

REVIEW ARTICLE

Carbon source regulation of antibiotic production

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Antibiotics are low-molecular-mass products of secondary metabolism, nonessential for the growth of producing organisms, but very important for human health. They have unusual structures and are most often formed during the late growth phase of the producing microorganisms. Their production arises from intracellular intermediates, which are condensed into more complex structures through defined biochemical pathways. Their synthesis can be influenced by manipulating the type and concentration of nutrients formulating the culture media. Among them, the effect of the carbon source has been the subject of continuous studies for both industry and research groups. Glucose and other carbohydrates have been reported to interfere with antibiotic synthesis and this effect depends on the rapid utilization of the preferred carbon source. Different mechanisms have been described in bacteria and fungi to explain the negative effects of carbon catabolites on antibiotic production. They show important differences depending on the microbe being considered. Their understanding and manipulation have been useful for both perfecting fermentation conditions to produce anti-infectives and for strain improvement. To improve the production of antibiotics, carbon source repression can be decreased or abolished by mutations resulting in antimetabolite resistance. Enzymes reported as regulated by the carbon source have been used as targets for strain improvement. During the last few years, important advances have been reported elucidating the essential aspects of carbon source regulation on antibiotic production at biochemical and molecular levels. The aim of this review is to describe these advances, giving special emphasis to those reported for the genus *Streptomyces*.

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INTRODUCTION

Antibiotics are low-molecular-mass (< 1500 kDa) products of secondary metabolism, usually produced during the late growth phase (idiophase) of a relatively small group of microorganisms. Antibiotics are not essential for the growth of producing cultures but serve diverse survival functions in nature.¹ In addition, antibiotics are very important for the health, nutrition and economics of our society.²

Owing to the use of antibiotics and other secondary metabolites, the average life expectancy in the United States increased from 47 years in 1900–1974 (males) to 80 years (females) in the year 2000.³ Probably, the most important use of secondary metabolites has been as anti-infective drugs. In the year 2000, anti-infective secondary metabolites marketed 55 billion dollars,⁴ but in the year 2007, the market for antibiotics increased to 66 billion dollars.⁵

If modern medicine is to continue in its present form, novel families of antibiotics must enter the marketplace at regular intervals.

Recently, new antibiotics have been introduced to the anti-infectious disease market, for example, augmentin, clarithromycin and ceftriaxone. However, within the next 10 years, aggressive screening programs for the selection of novel natural and modified chemical compounds will be necessary to produce novel antibiotics against resistant bacteria.

In addition to the screening programs for new compounds with antibacterial activity, during the last few decades, the pharmaceutical industry has extended these programs to other disease areas^{6–8} such as anticancer drugs, namely bleomycin, dactinomycin, doxorubicin and staurosporin;⁹ antimycotic agents, namely anidulafungin;¹⁰ and antihelminthic compounds, namely ivermectin, milbemycin and spinosyns.¹¹ In addition, the National Cancer Institute has invested considerable effort in evaluating the inhibitory activity of pepstatin on HIV-1 (human immunodeficiency virus-1). Pepstatin A is a small pentapeptide produced by several *Streptomyces* species with a unique

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This work was written to honor Professor Arnold L Demain, on the 60th anniversary of his very productive scientific and personal life. During this time, he has been strongly devoted to the study of secondary metabolism and has guided more than 100 students throughout the industrial microbiology and biotechnology world. In 1995, most of these students gathered to celebrate the First Army's Army and Friends Symposium at MIT in the United States, an academic event that has been repeated every 2 years for the past 15 years.

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hydroxyamino acid (statine) that sterically blocks the active site of HIV-1 protease.^{12,13}

The outstanding role of microorganisms in the production of antibiotics is notorious. At present, with <1% of the microbial world having been explored, the advances in techniques for microbial cultivation and extraction of nucleic acids from soil and marine habitats are allowing access to a vast untapped reservoir of genetic and metabolic diversity.¹⁴

The main biosynthetic pathways involved in antibiotic production are those forming peptides, polyketides, isoprenes, oligosaccharides, aromatic compounds and β -lactam rings. Knowledge of these pathways varies from those cases in which the amino-acid sequences of the enzymes and nucleotide sequences of the genes are known (for example, for penicillins and cephalosporins), to those in which even the enzymatic steps are still unknown.¹⁵

Antibiotics are formed by enzymatic pathways that occur through individual proteins, free or complexed, or through parts of large multifunctional polypeptides carrying out a multitude of enzymatic steps, for example, polyketide synthases and peptide synthetases.¹⁶ The genes encoding these enzymes are usually chromosomal. For example, the 30 biosynthetic *str/sts* and *blu* genes of streptomycin biosynthesis (and of related antibiotics such as 5'-hydroxystreptomycin and bluensomycin) that were cloned from *Streptomyces griseus* and *Streptomyces glaucescens* were all found in a chromosomal region of 30–40 kb.¹⁷ Only a few biosynthetic genes have been shown to be plasmid borne, for example, those encoding methylenomycin A synthesis in *Streptomyces coelicolor* and antibiotic AS-48 in *Enterococcus faecalis* S-48.¹⁸ Whether chromosomal or plasmid borne, antibiotic formation genes are usually clustered, especially in prokaryotes, but not necessarily as single operons. Antibiotic clusters in actinomycetes have different sizes. For instance, although actinorhodin and other simple aromatic polyketides are encoded in chromosomal regions of 20 kb or less, regions of 90–100 kb are required to encode for rapamycin and rifamycin and other complex polyketides. In the latter case, 50–80 kb of DNA are required for the modular polyketide synthetases (PKSs) themselves.¹⁹ Clusters of fungal biosynthetic genes have also been found for biosynthetic processes of sterigmatocystin formation by *Aspergillus nidulans*.²⁰

Antibiotic formation usually occurs during the late growth phase of the producing microorganism. The temporal nature of their formation is certainly genetic, but expression can be influenced greatly by environmental manipulations. Therefore, synthesis of antibiotics is often brought on by exhaustion of a nutrient, addition of an inducer and/or by a decrease in growth rate.²¹ These events generate signals that cause a cascade of regulatory events resulting in chemical (secondary metabolism) and morphological differentiation (morphogenesis) of the microbial antibiotic producers. The signal is often a low-molecular-weight butyrolactone inducer that acts by binding to and inactivating a regulatory protein (repressor protein/receptor protein) that normally prevents secondary metabolism and morphogenesis during rapid growth and nutrient sufficiency.²² Formation of antibiotics is also regulated by nutrients (such as nitrogen, phosphorous and carbon source), metals, growth rate, feedback control and enzyme inactivation.²³ Among these nutrients, the effect of carbon source on antibiotic production has been the subject of continuous study for both industry and research groups, not only from fermentation but also from biochemical and molecular biological stand points. In this work, we review the regulation of antibiotic biosynthesis by carbon source, and the mechanisms involved, giving special emphasis to antibiotics produced by the genus *Streptomyces*. We describe how some proteins and protein complex systems are crucial for carbon

source effect on antibiotic biosynthesis. These proteins and systems can be grouped as follows: (1) the phosphoenolpyruvate: phosphotransferase system (PTS), one of the most important mechanisms utilized for both Gram-negative and Gram-positive bacteria with low guanine and cytosine (GC) content; (2) CarI (for *N*-3-(oxohexanoyl)-L-homoserine lactone (OHHL) synthesis), a key factor used for *Erwinia*; (3) Bld and RegI, transcriptional regulators in *Streptomyces*; (4) other Bld proteins (such as BldA, BldG and BldH) for *Streptomyces*; (5) the Glk enzyme for the genus *Streptomyces*; (6) the SCO2127 protein, which propels production of metabolic signals responsible for carbon catabolite repression (CCR) in *Streptomyces*; (7) 3,5-cyclic adenosine monophosphate (cAMP) and its binding protein (cAMP receptor protein (CRP)), very important for Gram-negative bacteria and for the synthesis of some antibiotics in *Streptomyces*; (8) the CreA zinc-finger repressor in fungi; and (9) the catabolite control protein A (CcpA) binding to *cre* elements in *Bacillus*. In addition, direct carbon source inhibition of key enzymes involved in secondary metabolic pathways will also be documented.

REGULATION BY CARBON SOURCE

In bacteria and other microorganisms, glucose, usually an excellent carbon source for growth, interferes with the formation of many antibiotics 'too much of a good thing can be bad.'²⁴ In media containing mixtures of rapidly and slowly used carbon sources, the former is used first to produce cells but little or no antibiotics are synthesized. After the rapidly assimilated compound is depleted, the 'second-best' carbon source is used for idiolite formation.

Regulation by the carbon source depends on the rapid utilization of the preferred carbon source. One distinctive characteristic of secondary metabolism is its association with low growth levels;²⁴ thus, low concentrations of the repressing carbon source in the culture medium yield low growth rates and eliminate the interference of antibiotic biosynthesis.

Different mechanisms have been described in bacteria to explain the negative carbon catabolite effects on secondary metabolite production. These mechanisms show important differences depending on the type of bacteria being considered. For a detailed review about these mechanisms, refer to the studies by Deutscher²⁵ and Görke and Stülke.²⁶ A few characteristics of these mechanisms will be mentioned in this review. However, major emphasis will be given to the advances in the mechanism of carbon catabolite repression (mechanisms that inhibit synthesis at the level of DNA transcription) in the genus *Streptomyces*.

CARBON SOURCE REGULATION OF ANTIBIOTIC PRODUCTION IN GRAM-NEGATIVE BACTERIA

Gram-negative bacteria produce a particular type of secondary metabolites. In some cases, their production is sensitive to carbon source repression. One example is carbapenem biosynthesis in *Erwinia carotovora*.²⁷ In this microorganism, glycerol suppresses (a mechanism that arrests metabolites formation and may be the consequence of repression or inhibition) carbapenem biosynthesis by repressing transcription of the *carI* gene responsible for the synthesis of OHHL, a quorum-sensing molecule that induces antibiotic formation. Limitation of OHHL affects the expression of carbapenem biosynthetic genes (*carA-H*) by preventing formation of the complex between CarR (DNA-binding transcriptional activator of *carA-H*) and OHHL. The formation of this complex (CarR–OHHL) is a necessary step for transcriptional activation of the carbapenem biosynthetic genes in *E. carotovora*.^{28,29} On the other hand, glycerol does

not affect transcription of either *carR* or *hor* (transcriptional activator of antibiotic and pigment production) genes.²⁷ Carbapenem-like antibiotics, are also produced by other Gram-negative bacteria such as *Erwinia herbicola*, *Serratia* sp. strain American Type Culture Collection 39006 and *Photobacterium luminescens* strain TT01.³⁰ Interestingly, in *P. luminescens*, the *carR* gene is not present, suggesting a different mechanism for the regulation of antibiotic formation. In addition, in contrast to *E. carotovora*, *P. luminescens* carbapenem-like synthesis is not induced by the quorum-sensing molecule *N*-acyl-homoserine lactone.³⁰

An additional example of carbon regulation has been reported in the ethanol-utilizing strain, *Serratia marcescens*. In this microorganism (strain S389), Cang *et al.*³¹ observed that a low glucose concentration (27.7 mM) added to the growth medium, almost completely repressed formation of the red tripirrole antibiotic, prodigiosin. Other carbon sources such as galactose, fructose and sucrose also repressed antibiotic production. As mentioned above, *Serratia* also produces the antibiotic carbapenem. It is known that biosynthesis of both compounds (that is, carbapenem and prodigiosin) are under quorum-sensing control.^{32,33} These secondary metabolites are controlled by the *smaR* quorum-sensing locus, which represses both compounds when levels of *N*-acyl-homoserine lactone are low. Quorum sensing controls the production of prodigiosin, at least in part, by modulating transcription of three genes encoding regulatory proteins. Surprisingly, one of these proteins is a novel putative adenylate cyclase,³⁴ which likely causes a decrease in cellular cAMP concentration affecting pigment production.

