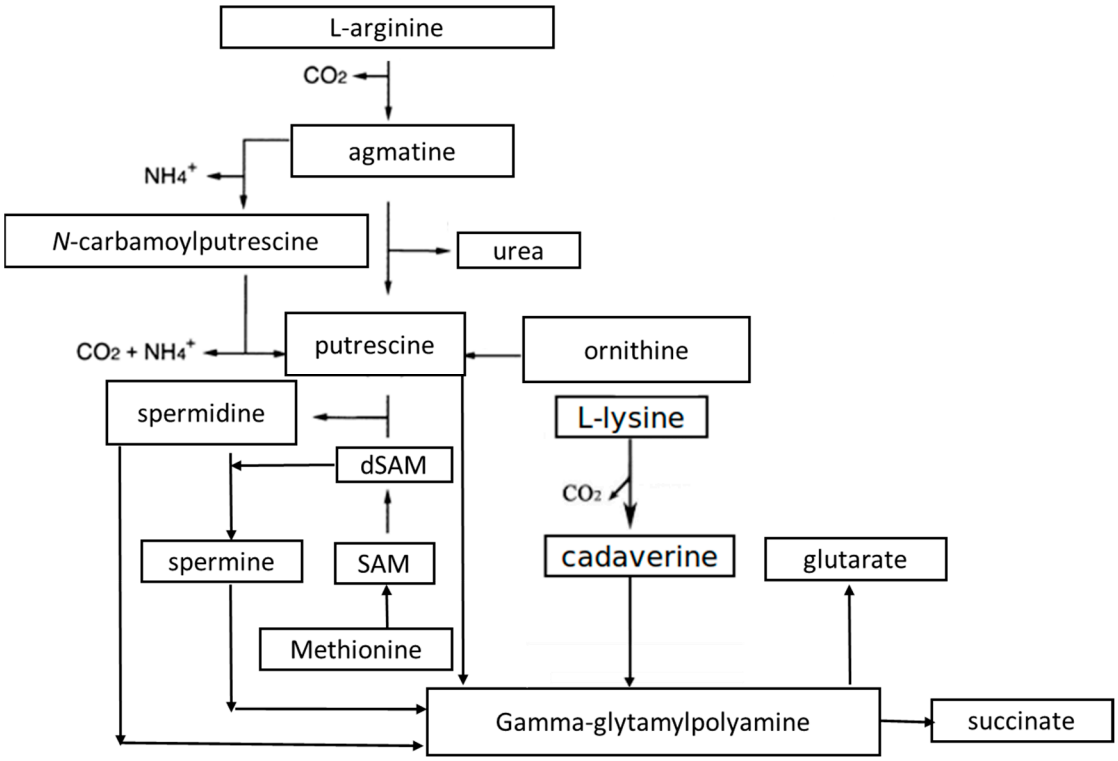

Figure 4. Combined scheme of polyamine biosynthetic and utilization pathways in *Streptomyces* (modified after [86]).

In *S. coelicolor*, polyamines putrescine, cadaverine, spermidine, and spermine caused delayed aerial mycelium and spore formation in media with these polyamines as the sole nitrogen source. Furthermore, polyamines induced prodigiosin (red antibiotic) production while abolishing actinorhodin (blue antibiotic) production in *S. coelicolor* M145 [140]. The deletion of the key gene for polyamine utilization—*glnA3*—resulted in a defect in the formation of aerial mycelium and sporulation in a medium with glutamate as a nitrogen source. Moreover, enhanced actinorhodin and prodigiosin production was observed in *S. coelicolor* Δ*glnA3* grown in media with nitrate, glutamine, and ammonium as sole nitrogen sources [140]. This indicates an impact of polyamine utilization on secondary metabolite production and morphological differentiation in *S. coelicolor* (Table 3).

In *S. tsukubaensis*, a direct effect of polyamine supplementation on tacrolimus (FK-506) production was reported. The presence of the polyamine putrescine in the production medium resulted in inhibited biomass accumulation and FK-506 production compared with the parental strain but higher compared with media supplemented with spermidine or spermine. Interestingly, very poor growth of *S. tsukubaensis* was observed in media supplemented with high amounts of spermidine and spermine (25 mM), while *S. coelicolor* was able to tolerate up to 50 mM spermine and 100 mM spermidine in complex and defined media [97] (Table 3).

Table 3. List of selected *Streptomyces*-derived and actinobacterial secondary metabolites with reported production change during fermentation. An increase or decrease in production was achieved through the addition of specific concentrations of different nitrogen sources (ammonium, nitrate, amino acids, polyamines), and yield change is indicated (if quantified in the study).