Among the mechanisms of CCR in Gram-negative bacteria, the phosphoenolpyruvate: PTS can be mentioned as one of the most important. PTS utilizes a protein phosphoryl transfer chain to transport and phosphorylate its sugar substrates. In *Escherichia coli*, PTS consists of four high-energy phosphoprotein intermediates and five protein domains. One of these proteins, EIIGlc is phosphorylated by a phosphoprotein (HPr) on His90. Subsequently, EIIGlc~P transfers its phosphate from His75 to a high-affinity homodimeric protein EIIB/C found in the cell membrane. The IIC domain crosses the membrane eight times harboring the sugar-binding site. The hydrophilic domain IIB transfers the phosphate group from EIIGlc~P to glucose, to form glucose 6-phosphate (Figure 1).²⁵

In addition to transferring the phosphate group to EIIB/C, EIIGlc~P activates adenylate cyclase. The activated form of this enzyme synthesizes cAMP, necessary for the synthesis of inducible enzymes and its intracellular levels mediate CCR. To activate transcription, cAMP binds to the DNA promoter region through the CRP.

In the presence of glucose, sugar is transported into the cell and concomitantly phosphorylated. This event causes dephosphorylation of EIIGlc~P, mediates inducer exclusion and deactivates adenylate cyclase. Inactivation of adenylate cyclase causes a reduction in cytoplasmic cAMP concentration and promotes dissociation of the cAMP-CRP complex from the DNA and deactivation of transcriptional initiation. The gene for EIIGlc is known as *crr*, because mutants of *E. coli* lacking this gene are resistant to CCR.²⁵

While studying *N*-acyl amino-acid synthase clones derived from environmentally obtained DNA, Clardy and Brady³⁵ discovered an

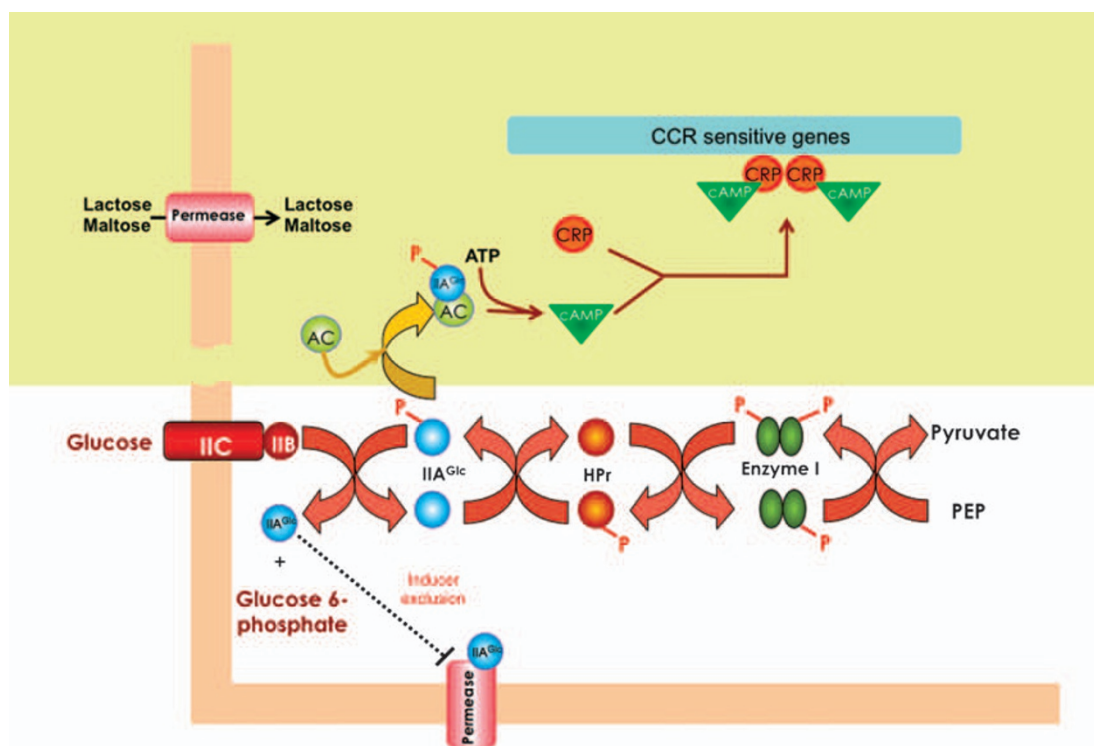


Figure 1 Model for carbon catabolite repression (CCR) mechanism in *Escherichia coli*. In white background is shown a cell under repressing conditions and in light yellow under non-repressing conditions. The phosphotransferase system (PTS) comprising Enzyme I, HPr and IIA^{Glc} phosphorylating enzymes, is the most important CCR mechanism in *E. coli*. In the presence of glucose, PTS internalizes and phosphorylates the sugar, rendering dephosphorylated IIA^{Glc}, which in turn exerts inducer exclusion of other non- or less-repressing carbon sources. On the contrary, when glucose is absent, IIA^{Glc} remains phosphorylated and activates the adenylate cyclase (AC). AC synthesizes cAMP, which binds to the cAMP receptor protein (CRP), activating the transcription of CCR-sensitive genes.

N-acyl amino-acid synthase (NasP) containing the cAMP-binding domain. Studies on the regulation of the antibiotic *N*-acylphenylalanine showed that antibiotic biosynthesis is cAMP dependent. In addition, using affinity chromatography, these authors confirmed a direct interaction between NasP and cAMP.

Pseudomonas species produce several types of antifungal compounds, of which the most studied are pyoluteorin, pyrrolnitrin, phenazines and 2, 4-diacetylphloroglucinol (Phl). In addition to its antifungal activity, Phl has shown an interesting bactericidal activity against methicillin-resistant *Staphylococcus aureus*.³⁶

Phenazines are heterocyclic compounds the effects of which are attributed to their redox activity. For example, the 2-hydroxy-phenazine-1 carboxylic acid produced by *Pseudomonas aureofaciens* is believed to destroy competing fungi through production of reactive oxygen species.³⁷ The expression of phenazine biosynthetic genes is regulated by multiple mechanisms, which are strongly influenced by environmental conditions. One of the primary factors governing phenazine production is population density, and in *Pseudomonas aeruginosa*, this dependency is affected by at least three quorum-sensing systems.³⁸

In several species of *Pseudomonas*, phenazine production is affected by the carbon source. For example, in *Pseudomonas chloraphis*, the greatest production of phenazine 1-carboximide was obtained using L-pyroglyutamic acid and glucose. However, fructose, sucrose and ribose had negative effects on phenazine 1-carboximide production.³⁸ Similar results were observed in a mutant of *Pseudomonas* sp. M18G, in which the greatest production of phenazine 1-carboxylic acid was achieved with glucose and ethanol as carbon sources.³⁹

In fluorescent *Pseudomonas*, Krebs cycle intermediates, such as succinate, induce catabolite repression for the utilization of non-preferred carbon sources, such as glucose. cAMP and its respective

cAMP-binding protein do not seem to be responsible for catabolite repression mechanisms in this genus.⁴⁰

A master regulator of carbon metabolism in *Pseudomonas* is an RNA-binding protein named Crc, which, under repressive conditions, inhibits the translation of mRNAs by binding to a sequence just downstream of the AUG start codon (Figure 2).⁴¹ Conversely, under nonrepressive conditions, a small RNA named CrcZ binds to the Crc protein to relieve mRNAs from catabolite repression. A two-component system CbrA/CbrB, which is sensitive to the carbon source, controls the expression of CrcZ.⁴⁰

In the same manner, other RNA-binding proteins such as RsmA (repressor of secondary metabolism) from *Pseudomonas fluorescens* avoid translation by attachment to mRNAs related to the synthesis of extracellular antifungal secondary metabolites. Translation of mRNAs can continue when the cell expresses high quantities of small RNAs that compete for binding to RsmA, which is similar to the carbon storage regulator, CsrA from *E. coli* and the regulation of sRNA expression is promoted by a two-component system named GacS/GacA.⁴²

CARBON SOURCE REGULATION OF ANTIBIOTIC PRODUCTION IN GRAM-POSITIVE BACTERIA

Gram-positive bacteria are characterized by having multiple layers of peptidoglycans as well as proteins and/or teichoic acids as part of their cell-wall structure. In addition, the GC content of the DNA significantly varies among these bacteria. Thus, the phylum *Firmicutes* corresponds to Gram-positive bacteria with low GC content, whereas the phylum *Actinobacteria* contains high GC content in their DNA structure. *Firmicutes* include the classes Bacilli, Clostridia and Erysipelotrichi.⁴³ The phylum *Actinobacteria* is constituted by the class *Actinobacteria* and includes the order Actinomycetales.⁴⁴ The

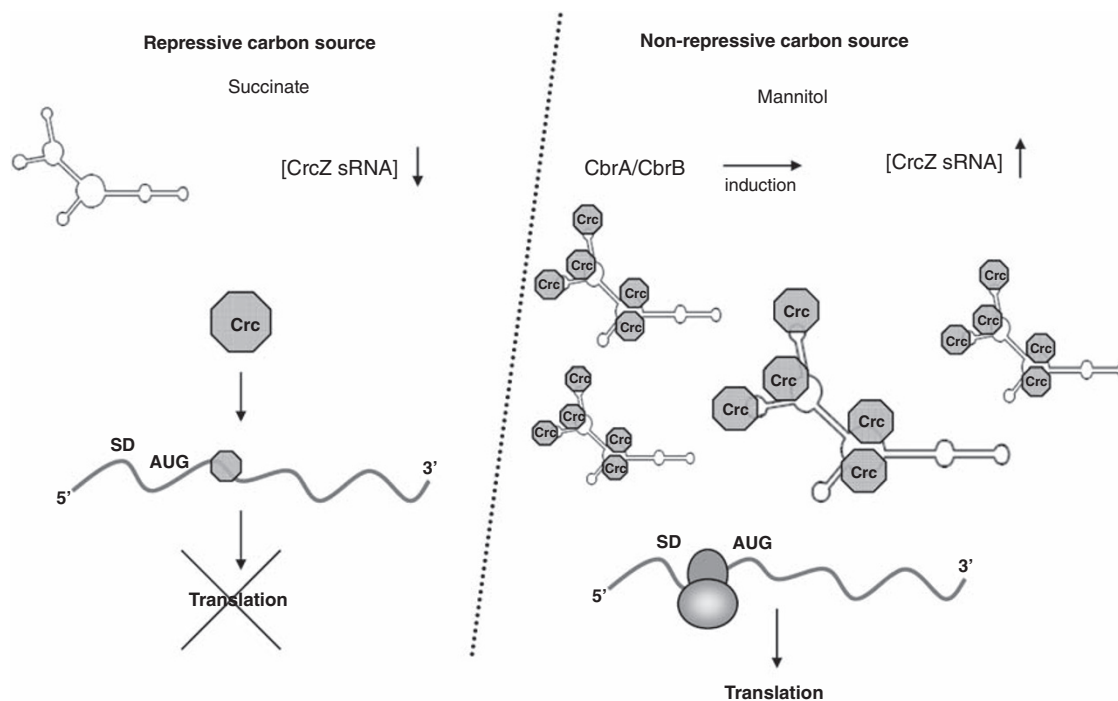


Figure 2 Schema of catabolite repression exerted by Crc/CrcZ. When a preferred carbon source such as succinate is present in the culture medium, there is a low level of CrcZ sRNA; thus Crc attaches to catabolite repression-sensitive mRNAs, inhibiting translation. Conversely, in the presence of a non-preferred carbon source such as mannitol, the expression of CrcZ sRNA is increased due to the induction of the CbrA/CbrB two-component system. The larger amount of CrcZ is then capable of binding to Crc protein, releasing mRNAs and allowing translation. Modified from Sonnleiter *et al.*⁴⁰

phylum *Actinobacteria* represents one of the largest and most diverse groups of bacteria, encompassing many important and well-characterized organisms, including *Streptomyces*, *Bifidobacterium*, *Corynebacterium* and *Mycobacterium*.

Gram-positive bacteria have been reported as being able to produce antibiotics either through ribosomal or nonribosomal mechanisms. In this regard, the orders Bacillales, Lactobacillales and Actinomycetales deserve special mention.

Bacillales

The order Bacillales belongs to the class Bacilli and produces a full range of secondary metabolites with antimicrobial and toxin activities. These compounds are often, but not always, polypeptides. Known antibiotic producers from the Bacillaceae family the synthesis of which is subject to CCR include *Bacillus cereus* that synthesizes cerealexin and zwittermicin; *Bacillus circulans* and *Brevibacillus laterosporus* that produce circulin; *Paenibacillus polymyxa* that produces both polymyxin and colistin; and *Brevibacillus brevis* that makes gramicidin and tyrothricin. *Bacillus licheniformis* producing bacitracin, *Bacillus pumilus* pumulin and *Bacillus subtilis* makes polymyxin, diffcicidin, subtilin and mycobacillin. In fact, around 4–5% of the *B. subtilis* genome is devoted to antibiotic production.⁴⁵ In this regard, it has been reported that in plant-associated *B. amyloliquefaciens* FZB42, as much as 8.5% of the genome appears to be devoted to antibiotic production.⁴⁶

Glucose is the preferred substrate in *B. subtilis*, and its presence represses the utilization of other alternative substrates. On the basis of physiological and transcriptional data from cointilization experiments with eight different carbon substrates, Kleijn *et al.*⁴⁷ demonstrated that malate is a second preferred carbon source for *B. subtilis*, which is rapidly cointilized with glucose and strongly represses the uptake of alternative substrates.

The mechanism of CCR in *B. subtilis* and other *Firmicutes* such as *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Lactobacillus*, differs from that of enterobacteria. These bacteria essentially possess the same protein constituents of the PTS as are found in *E. coli*. Therefore, the PTS components form a protein phosphorylation cascade, which uses phosphoenol pyruvate as a phosphoryl donor. However, these Gram-positive bacteria possess a bifunctional enzyme, the HPr kinase/phosphatase (HprK/P), which can be activated by several metabolites (fructose 1,6-diphosphate, gluconate-6-P and 2-phosphoglycerate) when grown in the presence of glucose. Activated HprK/P catalyzes phosphorylation of HPr at Ser-46 and dephosphorylation of P-Ser-HPr. Phosphorylated HPr binds to a pleiotropic regulator, CcpA, to allosterically promote catabolite repression and to prevent inducer accumulation by uncoupling sugar transport from H⁺ symport.²⁵ CcpA functions as a pleiotropic regulator by binding to the so-called catabolite-responsive elements (*cre*), which are located either upstream in the promoter regions or in open-reading frames.⁴⁸ It is interesting to note that polyclonal antibodies against CcpA from *Bacillus megaterium* share antigenic determinants with CcpA in many other Gram-positive bacteria, including bacilli, staphylococci, streptococci, lactic acid bacteria and some actinomycetes.⁴⁹ In addition, there is also another regulatory protein, CodY, able to repress genes which allow cell movement to places with higher nutrient concentrations, to breakdown these nutrients and transport the resulting products into the cell. The binding of CodY to certain DNA consensus sequences (CodY box), is facilitated by its interaction with GTP or to branched chain amino acids, specially isoleucine and valine. However, the consensus sequences for the binding of CodY to DNA have not been completely established.⁵⁰

Finally, it has been demonstrated in *B. subtilis* that subtilin production is controlled by both the culture density (through a quorum-sensing mechanism) and the microbial growth phase.^{45,51}

Lactobacillales

Lactic acid bacteria display numerous antimicrobial activities; among them, bacteriocins are especially important because of their use as food preservatives.⁵² Bacteriocins are antimicrobial peptides or proteins synthesized ribosomally (which distinguishes them from enzymatically synthesized peptide antibiotics) by bacteria. They have a specific killing mechanism that usually acts upon closely related species. It is generally accepted that bacteriocins kill cells by permeabilizing the cytoplasmic membrane and generating pores that allow leaking of cellular components.⁵³

Production of bacteriocins involves a quorum-sensing mode of regulation, mediated by a secreted peptide pheromone (induction peptide), a histidine protein kinase and a response regulator. The secreted pheromone, which in most cases is constitutively produced at a low level during exponential growth phase, serves as a means to measure the cell density of a producing culture. At a certain cell density, the induction peptide reaches a critical threshold concentration that triggers a series of phosphorylation reactions on the histidine protein kinase and response regulator, resulting in a phosphorylated response regulator. The latter binds to regulated promoters of the bacteriocin regulon to activate all genes involved in bacteriocin biosynthesis.⁵⁴ Although this quorum-sensing induction mechanism has been demonstrated for many different systems, there are additional factors to be considered. For instance, Cheigh *et al.*⁵⁵ studied nisin Z production by *Lactococcus lactis* subsp. *lactis* A164 grown in various carbon sources, and observed that cells grown in sucrose or glucose generated greater cell mass than did those grown with lactose. However, even at high cell density, the culture yielded lower nisin Z activity along with weaker nisZ transcripts. These data indicate that lactose increases transcription of the nisZ structural gene, whereas glucose and fructose reduce nisin production.

In batch culture production of pediocin (another peptide antibiotic) by *Pediococcus acidilactici* NRRL B-5627, expression is inhibited by increasing glucose concentrations in the growth medium.⁵⁶ In *Streptococcus salivarius*, the production of two lantibiotics (with 2.368 and 2.74 kDa), known as salivaricin A2 and salivaricin B (SboB), has been studied. SboB is encoded by an eight-gene cluster and its production can be reduced in agar cultures supplemented with either glucose or lactose at 1% (wt/vol) rather than sucrose, indicating that the SboB locus is susceptible to CCR.⁵⁷ Furthermore, the bacteriocin bovicin HC5 produced by *Streptococcus bovis* was also catabolite repressed by glucose at dilution rates higher than 0.6 per h.⁵⁸

Growth studies with *Lactobacillus brevis* have shown that this microorganism simultaneously consumes numerous carbon sources and appears to lack the normal hierarchical control of carbohydrate utilization. Analysis of several independent *L. brevis* isolates indicates that cointilization of xylose and glucose is a common trait for this species. Moreover, carbohydrates that can be used as a single carbon source are simultaneously utilized with glucose. Proteomic and mRNA analyses showed that genes in the *xyl* operon were expressed in cells grown on xylose or on glucose/xylose mixtures but not in those grown on glucose alone. However, expression levels of XylA and XylB proteins in cells grown on a glucose/xylose mixture were reduced 2.7-fold from that observed in cells grown solely on xylose. These results suggest that regulation of xylose utilization in *L. brevis* is not as stringently controlled as in other lactic acid bacteria, in which CCR operates to prioritize carbohydrate utilization more rigorously.⁵⁹

In lactic acid bacteria, Chaillou *et al.*⁶⁰ demonstrated that transport and phosphorylation of glucose is undertaken by the mannose PTS (HPr, EI and an EII^{Man} complex). Mutations in the EII^{Man} complex inactivate it and elicit loss of the preferential consumption of glucose over other carbon sources, such as lactose in *Lactobacillus casei* or xylose in *Tetragenococcus halophila*. The use of mutants affecting mannose PTS expression in *S. salivarius* has a pleiotropic effect on various metabolic enzymes, as well as on urease activity and on an inducible fructose PTS activity.⁶⁰ Interestingly, deletion of EII^{Man} or *mpt* (mannose-specific PTS) also produces resistance or immunity to different class II bacteriocins.^{54,61–63} In addition, in *Streptococcus gordonii*, CcpA is implicated in penicillin tolerance both *in vitro* and *in vivo*, linking antibiotic survival to bacterial sugar metabolism.⁶⁴ Consequently, it will be of great interest in future work to analyze the expression of bacteriocin operons with regard to the carbon source used, because it seems possible that the carbon source regulates bacteriocin production by mediating the formation of enzymes involved in modification, immunity or signal transduction.

In addition, a role for CcpA in the transcriptional regulation of the *xyl* regulon in *Lactobacillus pentosus* has been demonstrated. However, no information on the relationship between activity of the EII^{Man} complex and CcpA-dependent CCR mediated by glucose is available for *S. salivarius*. In addition, in *S. gordonii*, CcpA is implicated in penicillin tolerance both *in vitro* and *in vivo*, linking antibiotic survival to bacterial sugar metabolism.⁶⁴

On the other hand, CCR in *Streptococcus mutans* can be independent of CcpA and the serine-phosphorylated HPr functions in concert with particular PTS permeases to prioritize carbohydrate utilization by modulating sugar transport and transcription of catabolic operons.⁶⁵

Actinomycetales

The Actinomycetales are primarily aerobic and chemo-organotrophic microorganisms with high GC content. They comprise filamentous, often branching and morphologically diverse bacteria.⁶⁶ Most of them are Gram-positive eubacteria, although some species with complex cell-wall structures do not show clear Gram staining. A great number of these species are saprotrophic, but a few are pathogenic in plants or animals, including humans. Actinomycetales include families such as streptomycetaceae, nocardiaceae, corynebacteriaceae, pseudonocardiaceae, etc.,⁶⁷ and have received special attention either because of their industrial applications or because of their capability for causing human diseases. Owing to their importance as antibiotic producers, streptomycetaceae, nocardiaceae and corynebacteriae deserve special attention.