Compound	Producer	Nitrogen Source Tested	Effect on Production	Reference
Tylosin	*Streptomyces fradiae NRRL 2702*	Ammonium (20 mM/L)	Decrease (~2-fold)	[117]

Table 3. *Cont.*

Compound	Producer	Nitrogen Source Tested	Effect on Production	Reference
Leucomycin	*Streptomyees kitasatoensis*	Ammonium (2 mM/L)	Decrease (50%)	[145]
NAI-107	*Microbispora ATCC PTA-5024*	Ammonium (25 mM/L)	Increase (~0.2 fold)	[114]
Neomycin B	*Streptomyces fradiae SF-2*	Ammonium (60 mM/L)	Increase (0.54–3.3 fold)	[122]
Streptonigrin	*Streptomyces flocculus (ATCC 13257)*	Ammonium (0.5–2 g/L)	Increase (2-fold)	[123]
AK-111-81	*Streptomyces hygroscopicus*	Ammonium (0.15%)	Increase (6-fold)	[146]
SBR-22	*Streptomyces psammoticus BT-408*	Ammonium (2.5 g/L)	Increase (1.2-fold)	[147]
SA-53	*Streptomyces anandii var. Taifiensis*	Ammonium (280 mg/L)	Increase (2-fold)	[148]
Azalomycin	*Streptomyces hygroscopicus*	Nitrate	Increase	[149]
Erythromycin	*Saccharopolyspora erythraea*	Nitrate (15 mM/L)	Increase	[150]
Lividomycin	*Lividomycin poducer M814*	Nitrate	Increase	[151]
Lincomycin	*Streptomyces lincolnensis*, *Streptomyces* sp. *MS-266 Dm4*	Nitrate (23.5 mM/L)	Increase	[152]
Rifamycin B and SV	*Amycolatopsis mediterranei*, *Amycolatopsis mediterranei U32*	Nitrate (12.5–80 mM/L)	Increase (4-fold)	[126,153]
Meroparamycin	*Streptomyces MAR01*	Nitrate (19.8 mM/L)	increase	[124]
Cephamycin	*Streptomyces clavuligerus*	Amino acids (lysine, 14.6 g L^{-1})	Increase (6-fold)	[144]
Tacrolimus (FK-506)	*Streptomyces tsukubaensis*	Amino acids (lysine, 2.5 g/L)	Increase (30%)	[56,154]
Rapamycin	*Streptomyces hygroscopicus*	Amino acids (lysine, 10 g/L)	Increase (150%)	[155]
Leucomycin	*Streptomyees kitasatoensis*	Amino acids (1%)	Increase (2–4 fold)	[145]
Prodigiosin	*Streptomyces coelicolor*	Polyamines (25 mM/L)	Increase	[140]
Actinorhodin	*Streptomyces coelicolor*	Polyamines (25 mM/L)	Decrease	[140]
Tacrolimus (FK-506)	*Streptomyces tsukubaensis*	Polyamines (25 mM/L)	Decrease (3-fold)	[97]

3. Perspectives for Secondary Metabolite Discovery in *Streptomyces*

In the current review, different approaches for the discovery and production of secondary metabolites in *Streptomyces* were summarized. Recent advances in engineering strategies and the possibility of obtaining fully sequenced genomes of strains of interest using Next-Generation Sequencing (NGS) revealed the so far unavailable potential of *Streptomyces* sp. as a reservoir for novel valuable compounds [156]. On the one hand, during the last decades, genetic tools based on the CRISPR/Cas system have offered different new strategies to optimize secondary metabolite production and activate silent BGCs, but further optimization of the CRISPR/Cas system for the engineering of Streptomycetes is still

ongoing [46]. On the other hand, recent advancements in the introduction of "super-host" strains for the heterologous expression of BGCs (e.g., *S. coelicolor*, *S. lividans*, *S. albus*, *S. avermitilis*, *S. chattanoogensis*) represent a further possibility to overcome existing limitations [69]. However, super-host optimization has diverse challenges as well, for instance, some metabolites were not produced significantly [69].

Possibilities for genetic manipulation of *Streptomyces* were combined with classical fermentation optimization efforts in multiple recent studies as well. In particular, the investigation of nitrogen supply from amino acids and amino acid-derivative compounds, such as polyamines, provided new insights into secondary metabolite discovery [56,97,140]. However, different nitrogen sources under certain concentrations can decrease secondary metabolite production and can even be toxic to cells when in excess (Table 3), which requires strict control of nutrient supply during fermentation. The application of novel bioinformatics tools, techniques for genetic engineering, and nutrient supply strategies led to recently reported advancements in natural product discovery in *Streptomyces* sp. and an increased number of new compounds of interest [3,157,158].

4. Conclusions

The complex biosynthesis of secondary metabolites in *Streptomyces* wild-type producers naturally results in rather low titers. Nevertheless, biosynthetic steps in primary and secondary metabolism as well as their regulation can be suitable targets for engineering. Furthermore, the dependence of secondary metabolism in *Streptomyces* sp. on a precursor supply coming from primary metabolism is crucial and can be optimized for biotechnological needs in addition to the application of available genetic manipulation techniques. One example of this is the importance of cellular nitrogen metabolism for the supply of relevant nitrogen-containing compounds as precursors into secondary metabolism or its induction by these compounds. Ammonium, nitrate, amino acids, and, recently, polyamines have been demonstrated to strongly influence the production of secondary metabolites in different *Streptomyces* strains. A combination of metabolic engineering strategies targeting relevant genes for nitrogen supply with feeding strategies can be a very effective strategy in *Streptomyces* strains for the optimization of the production of compounds of interest.

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