Streptomyces

The *Streptomyces* genus has been found in different environments, such as soil, fresh water and seawater. Frequently, these environmental conditions turn out to be complex and adverse. Considering the fact that streptomycetes are nonmotile microbes, they cannot escape and must face changing environmental conditions. To develop, they must compete with other microorganisms for nutrients present in the environment. For this purpose, they have the ability to produce a wide range of secondary metabolites, such as antibacterial and antifungal substances.⁶⁸

Nutrients in nature are usually present in a wide range of complexities; therefore, microorganisms must have the necessary tools to succeed in their utilization. For this purpose, 819 potentially secreted proteins have been predicted to operate in *S. coelicolor*. Among them, cellulases/endoglucanases, chitinases/chitosanases, amylases, proteases/peptidases and pectate lyases are of special importance and many of

these enzymes also have commercial interest.⁶⁹ In addition, because of their capability to degrade multiple natural polymers, streptomycetes have an important role in soil ecology. Very often, the complex and changing environmental conditions can produce microbial stress. To confront this, streptomycetes are armed with many proteins (614 for *S. coelicolor*), which help them survive under adverse conditions. These protective proteins include transporters, drug efflux proteins and hydrolases.⁶⁹

The complex life cycle of streptomycetes, their high physiological versatility and ability to produce a great variety of secondary metabolites is reflected in their genome size. With sizes of 8.54, 8.7 and 9.03 Mb, the genomes of *S. griseus*, *S. coelicolor* and *Streptomyces avermitilis*, respectively, are among the largest genomes found in the microbial world.^{70,71} Moreover, streptomycetes have higher GC content (>70%) than nearly all other organisms. Unlike most other eubacterial chromosomes, the chromosome of this genus is linear with terminal-inverted repeats and covalently bound terminal proteins at the 5' end. Housekeeping genes are mainly found in the central region of the linear chromosome, whereas nonessential functions are often located near its ends.¹⁹ It is in this region where genes coding for secondary metabolites or transposons are located.⁷²

The ability to produce secondary metabolites, including antibiotics and bioactive compounds, is one of the most interesting properties of *Streptomyces*. This genus produces ~70% of the clinically useful antibiotics and anticancer agents.⁷³

For antibiotic production, common intracellular intermediates such as amino acids, sugars, fatty acids, terpenes, etc. are condensed into more complex structures by defined biochemical pathways.⁷⁴ Nearly 5% of the streptomycete genome (between 23 and 30 gene clusters) is devoted to the synthesis of secondary metabolites.⁷⁵ The highest percentage reported is 6.43% for the *S. avermitilis* genome, as this microorganism has the highest proportion of predicted secondary metabolite gene clusters of all bacterial genomes sequenced to date.⁷⁴

Streptomyces antibiotics are manufactured by microbial fermentation. The onset of biosynthesis is responsive to environmental cues including phosphate ions⁷⁶ and oxygen concentration, the nature and levels of the carbon and nitrogen source, as well as typical variables, such as temperature, light and pH. Several precise regulatory mechanisms are involved in the onset, maintenance and conclusion of antibiotic production.²³ Among these mechanisms, carbon source regulation is one of the main factors required for controlling secondary metabolism.

It is well known that several sugars are commonly used as carbon sources for growth and secondary metabolite production, but some of them are preferred by this genus. At the molecular level, the preferential use of one carbon source over the other and the synthesis of secondary metabolites responds either to prevention of transcriptional activation⁷⁷ or to repression. Repression by the carbon source is commonly known as CCR.^{78–80} This is a phenomenon usually caused by glucose, but in different organisms, other rapidly metabolized carbon sources can cause repression and, indeed, sometimes repress the catabolism of glucose itself.²³

More than 20 examples of antibiotics are reported to be suppressed by the carbon source. Glucose and other carbohydrates, such as glycerol, maltose, mannose, sucrose and xylose, have been found to interfere with the synthesis of antibiotics (Table 1). For instance, glucose depresses the formation of β -lactam antibiotics and macrocyclic polyketides. Cephamycin C is a β -lactam antibiotic produced by *Streptomyces clavuligerus* the synthesis of which is hindered by glycerol through the repression of the enzymes cephamycin C synthetase and expandase. Moreover, expandase activity is inhibited by phosphory-

Table 1 Carbon sources interfering with secondary metabolism in actinomycetes

Idiolite	Microorganism	Interfering carbon source	Target	Reference
Actinomycin IV	<i>Streptomyces parvulus</i>	Glc, Gly	Hydroxykynureninase Kynurenine formamidase II Tryptophan pyrrolase(R)	Brown <i>et al.</i> ²⁰⁵ Brown <i>et al.</i> ²⁰⁵ Brown <i>et al.</i> ²⁰⁵
Actinorhodin	<i>Streptomyces coelicolor</i>	Glc	AfsR2, SCO6569	Kim <i>et al.</i> , ⁸⁵ Im <i>et al.</i> ⁸⁶
Cephamicin C	<i>Streptomyces clavuligerus</i>	Gly	Cephamicin C synthetase and expandase	Lebrihi <i>et al.</i> ⁸¹
Cloramphenicol	<i>Streptomyces venezuelae</i>	Glc	Arylamine synthetase (R)	Bhatnagar <i>et al.</i> ²⁰⁶
Erythromycin	<i>Saccharopolyspora erythraea</i>	Glc, Gly	S-AMEMT (I)	Bermudez <i>et al.</i> ¹⁵¹
		Glc	Methylmalonyl-CoA-mutase	Bermudez <i>et al.</i> ¹⁵¹
		Glc	Type I polyketide synthase	Reeve and Baumberg ¹⁵³
Kanamycin	<i>Streptomyces kanamyceticus</i>	Glc	N-AKAH (R)	Satoh <i>et al.</i> ¹⁴¹
Neomycin	<i>Streptomyces fradiae</i>	Glc	Alkaline phosphatase (R)	Bandyopadhyay and Majumdar ⁸⁸
Puromycin	<i>Streptomyces alboniger</i>	Glc	O-demethylpuromycin O-methyltransferase (R)	Sankaran and Pogel ²⁰⁷
Streptomycin	<i>Streptomyces griseus</i>	Glc, Man	Mannosidostreptomycinase (R)	Demain and Inamine ⁸⁷
Tetracycline	<i>Streptomyces aureofaciens</i>	Glc	ATC oxygenase (I)	Erban <i>et al.</i> ²⁰⁸
Tylosin	<i>Streptomyces fradiae</i>	Glc	MMCAC and PCAC (R)	Vu-Trong <i>et al.</i> ²⁰⁹

Abbreviations: ATC oxygenase, Anhydrotetracycline oxygenase; Glc, glucose; Gly, glycerol; I, inhibition; Man, mannose; MMCAC, Methylmalonyl-CoA carboxyltransferase; N-AKAH: N-Acetylkanamycin amidohydrolase; PCAC, Propionyl-CoA carboxylase; R, repression; S-AMEMT, S-adenosylmethionine erythromycin-O-methyl transferase.

lated intermediates of glycolysis, such as glucose 6-phosphate and fructose 1–6 bis-phosphate.⁸¹ In addition to cephamycin C, *S. clavuligerus* produces clavulanic acid, a β -lactamase inhibitor. Although structurally related, these two β -lactams are derived from different biosynthetic precursors. Surprisingly, although glycerol abolishes cephamycin C production, it concomitantly increases clavulanic acid formation. Indeed, as this actinomycete cannot utilize glucose,⁸² glycerol is a good carbon source for clavulanic acid fermentation.⁸³

Macrocyclic polyketides produced by type I and II PKSs are highly significant secondary metabolites because of their clinical applications. Examples produced by type I PKSs comprise rifamycin and erythromycin, useful against mycobacterial infections; FK506, rapamycin and avermectin are used as immunosuppressant, antitumor and veterinary agents, respectively.⁸⁴ Polyketides produced by type II PKSs include actinorhodin, tetracenomycin, anthracyclines and tetracyclines.⁸⁴ The production of polyketides is also suppressed by different carbon sources. For instance, glucose inhibits actinorhodin production in *Streptomyces lividans* by repressing the synthesis of *afsR2* mRNA that encodes for a global regulatory protein involved in the stimulation of secondary metabolite biosynthesis. As expected, no repression is observed when glucose is substituted by glycerol in this microorganism.⁸⁵ As in *S. lividans*, this gene is necessary in *S. coelicolor* for actinorhodin production. Using two-dimensional gel electrophoresis, it has recently been demonstrated that AfsR2 binds to SCO6569, suggesting that this protein is an AfsR2-dependent downregulator for actinorhodin biosynthesis in *S. coelicolor*.⁸⁶

Additional examples of antibiotics the production of which is regulated by the carbon source include the aminoglycoside antibiotics (such as streptomycin, kanamycin, istamycin, neomycin and gentamicin) by repression of biosynthetic enzymes.^{17,24} Glucose suppresses streptomycin and neomycin production by *S. griseus* and *Streptomyces fradiae*, respectively. The mechanism involves mannosidostreptomycinase and alkaline phosphatase repression for streptomycin and neomycin biosynthesis, respectively.^{87,88} For gentamicin production, the glucose effect seems to occur at a step beyond antibiotic intermediate 2-deoxystreptamine.⁸⁹

Actinomycin production by *Streptomyces antibioticus* is also subject to control by the carbon source. In this microorganism, glucose transcriptionally represses hydroxykynureninase, an enzyme of the antibiotic pathway. In a control culture of *S. antibioticus* with galactose as the carbon source, the specific mRNA is low during the trophophase and high in the idiophase. When this culture is compared with that in a glucose medium, the mRNA is much lower and accordingly, the increase in enzyme activity observed in the control by 48 h is almost completely repressed by glucose or glycerol even after 96 h incubation.⁹⁰ In addition to hydroxykynureninase, the phenoxazinone synthase is repressed by glucose. This 88 000 Mr enzyme catalyzes the condensation of 4-methyl-3-hydroxyanthraniloyl pentapeptide to produce actinomycin. Compared with control cells grown in galactose, the specific activity of phenoxazinone synthase and its mRNA levels is decreased in a glucose medium.⁹¹

There are more examples of antibiotics the synthesis of which is impaired by the carbon source, but with scarce information about the target enzymes. This is the case for the synthesis of retamycin, an anthracycline antitumoral complex produced by *Streptomyces olindensis* using a polyketide pathway. This compound can be produced in a chemostat using a phosphate-limited defined medium. Anthracycline production from this microorganism can be repressed by 139 mM glucose. Under these conditions, an increase in the excretion of organic acids (such as pyruvate, citrate, succinate and lactate), with reduction in the biomass yield is observed, suggesting that flux through the glycolytic pathway has an important role in the repression of the biosynthesis of this antitumoral agent.⁹²

Doxorubicin also belongs to the anthracycline family of antitumor compounds. Its synthesis can be hindered by glucose and galactose in *Streptomyces peucetius* var. *caesius*, a strain derived by mutation from the daunorubicin producer *S. peucetius*. The glucose effect is observed when sugar is added initially or after 24 h fermentation, but not when added during the stationary growth phase. In addition, in a resting cell system containing protein-synthesis inhibitors, anthracycline formation is not affected by high glucose concentrations, suggesting a repressive rather than an inhibitory effect as the possible regulatory mechanism.⁹³

Nystatin belongs to the family of aminoglycosides. When *Streptomyces noursei* American Type Culture Collection 11455 is supplied with glucose as the carbon source, although high nystatin titers are achieved because of high biomass concentration, glucose tends to negatively influence nystatin production.⁹⁴ Conversely, glycerol supports a high specific growth rate and high nystatin yields and therefore represents a better choice of carbon source than glucose.

Spiramycin is a 16-membered macrolide antibiotic produced by *Streptomyces ambofaciens* used to treat toxoplasmosis.⁹⁵ Antibiotic production is regulated by glucose and glycerol.⁹⁶ Although glycerol increases growth rate and internal adenosine triphosphate (ATP), it depresses spiramycin production. Specific spiramycin production is increased 10-fold by fed-batch cultures with glycerol and ammonium feeding.⁹⁷ In a batch culture under ammonium depletion, excess glucose led to pyruvate and α -ketoglutarate accumulation and maintained an antibiotic production phase from these acids after glucose exhaustion.⁹⁸

One of the conclusions from the above-mentioned information is that not all secondary metabolite genes are equally sensitive to carbon source regulation control. For instance, actinorhodin production is sensitive to glucose concentrations in the range of 100 mM; however, higher concentrations are necessary to repress doxorubicin production.⁹³

Mechanism of CCR in *Streptomyces*

CCR control by PTS. The mechanism for CCR in *Streptomyces* is not clearly understood and there is probably more than one mechanism involved. The possible mechanisms have been studied primarily in *S. coelicolor*, by far one of the better known biological models.^{19,99} One of these mechanisms involves the PTS described above.^{100,101} In *S. coelicolor*, there are at least four PTS transporters, all of which use the universal PTS energy-coupling enzyme I (EI, encoded by *ptsI*) and HPr (encoded by *ptsH*).^{102–104} Two of the transporters have been characterized in detail, and are responsible for the transport of D-fructose and N-acetylglucosamine.^{102,105} Glucose is not transported by the PTS, but through the specific GlcP permease, which is probably the main reason why PTS is not involved in glucose repression.¹⁰⁶

Recently, a pleiotropic effect of N-acetylglucosamine (GlcNAc) uptake on development and production of the blue-pigmented polyketide antibiotic actinorhodin has been reported in *S. coelicolor*.¹⁰⁷ This effect depends on the nutritional conditions: under famine (poor) conditions, GlcNAc stimulates development and antibiotic production, whereas under feast (rich) conditions, it blocks development.¹⁰⁸ In nature, under starvation conditions, the most likely source of GlcNAc is the autolytic degradation of the bacterial cell wall, whereas under rich conditions, the most likely source is chitin. Hence, GlcNAc signals the nutritional status of the habitat and controls the process of programmed cell death that coincides with development. GlcNAc enters the cell and is phosphorylated through the GlcNAc-specific phosphoenolpyruvate-dependent PTS permease complex that is composed of the general phosphotransferases EI, HPr and IIA^{CrT} and the specific IIB and IIC components NagF and NagE2, respectively.¹⁰⁷ Phosphorylated GlcNAc (GlcNAc-6-P) is further deacetylated by NagA (GlcNAc-6-P deacetylase) producing glucosamine-6-phosphate (GlcN-6-P), an allosteric effector of the global transcription factor DasR (deficient in aerial mycelium and spore formation).¹⁰⁸ DasR belongs to the GntR family and controls actinorhodin production by binding to an operator site (*dre* for DasR-responsive element) overlapping the promoter for *actII-ORF4*, the pathway-specific activator gene for the actinorhodin biosynthetic cluster. In addition, DasR represses *nagF* and *nagE2* genes, which encode the structural IIB and IIC domains of the GlcNAc permease.

The effector molecule is GlcN-6-P, thus upon binding to GlcN-6-P, DasR is released from its target genes,¹⁰⁹ relieving the inhibition of antibiotic production and morphological differentiation. This effect is absent in a mutant defective in GlcNAc transport.¹⁰⁹ Therefore, transcription of *nagE2* is induced by GlcNAc but repressed by DasR.

As mentioned above, besides its role in the control of primary and secondary metabolism, DasR also controls the onset of morphological differentiation.^{109,110} The manner by which it does that is yet unclear, but it is most likely primarily due to a complete upset of primary metabolism rather than the control of developmental genes, such as the *bld* or *whi* genes (GP van Wezel, personal communication). As an example, one of the major targets of DasR control besides the *pts* and *nag* regulons is the *dasABCD* chitobiose utilization operon that lies immediately adjacent to (and is oppositely oriented toward) *dasR*. Similar to the *pts* genes, *dasA* is required for sporulation, and *dasA* deletion mutants have a bald phenotype.¹¹¹ Extracellular complementation experiments with different *bld* mutants showed that the *dasR* mutant is arrested at a very early stage of the developmental program.¹⁰⁹ A complete GlcNAc-dependent signaling model was recently proposed for *Streptomyces* by Nothaft *et al.*¹⁰⁷ from the perspective of the environmental nutritional status to the onset of antibiotic production through DasR. The start of the signaling cascade is the transport of GlcNAc by NagF, and it culminates in the control of pathway-specific activators for antibiotic production (such as ActII-ORF4). Interestingly, both are subject to multilevel control¹⁰⁷ as both *nagE2* and *actII-ORF4* transcription are activated by AtrA, a TetR-family regulator originally identified as an activator of actinorhodin biosynthesis.⁷⁷

The evidence provided points to the function of PTS proteins as key elements in CCR exerted by fructose and GlcNAc in *Streptomyces*.¹¹⁰ However, deletion of the *ptsH* gene encoding HPr has no effect on glucose repression of agarase, galactokinase and glycerol kinase, indicating that PTS responds only to the presence of fructose and GlcNAc.^{105,112,113} Therefore, at least one additional mechanism is necessary to explain how glucose and other carbon sources exert CCR.

The role of *bld* and *regI* in CCR. In addition to the PTS system, the analysis of *Streptomyces* mutants insensitive to CCR has suggested the involvement of the products of a number of genes such as *bld* and *regI* in the mechanism of CCR.^{114,115} In *S. coelicolor*, the synthesis of antibiotic and the formation of aerial hyphae initiate at approximately the same time during microbial development, and there is some evidence suggesting that both events are coordinated at the molecular level. In this regard, BldB has been characterized as a protein involved in aerial hyphae formation.¹¹⁴ Interestingly, it has been observed that after rapid growth has ceased, culture supernatants become protein rich, supporting the idea that protein secretion is also a stationary-phase phenomenon.¹¹⁶

It is known that on different carbon sources, BldB null mutants have a bald phenotype, that is, lacking an obvious aerial mycelium, are pleiotropically blocked for antibiotic biosynthesis¹¹⁰ and defective in CCR.^{117–119} Thus, *bldB*, which encodes a small protein containing a putative DNA-binding helix-turn-helix motif, seems to link secondary metabolite production and development to carbon source-dependent gene regulation. In addition to *bldB* mutants from *S. coelicolor*, other *bld* mutants from this microorganism that fail to develop normal aerial mycelia and to produce antibiotics have been isolated by ultraviolet irradiation. The mutants are grouped into four distinct phenotypic classes. In each class, the pleiotropic phenotype is due to a single mutation and separately involves the *bldA*, *bldB*, *bldG* and *bldH* genes. Among them, *bldA*, *bldG* and *bldH* are repressed during growth

on media containing glucose or cellobiose. Repression is partially relieved using alternative carbon sources (arabinose, galactose, glycerol, mannitol and maltose) for *bldA* and *bldG* mutants and totally relieved for *bldH* mutants.¹¹⁴ Therefore, all bld mutants isolated, exhibit carbon source-dependent differentiation.

The *bldA* gene encodes for a tRNA capable of efficient translation of the uncommon UUA (leucine) codon, which is rarely present in the *Streptomyces* genome as TTA. Interestingly, it has been shown that the gene for a *bldA*-dependent protease inhibitor proved to be dependent on the TTA-containing pleiotropic regulatory gene *adpA*.¹¹⁶ *adpA*, also known as *bldH*, mediates the effects of *bldA* on morphological differentiation and antibiotic production.¹¹⁴ With regard to *bldG*, it has been proposed that this gene encodes a putative anti-anti-sigma factor that might control transcription of both aerial mycelia formation and antibiotic formation.¹²⁰

Using transposon mutagenesis, Chouayekh *et al.*¹²¹ isolated *S. lividans* TK24 mutants the α -amylase expression of which was resistant to glucose catabolite repression. Mutant characterization showed interruption of the *sblA* gene (equivalent to SCO0479 of *S. coelicolor*). This gene was predicted to encode a bifunctional enzyme of 274 amino acids with phospholipase C-like and phosphoinositide phosphatase activities. In addition, the *sblA* mutant strain was shown to enter prematurely into the stationary phase when grown on minimal medium containing glucose as the main carbon source. When cultured in a complex glucose-rich liquid medium, the wild-type strain showed diauxy growth, resuming growth after the transition phase. However, the *sblA* mutant does not resume growth after the transition phase, when cultured in the same medium, and sporulates much earlier than the wild-type strain when plated on solid-rich medium. These phenotypic differences were abolished when glucose was omitted from the rich medium and correlated with reduced ability for glucose uptake of the *sblA* mutant strain. Cleavage of phosphoinositides by *SblA* seems necessary to trigger the glucose-dependent renewed growth that follows the transition phase through an unknown mechanism. The temporal control of *sblA* expression was shown to depend on two operator sites. One site, located just upstream of the -35 promoter region, likely constitutes a repressor-binding site. The other site, located 170 bp downstream of the GTG *sblA* translational start codon, may be involved in regulation of the degradation of the *sblA* transcript.

Involvement of glucose kinase in CCR. D-glucose is one of the easiest utilizable carbon sources for growth in the genus *Streptomyces*. High concentrations of sugar elicit CCR over the use of alternative carbon sources and the synthesis of several secondary metabolites. Glucose repression in *S. coelicolor* operates at the transcriptional level to repress enzymes involved in the use of glycerol, arabinose, fructose and galactose¹²² and this effect seems to be due to either intermediates of carbohydrate catabolism, for example, fructose 1,6-diphosphate and glucose 6-phosphate^{123,124} or enzymes of the glucose catabolic pathway, such as glucose kinase.^{125–128} In this regard, it has been reported that mutants of *S. coelicolor* resistant to the nonutilizable glucose analog, 2-deoxyglucose (DOG), appear to be generally deficient in glucose repression.¹²² These mutants (Dog^R) can utilize glycerol, arabinose, fructose and galactose in the presence of glucose. Of these mutants, 85% cannot grow on glucose. The loss of glucose repression in Dog^R mutants has been correlated with the absence of an ATP-dependent glucose kinase (Glk) activity.¹²⁹ Complementation of these mutants with the *glk* gene (*glkA*) restores not only Glk activity but also DOG sensitivity and partially restores glucose repression.^{125,126} Similarly, when the *S. coelicolor glkA* gene, is introduced

into a *S. lividans* Dog^R mutant (unable to utilize glucose and the chitinase production of which is resistant to glucose repression), sensitivity to DOG and the ability to utilize glucose are restored, but glucose repression of chitinase production is only partially recovered.¹²⁸ Interestingly, when *S. coelicolor* Dog^R mutants were complemented with the *glk* gene of the Gram-negative *Zymomonas mobilis*, Glk activity and glucose utilization are restored, but not glucose repression.¹³⁰ These results suggested a regulatory role, different from Glk catalytic activity, necessary for CCR in this microorganism. The regulatory role for Glk in glucose repression of *S. coelicolor* was further supported by the experiments conducted by Kwakman and Postma.¹²⁷ These authors demonstrated that the CCR of glycerol kinase and agarase is relieved in cultures of Dog^R mutants, grown in a series of carbon sources, such as galactose and glycerol, which are not metabolized through Glk. These results strongly suggested that catabolite repression is not regulated by the flux through Glk and that the protein itself has a regulatory role in CCR.¹²⁷

With regard to the possible mechanism by which Glk exerts a regulatory effect on CCR, it has been observed that this enzyme does not contain obvious DNA-binding sites and thus, it is unfeasible that it could directly regulate transcription. Therefore, it has been proposed that GlkA can transmit its CCR signal by interaction with transcription factors.¹²⁶ These may include pathway-specific regulators such as MalR and GylR, repressors of the maltose and glycerol operons, respectively, or pleiotropic regulators such as BldB.^{131,132} To verify such relationship, MalR, GylR and BldB proteins were heterologously overproduced, purified and assayed for interaction with GlkA. This was approached using several means, including surface plasmon resonance, pull-down assays, a peptide library server, immunoprecipitation and *in vivo* using two-hybrid technology.¹³³ However, these attempts did not allow identification of a GlkA-interacting protein making interaction between Glk and these transcriptional regulators unlikely. On the other hand, apparent binding between Glk and the glucose transporter GlcP has been reported,¹³⁴ suggesting that a Glk–GlcP complex is required for efficient glucose metabolism.

Implication of SCO2127 in CCR. Thus, there is mounting evidence that Glk itself is not sufficient to elicit CCR in the genus *Streptomyces*. For instance, it has been previously shown in *S. coelicolor* that resistance to DOG yields Dog^R mutants with decreased glucose kinase activity and reversal of glucose repression.¹²² A derivative of the temperate phage ϕ C31 containing *glkA* alone is not enough to completely restore the wild-type phenotype when used to lysogenize a *S. coelicolor glk* null mutant with the Dog^R phenotype. Complete restoration to the wild-type phenotype is observed with the temperate phage ϕ C31 KC896 containing both *glkA* and *sco2127* genes.¹²⁶ Additional evidence has been supplied by Flores *et al.*¹³⁵ showing no differences in the Glk levels between the parental strain of *Streptomyces kanamyceticus* and a mutant derived from this microorganism, insensitive to CCR for α -amylase and kanamycin formation. Therefore, these results suggest the need for additional mechanisms, in addition to Glk, to explain the CCR mechanism exerted by glucose.

In addition to DOG-resistant mutants from *S. coelicolor*¹²² and *S. lividans*,¹²⁶ Dog^R mutants have also been isolated from *S. peucetius* var. *caesius*. This microorganism, with an 8.16-Mb genome size, is a doxorubicin producer.¹³⁶ In addition to presenting CCR insensitivity and low Glk activity, Dog^R mutants also show difficulties in transporting glucose.⁹³ As previously reported for a Dog^R mutant from *S. coelicolor*,¹²⁵ a partial reversion of the Dog^R phenotype is observed when transforming the mutant with *glkA*. However, a complete reversion of this phenotype is obtained when the mutant is

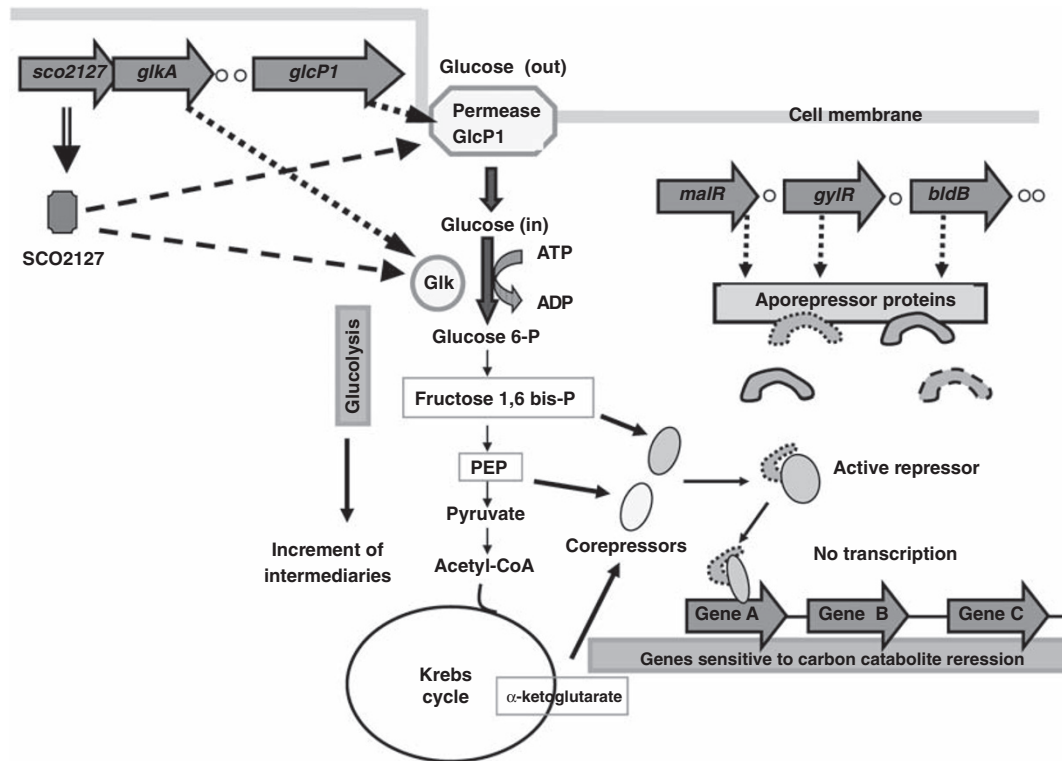


Figure 3 Hypothetical model explaining the stimulatory effect of SCO2127 in *S. coelicolor*. Abbreviations: *bldB*, gene encoding for aerial hyphae formation aporepressor; GlcP1, glucose permease; Glk, glucose kinase; *gylR*, gene encoding for glycerol aporepressor; *malR*, gene encoding for maltose aporepressor; PEP, phosphoenolpyruvate. The interrupted arrow (--->) indicates enzyme activation. (--->) indicates gene expression.

transformed with the *sco2127* gene alone.^{137,138} Thus, in the recombinant strain, glucose uptake and Glk activity values are reverted back to the level of the original strain or even higher, and the recombinant regains sensitivity to CCR. These results were surprising considering that the *sco2127* region does not seem to encode for either a glucose permease¹⁰⁶ or a Glk,¹²⁶ and also lacks DNA-binding motifs. Dot-blot analysis of the *S. peucetius* var. *caesius* Dog^R mutant suggested that *sco2127* encodes a protein, which stimulates transcription of *glk* and probably that of the glucose permease gene as well.¹³⁷ In conclusion, these data suggest participation of an integral regulatory system that is initiated by an increase in glucose incorporation and its metabolism, resulting in increased synthesis of catabolites, which may be involved in eliciting CCR in this microorganism. In agreement with this possibility, among several products of glucose metabolism, fructose 1,6 bis-phosphate and phosphoenolpyruvate exert CCR on anthracycline formation in *S. peucetius* var. *caesius*¹²³ with fructose 1,6 bis-phosphate being the most effective (Figure 3). Therefore, it is feasible that phosphorylated sugars can mediate CCR. In this regard, it is known that 2-deoxyglucose, but not 3-O-methylglucose, interferes with tylosin production by *S. fradiae* in a process sensitive to glucose and phosphate. It has been reported that 2-deoxyglucose is translocated and phosphorylated by most bacteria, whereas 3-O-methylglucose is imported but rarely phosphorylated, supporting glucose phosphorylation as a necessary step for CCR.²⁴

The *sco2127* region (576 pb) is located upstream of the *glkA* gene and no evident function has been conferred to its possible expression product. As *sco2127* lacks DNA-binding motifs, direct regulation of the *glk* promoter cannot be expected. Recently, the protein was overexpressed and purified and polyclonal anti-SCO2127 antibodies were produced. The antibodies were able to detect SCO2127 from

crude extracts of both *S. coelicolor* and *S. peucetius* var. *caesius*, yielding a single protein band of 34 kDa in western blot analyses.¹³⁹ SCO2127 and its corresponding ortholog from *S. peucetius* var. *caesius* show a 61% identity in amino-acid composition (JK Sohng, personal communication). When the protein was monitored in *S. coelicolor* cultures grown in the presence of 50 mM glucose, SCO2127 was detected at the beginning of fermentation and during the logarithmic growth phase, but decreased at later stages of microbial growth. Its production during logarithmic growth agrees with its predicted involvement in CCR, as SCO2127 expression is closely linked to glucose concentration present in the culture medium.¹³⁹ Detection of SCO2127 at the beginning of fermentation may be related to its possible role in carbohydrate transport¹³⁸ as the seed cultures were grown in the presence of 50 mM mannitol. On the other hand, cultures grown in 100 mM glucose showed SCO2127 even at later stages of microbial growth coinciding with the presence of 40% of the initial available glucose.

It seems reasonable to think that stimulation of both activities allow efficient glucose consumption with the concomitant production of glucose catabolites, compounds presumably involved in eliciting CCR.¹²³ However, a direct effect of SCO2127 on the activation of Glk or GlcP cannot be discarded. In this regard, the apparent binding of Glk to the major glucose transport system of *S. coelicolor* (GlcP) reported by van Wezel *et al.*¹³⁴ might be mediated by SCO2127, increasing the efficiency of glucose metabolism.

Participation of cAMP and CRP in CCR. For a long time, the existence cAMP in *Streptomyces* was in doubt. However, cAMP and its binding protein are present in this genus. In addition, 3,5-cyclic guanosine monophosphate is also present in streptomycetes.¹⁴⁰ There are several examples suggesting the participation of cAMP in CCR. In

S. kanamyceticus, cAMP relieves glucose repression of *N*-acetylkanamycin amidohydrolase.¹⁴¹ In addition, dibutyryl cAMP supplementation to cultures of *S. fradiae*, under conditions of growth limitation by ferric ammonium citrate, stimulates tylosin production by 25–30%.¹⁴² In the same line, variants of this microorganism with high tylosin production contain 20–50% more cAMP than the parental strain.²⁴

Spore germination, aerial mycelium formation and actinorhodin production are also dependent on cAMP in *S. coelicolor*.¹⁴³ Disruption of *cya*, the gene encoding adenylate cyclase in *S. coelicolor* generated a mutant (BZ1) unable to produce cAMP and defective in aerial mycelium formation.¹⁴³ Both wild-type and mutant cultures showed acidification of the medium from 7 to 4.5, but only the parent could partially neutralize the medium during formation of aerial mycelia. Addition of exogenous cAMP or the use of a pH 7 buffer allows the mutant to make aerial mycelia and increases actinorhodin production. Conversely, synthesis of undecylprodigiosin did not respond to cAMP in the presence or absence of buffer. This effect fits well with the selective repression of actinorhodin production in cultures grown in high glucose.¹⁴⁴ In addition, the existence of CRP in *S. coelicolor* has been demonstrated. Thus, SCO3571 (*crp*) encodes for a protein of 224 amino acids that clusters to the CRP subfamily.¹⁴⁴ As reported for *cya* mutants from *S. coelicolor*,¹⁴³ strains defective in *crp* also showed a lack of germination and actinorhodin production. However, synthesis of several glucose-repressive enzymes (such as α -amylase, mannanase, xylanase and cellulase), induced by their specific substrates was glucose repressed in the *crp* mutant, suggesting that CRP has no role in CCR in *S. coelicolor*.¹⁴⁵

In contrast to *cya* mutants, addition of exogenous cAMP does not restore the *crp* mutant phenotype, indicating that CRP participation is not due to a lack of cAMP biosynthesis. Although it has been suggested that *S. coelicolor* may use a *Cya*–cAMP–CRP system to trigger morphogenesis and actinorhodin formation,¹⁴⁵ further research is necessary to unequivocally demonstrate the mechanism of CRP participation.

Contribution of ATP and adenosine in CCR. Another nucleotide involved in the regulation of secondary metabolite biosynthesis is ATP. Li *et al.*¹⁴⁶ reported the effect of extracellular ATP (exATP) on antibiotic formation by *S. coelicolor*. When this microorganism is grown in the presence of 10 mM exATP, actinorhodin concentration increases by 90% compared with a culture grown in the absence of the nucleotide. Conversely, only a 23% increase in undecylprodigiosin is observed. These authors also claim that stimulation by exATP is extended to antibiotics produced by other streptomycetes, including *S. lividans*, *S. griseus*, *Streptomyces violaceoruber* and *S. avermitilis*.¹⁴⁶ The authors suggest that exATP acts as a signaling molecule at the bacterial surface leading to intracellular signaling events and induction of transcriptional activators of antibiotic production. However, in higher exATP, cultures exhibit high intracellular ATP levels and a decreased production in both actinorhodin and undecylprodigiosin.

The nucleoside adenosine also seems to have a role in the production of actinorhodin. Adenosine kinase phosphorylates adenosine to produce AMP using ATP as the main phosphoryl donor. In *S. lividans* mutants lacking adenosine kinase, no AMP is produced and adenosine is accumulated.¹⁴⁷ Under these conditions, production of actinorhodin is suppressed in the mutant and a loss of sporulation is observed. Conversely, production of undecylprodigiosin (the red antibiotic) is enhanced. A promoter-probe assay verified the repressive effect of adenosine on transcription of the pathway-specific activator, *actII-ORF4* of actinorhodin production and induction of the undecylprodigiosin promoter.¹⁴⁷ No connection between the ATP and

adenosine effects on antibiotic production are visualized as *S. coelicolor cya* mutants, unable to produce cAMP, are expected to accumulate intracellular ATP, which might phosphorylate adenosine avoiding repression of actinorhodin production. Conversely, accumulation of intracellular ATP could be expected to occur in a *S. lividans adk* mutant, favoring cAMP and actinorhodin formation, which is not the case.

Saccharopolyspora. *Saccharopolyspora erythraea* is used for industrial-scale production of the antibiotic erythromycin A. Derivatives of this antibiotic have a crucial role in medicine for the treatment of infectious diseases and as a gastrointestinal motor stimulator.⁵ Its chromosome comprises 8.2 Mb pairs, predicted to encode 7264 genes.¹⁴⁸ As reported for other actinomycetes, *S. erythraea* has a circular chromosome. Its genome contains at least 25 gene clusters for production of known or predicted secondary metabolites, and at least 72 genes have been predicted to confer resistance to a range of common antibiotic classes.¹⁴⁸ Erythromycin is produced from propionyl-CoA or succinyl-CoA, through a process of assembly that involves at least 28 active sites arranged along three large proteins. This is followed by hydroxylation and glycosylation processes involving 18 additional proteins. Finally, microbial ribosomes are protected from high antibiotic-specific toxicity by specific methylation of the rRNA.¹⁴⁹

Glucose transiently represses antibiotic formation¹⁵⁰ probably by repressing methylmalonyl-CoA-mutase.¹⁵¹ Although this enzyme is repressed by glucose, methylmalonyl-CoA decarboxylase production is not affected by the carbon source, suggesting that the negative effect of glucose on erythromycin production could be due, in part, to lower pools of succinyl-CoA and methylmalonyl-CoA.¹⁵¹ Recently, engineering *S. erythraea* through duplication of the methylmalonyl-CoA mutase led to a 50% increase in erythromycin production.¹⁵² Moreover, glucose or glycerol inhibits *S*-adenosylmethionine erythromycin *O*-methyl transferase activity. Reeve and Baumberg¹⁵³ measured the effect of glucose on the transcription of the *eryAI* gene, encoding the type I polyketide synthase. With an increase in glucose concentration, they found a decrease in the lag before the onset of erythromycin production, but a decrease in the final levels of *eryAI* expression.

Substitution of glucose by cane molasses accompanied by a reduction in ammonium sulfate concentration and supplementation of the medium with *n*-propanol has been utilized for increasing erythromycin production and reduction in costs of antibiotic production.¹⁵⁴

In *S. erythraea*, the erythromycin biosynthetic cluster lacks a regulatory gene.¹⁵⁵ However, a 17.7-kDa *bldD* ortholog has recently been described, which positively regulates all promoters in the erythromycin production cluster, suggesting the existence of a transcriptional activator of the *ery* gene cluster.¹⁵⁵ In *S. coelicolor*, *BldD* negatively regulates the expression of key developmental genes.¹⁵⁶ *bldD* mutants pleiotropically affect both formation of aerial hyphae and antibiotic production in this microorganism.¹⁵⁷ Similarly, *S. erythraea bldD* mutants produce a bald phenotype and sevenfold less erythromycin than the *S. erythraea* wild-type strain (NRRL2338).¹⁵⁵

Nocardia. Bacteria from the genus *Nocardia* produce a wide variety of secondary metabolites. Nocardicin A and B (*Nocardia* sp.), ryfamycin (*Nocardia mediterranea*), ansamitocin (*Nocardia brasiliensis*), 3-*O*-demethyl mutactimycin (*Nocardia transvalensis*), neo-nocardin (*Nocardia kuroishi*) and cephamycin C (*Nocardia lactamdurans*) are some examples of antibiotics produced by this genus.

Although reported as a good antibiotic producer, studies on the effect of carbon source on idiolite formation are limited in this genus.

For instance, it has been reported that various sugars and their metabolites inhibit cephamycin C production by *N. lactamdurans* resting cells.¹⁵⁸ At the biochemical level, glucose-6-phosphate and fructose-1,6-diphosphate inhibit deacetoxycephalosporin C (DAOC) synthase, one of the pathway enzymes dealing with antibiotic formation. With regard to cAMP levels, a similar behavior to that reported for several streptomycetes is observed, that is, high during growth and low during antibiotic production, suggesting that this nucleotide is not involved in derepressing secondary metabolism.

Corynebacterium. *Corynebacterium kutscheri* and *Corynebacterium xerosis* produce antimicrobials effective against bacteria and fungi. It has been established that antibiotic production in these species is greatly influenced by variation in the carbon source. Along this line, ribose and lactose suppress the antimicrobial activity of *C. kutscheri* and *C. xerosis*, respectively.¹⁵⁹

The corynebacteria have genes for the PTS-regulatory mechanism of CCR. Two energy-coupling proteins, E1 and Hpr, and permeases for glucose and fructose are present. Moreover, it contains two distinct genes encoding an IIAB^{P_{tx}}-like and a novel HPr-type protein of unknown function. In addition, a possible PTS gene target was elucidated as a possible regulatory gene encoding an antiterminator protein.¹⁶⁰

CARBON SOURCE REGULATION OF SECONDARY METABOLISM BIOSYNTHESIS IN FUNGI

As observed for actinomycetes, fungi also synthesize antibiotics in response to physiological stresses, such as nutrient limitation (carbon, nitrogen or phosphate sources). The growth medium markedly influences the production of antibiotics.¹⁶¹ In addition, the carbon source may exert complex regulation on gene expression and enzyme activities for polyketide synthesis.¹⁶²

Table 2 shows some examples of antibiotics produced by fungi that are repressed by different carbon sources, and their target enzymes.

Penicillins and cephalosporins are β -lactam antibiotics. The formation of hydrophobic penicillins has been reported in fungi only, notably *Penicillium chrysogenum* and *A. nidulans*, whereas the hydrophilic cephalosporins are produced by both fungi, for example, *Acremonium chrysogenum* (cephalosporin C) and bacteria.¹⁶³

In *P. chrysogenum*, glucose represses the transcription of penicillin biosynthetic genes *pcbAB*, *pcbC* and *penDE*¹⁶⁴ which encode for δ -(L- α -aminoadipyl)-L-cysteinyll-D-valine synthetase (ACVS), isopenicillin *N*-synthase and IPN acyltransferase, respectively. Whereas glucose negatively affects *pcbAB*, *pcbC* and *penDE* gene promoters, alkaline pH exerts a small positive effect on these promoters.¹⁶⁴ The *P. chrysogenum* *pcbC* gene was cloned downstream of the strong, inducible *Hansenula polymorpha* alcohol oxidase promoter (P_{AOX}) and was integrated at the P_{AOX} locus in the *H. polymorpha* genome and

cultivated on methanol medium to induce the P_{AOX} and then to mid-exponential growth phase. When glucose (0.5%) was added to the cultures, a treatment that results in complete repression of the P_{AOX} (30 min after glucose addition), the isopenicillin *N*-synthase protein levels in the cells decreased by >50%.¹⁶⁵

In the same manner, genes *aatA* and *ipnA* but not *acvA* (homologs to *penDE*, *pcbC* and *pcbAB*, respectively) are repressed by glucose in *A. nidulans*.^{166,167} A Cre consensus sequence 5'-SYGGRG-3', is located in the upstream region of the *ipnA* gene and the binding of CreA (CCR) has been confirmed *in vitro*.¹⁶⁸ However, it has been shown that *A. nidulans* *creA* mutants cultured in glucose, still present repression of *ipnA* transcription.¹⁶⁹

CreA is the major repressor protein regulating the CCR of genes involved in carbon metabolism in *A. nidulans*.¹⁷⁰ It binds to the consensus DNA sequence 5'-SYGGRG-3' (where S could be C or G; Y could be C or T and R could be A or G) in promoters of glucose-repressible genes, turning off their expression. Cre-binding sites are found in the promoters of *cre* genes from diverse filamentous fungi.¹⁷¹

The promoters of many glucose-repressible target genes have Cre-binding consensus sequences.¹⁷² The Cre1 protein (from the *cre1* gene) sequence shows high similarity to other glucose-repressor proteins from filamentous fungi, suggesting a similar regulation and a related role in CCR. Janus *et al.*¹⁷¹ identified a 114-bp minimal promoter with two Cre1-binding sites, the first minimal regulatory promoter sequence for the β -lactam antibiotic-producing fungus. The identification of 114-bp CT-rich sequences in the nontranscribed *cre1* upstream region suggests that it is required to trigger glucose-dependent gene expression.¹⁷¹

Mutations in *creB* and *creC* have shown little effect on carbon regulation of penicillin biosynthesis. All these results point to a second mechanism of carbon repression, which is *creA* independent.¹⁷³

In *A. chrysogenum*, glucose represses cephalosporin C formation. As this organism contains *cre1*- and *cre1*-binding sites upstream of the isopenicillin *N*-synthase (*pcbC*) and the DAOC/deacetylcephalosporin C synthase (*cefEF*) genes, it is considered that *cre1* might be involved in repression of antibiotic formation. Indeed, in the wild-type strain, glucose increased the level of *cre1* transcripts sixfold. As expected, in a commercial high-producing *A. chrysogenum* strain, glucose did not affect the *cre1* transcript levels. Therefore, it is feasible that *cre1* might be involved in glucose repression of cephalosporin C production and that during the program of strain improvement, this control mechanism could have been deregulated.^{171,174} CT-rich regions are putative activator sequences in close proximity to transcription start sites.¹⁷⁵ In *A. chrysogenum*, the CT-rich region contains two closely spaced binding sites (namely BS14 and BS15) that cooperatively bind to Cre1. The *cre1* promoter from *A. chrysogenum* displays a positive autoregulation upon glucose induction. This 114-bp minimal pro-

Table 2 Carbon sources interfering with secondary metabolism in fungi

Idiolite	Microorganism	Interfering carbon source	Target	Reference
Cephalosporin C	<i>Acremonium chrysogenum</i>	Glc	Deacetoxycephalosporin C	Jekosch and Kuck ²¹⁰
		Gly	Synthetase (expandase) (R)	Jekosch and Kuck ²¹⁰
		Mal	δ -ACVS (I), cyclase (R)	Zhang and Demain ¹⁷⁶
Penicillin	<i>Penicillium chrysogenum</i>	Glc	ACVS (R), IPN cyclase (R)	Theilgaard <i>et al.</i> ²¹¹
			IPN acyl-transferase (R)	Gutiérrez <i>et al.</i> ¹⁶⁴
	<i>Aspergillus nidulans</i>	Glc	ACVS (R), IPN synthase (R)	Brakhage <i>et al.</i> ¹⁶⁶
			IPN acyl transferase (R)	Litzka <i>et al.</i> ¹⁶⁷

Abbreviations: ACVS, δ -(L- α -aminoadipyl)-L-cysteinyll-L-valine synthetase; Glc, glucose; Gly, glycerol; I, inhibition; IPN, isopenicillin *N*-synthase; Mal, maltose; R, repression.

moter with two Cre1 consensus binding sites is an example of a minimal regulatory promoter sequence for this β -lactam antibiotic-producing fungus.¹⁷¹

Direct inhibition (a mechanism that restrains synthesis at the level of enzyme activity, therefore the effect is immediate) of the action of secondary metabolic enzymes may also be involved in carbon source control. Thus, glucose and a number of its phosphorylated metabolites inhibit ACVS, the first enzyme of cephalosporin synthesis, in crude extracts of *A. chrysogenum* and *S. clavuligerus*.¹⁷⁶ As no inhibition is observed with the purified enzyme, it seems that this phenomenon might be caused by competition for ATP between primary (Embden–Meyerhof pathway) and secondary metabolism. Accordingly, it has been found that inhibition of ACVS by sugars can be prevented by addition of more ATP. Conversely, both the crude and the purified forms of ACVS are inhibited by glyceraldehyde-3-phosphate (G3P). This effect seems to be due to the ability of G3P to chemically complex and remove cysteine, a substrate of ACVS. The *in vivo* significance of such inhibitory phenomena remains to be determined.

With regard to the mechanism of CCR in *A. nidulans*, carbon catabolism is a tightly regulated process, whereby the most energetically favorable carbon sources are used preferentially over less readily metabolized carbon sources. It has been demonstrated that a Glk (GlkA4) and a hexokinase (Hxk) participate equally in sugar signaling. Single *glkA4* and *hxkA* mutants were able to substitute for each other in their function in catalysis and in CCR. However, double mutants in *glkA4* and *hxkA* genes showed derepression in genes, such as *facA* for acetate catabolism, *xlnA* for xylan degradation and *alcA* and *alcR* for ethanol utilization.¹⁷⁷ These observations suggest that glucose phosphorylation could be the relevant signal for glucose repression in *A. nidulans*.¹⁷⁷ In addition, the *creA*, *creB*, *creC* and *cre1* genes¹⁷⁸ were identified as coding for regulatory proteins involved in CCR in *A. nidulans*. CreA and Cre1 contain two Cys₂His₂ DNA-binding zinc fingers similar to the *Saccharomyces cerevisiae* glucose-repressor protein Mig1.^{170,179,180} *creB* encodes a deubiquitinating enzyme, and *creC* encodes a WD40 protein that contains five WD40 repeats and a proline-rich region. CreC forms a complex with CreB.^{181,182} The proposed model for CCR through CreA modification or stability involves the CreB deubiquitination enzyme complexing with the CreC protein to modify or stabilize CreA under carbon-repressing conditions.¹⁸³

An additional gene implicated in the mechanism of *A. nidulans* CCR is *creD*. Mutations in this gene suppress the *creB15* and *creC27* mutant phenotypes, showing derepression of *facA* and *alcA* genes. The *creD* gene encodes a protein that contains arresting domains and PY motifs and interacts with the HECT (homologous to the E6-associated protein carboxyl terminus) ubiquitin ligase.¹⁸⁴ Although the elements of ubiquitination and deubiquitination networks have been disclosed, their direct targets still remain to be elucidated.¹⁷⁰ Recently, it was found that the mechanism of action of CreA in CCR does not require its degradation nor its cellular localization, but seems to entail some modification or interaction with another protein to act as a repressor.¹⁷⁰ In a study elucidating the kinetics of glucose uptake in *A. nidulans*, two energy-requiring glucose transport systems were identified: a high-affinity glucose-repressible system and a low-affinity glucose inducible system (MstE). Besides glucose, *mstE* expression is induced in the presence of other repressing carbon sources, and is dependent on the function of the transcriptional repressor CreA.¹⁸⁵

Pleuromutilin is a diterpenoid antibiotic produced by *Pleurotus mutilis*-04. Pleuromutilin is currently used as an intermediate for the production of tiamulin and valnemulin for veterinary use. In addition, retapamulin is a promising antibiotic with the potential for human use

in the treatment of Gram-positive bacterial infections.¹⁸⁶ Compared with glucose as the sole carbon source, soybean oil was beneficial for pleuromutilin production in shaker flask cultures.¹⁸⁷ This result suggested a suppressive effect of glucose on antibiotic production.

Fusarium fujikuroi produces the red pigment bikaverin, which displays antibiotic activity against some protozoa and phytopathogenic fungi.¹⁸⁸ Antibiotic production occurs during the stationary growth phase, after exhaustion of the nitrogen source. After 12 days of incubation, antibiotic production was stimulated by sucrose, but strongly suppressed in the presence of equivalent carbon concentrations, up to 8% glucose, by glucose, galactose, fructose, maltose, xylose and glycerol.¹⁸⁹ Sucrose is converted to glucose and fructose by invertase activity; however, neither fructose nor a mixture of glucose and fructose reproduced the sucrose stimulatory effect observed in *F. fujikuroi*, suggesting that the inducing signal is sucrose itself, and not a sucrose-derived metabolite.

STRAIN IMPROVEMENT BY AVOIDING OR REMOVING CCR

Although it has been more than 60 years since the first penicillin-overproducing mutant was obtained by X-ray treatment of *P. chrysogenum* X-1612,¹⁹⁰ conventional breeding (random mutagenesis and massive tracing of strains) remains the preferred method when molecular genetic tools have not been developed for the producer microorganism.¹⁹¹

Nutritional repression can be decreased by mutation to antimetabolite resistance. An example of a selective agent is DOG, utilized for enzymes and secondary metabolites, the synthesis of which is controlled by carbon source regulation. This is illustrated by the generation of spontaneous mutants from *S. peucetius* var. *caesius* resistant to DOG.¹³⁶ When one of these mutants is recombined with a mutant isolated as resistant to growth inhibition, as determined by high doxorubicin concentrations, the recombinant offspring show resistance to the mentioned compounds, insensitivity to CCR and improvement in antibiotic formation.¹⁹²

Although random mutagenesis and screening procedures have been widely used for genetic improvement in antibiotic production, there are certain disadvantages, such as the time necessary to obtain a favorable mutation. Thus, several rounds of improvement are normally required, before a good increase in antibiotic production is observed. In addition, there are risks of unwanted mutations resulting in crippled strains, and random approaches may not always produce the optimal solution in terms of specific mutations that are required for the desirable phenotype. However, there is a great potential in the use of metabolic engineering combined with advances in the biochemical knowledge and understanding of the biosynthetic pathways to successfully improve antibiotic production. On the basis of this, antibiotic production can be improved by metabolic engineering in several ways. A flux increase in the biosynthetic pathway can be improved by directed mutagenesis to elevate precursor availability or reduce by-product formation.¹⁹³ For instance, by cloning acetyl-CoA carboxylase into an expression vector and introducing it into a wild-type strain of *S. coelicolor*, the conversion of acetyl-CoA into malonyl-CoA was enhanced and channeled to actinorhodin production.¹⁹⁴ In a similar work, deletion of the *pfkA2* gene, corresponding to one of the three reported homologs of phosphofructokinase in *S. coelicolor*, increased actinorhodin and undecylprodigiosin production approximately four times as compared with the wild-type strain.¹²⁴ In addition, it is known that G3P and L-arginine are precursors in the biosynthesis of clavulanic acid in *S. clavuligerus*. G3P is converted into 1,3-diphosphoglycerate (1,3-BPG) by G3P dehydrogenases. These enzymes are encoded by genes *gap1* and *gap2*

in this microorganism. Therefore, to improve clavulanic acid production, the glycolytic pathway has been genetically engineered by targeted gene disruption of both *gap* genes. The procedure allowed accumulation of G3P and an increase in antibiotic production of more than twofold when L-arginine was fed to the cultures.¹⁹⁵

We have already mentioned some key enzymes for secondary metabolism that are regulated or repressed by the carbon source (Table 1). A few of these enzymes have been used as targets for strain improvement. For instance, in the case of cephalosporin production, a lead enzyme, DAOC synthase (or expandase), which is affected by CCR, has been chosen as a target to increase production of these antibiotics. An initial approach, carried out by Basch and Chiang¹⁹⁶ in an *A. chrysogenum* strain, increased the copy number of the expandase gene (*cefEF*) encoding for DAOC synthetase. In the resulting transformants, they observed a 40–70% reduction in deacetoxycephalosporin C levels and an increase in the cephalosporin C titer. A more recent study shows an increase in cephalosporin production based on the disruption of the *cefEF* gene of *A. chrysogenum*, followed by cloning and one-step replacement with the *cefE* gene from *S. clavuligerus*. Through this procedure, recombinant strains were obtained producing high titers of penicillin N, which is converted to DAOC. Deacylation of DAOC is carried out by two final enzymatic bioconversions catalyzed by D-amino acid oxidase and glutaryl acylase yielding 7-aminocephalosporanic acid, precursor of all clinically important semi-synthetic cephalosporin derivatives.¹⁹⁷

In *S. clavuligerus*, expandase was modified by site-directed mutagenesis to promote the conversion of penicillin G to 7-phenylacetylaminodeacetoxycephalosporonic acid, precursor of 7-amino-deacetoxycephalosporonic acid. The resulting mutants showed increased k_{cat}/K_m values and 13-fold increase in relative activity toward penicillin G.¹⁹⁸ Addition of different side chains to 7-aminodeacetoxycephalosporonic acid converts this compound into the oral cephalosporins: cephadrine, cephalixin and cephadroxil.

S-adenosyl-L-methionine (SAM) is most commonly used in living systems as a methyl donor involved in methylation reactions. The synthesis of SAM in the cell is solely catalyzed by an enzyme called SAM synthetase (MetK, ATP: L-methionine S-adenosyltransferase, EC 2.5.1.6). Overexpression of SAM especially enhances the production of a number of secondary metabolites, including actinorhodin and calcium-dependent antibiotics in *S. coelicolor*, pristinamycin in *Streptomyces pristinaespiralis*, granaticin in *S. violaceoruber*, oleandomycin in *S. antibioticus*, avermectin in *S. avermitilis* and doxorubicin in *S. peucetius* var. *caesius*.^{199–202} Although the overall mechanism underlying this activity is not completely understood, the obvious methylation of intermediates leading to the biosynthesis of antibiotics involving SAM provides a strong explanation for the high yield of these compounds.²⁰³

There have been notorious advances in the elucidation of the regulatory mechanisms of antibiotic formation at the biochemical and molecular levels. Thorough investigation of these mechanisms could allow us to increase the efficiency of metabolic pathways involved in antibiotic production, making the genetic improvement of industrial strains a more efficient and oriented task, compared with classical strategies.²⁰⁴

CONCLUDING REMARKS

The outstanding role of microorganisms in the production of antibiotics is notorious. By far, most of the industrially important antibiotics are produced by the genus *Streptomyces*. The complex life cycle, high physiological versatility and ability to produce a great variety of secondary metabolites by this genus, is reflected in its large

genome size. Antibiotics are usually formed during the late growth phase through enzymatic pathways carrying out various enzymatic steps. They are often brought on by exhaustion of nutrients, a situation that generates signals that cause a cascade of regulatory events resulting in chemical and morphological differentiation. Among the nutrients, the effect of the carbon source on secondary metabolite production has been the subject of this review.

Regulation by the carbon source depends on the rapid utilization of the preferred carbon source. Different mechanisms have been described in bacteria and fungi to explain the negative carbon catabolite effects on antibiotic production. These mechanisms show important differences depending on the microbe being considered.

With the available information, some proteins and protein complex systems have been found to be crucial for the carbon source effect on antibiotic biosynthesis. In addition, direct carbon source inhibition of key enzymes involved in secondary metabolite pathways has also been documented.

For a desired antibiotic to become a commercial reality, the genetic improvement in its production is a necessary step before getting the required titers for industrial production. For every new strain developed genetically, there are further opportunities to increase its titers. The relevance of the understanding of CCR mechanisms will provide not only scientific profit, but also rational strategies for selection of secondary metabolite overproducing strains. Both strain improvement and nutritional modification will preclude the scaling up into the pilot scale by biochemical engineers and later, into factory size fermentors.

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