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Biosynthesis and Metabolic Engineering of Pseudo-oligosaccharides

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Abstract

Pseudo-oligosaccharides are microbial-derived secondary metabolites whose chemical structures contain pseudosugars (glycomimetics). Due to their high resemblance to the molecules of life (carbohydrates), most pseudo-oligosaccharides show significant biological activities. Some of them have been used as drugs to treat human and plant diseases. Because of their significant economic value, efforts have been put into understanding their biosynthesis, optimizing their fermentation conditions, and engineering their metabolic pathways to obtain better production yields. A number of unusual enzymes participating in diverse biosynthetic pathways to pseudo-oligosaccharides have been reported. Various methods and conditions to improve the production yields of the target compounds and eliminate byproducts have also been developed. This review article describes recent studies on the biosynthesis, fermentation optimization, and metabolic engineering of high-value pseudo-oligosaccharides.

Introduction

Among the myriad of naturally occurring compounds, sugars play many important roles in biological systems. They are involved in both the morphology and physiological processes in all organisms, including bacteria, as well as viruses. Sugar association in biological systems is limitless, from components of bacterial cell walls (e.g., peptidoglycans and lipopolysaccharides), to signaling molecules for cellular functions (e.g., glycoproteins, glycolipids), to the genetic materials DNA and RNA. Sugars are involved in cell-cell adhesion (the basis for inflammation and cancer metastasis), cell recognition (e.g., viral entry), biofilm formation, energy generation, and many other processes.

In nature, many bacteria, particularly the actinomycetes, produce secondary metabolites whose chemical structures closely resemble sugars (glycomimetics) (1). These natural products normally contain one or more six-membered ring structures similar to pyranosugars (glucosimimetics), or five-membered ring structures similar to furanosugars (ribosimimetics). The natural glycomimetics, also known as pseudosugars or cyclitols, include the aminoglycoside antibiotics (e.g., kanamycin, neomycin, and gentamicin) (2), the pseudo-oligosaccharides (e.g., acarbose, validamycins, amylostatins, and trestatins) (1), and the aminocyclopentitol pactamycin (3). As sugars are important components of living organisms, sugar mimetics such as these usually show significant biological activities.

The pseudo-oligosaccharides are oligosaccharides in which one or more of their sugar moieties have been replaced with pseudosugar (cyclitol) units. These pseudosugars include

valienamine, an unsaturated aminocyclitol, and its epoxy, hydroxy, or dihydro forms. While the function(s) of pseudo-oligosaccharides in nature remain elusive, a number of them have been used to treat human and plant diseases. For example, the pseudo-tetrasaccharide acarbose, a potent α -glucosidase inhibitor produced by an *Actinoplanes* sp. soil bacterium, is clinically used to treat type 2 non-insulin-dependent diabetes mellitus (NIDDM) (4). This pseudosugar-containing oligosaccharide reduces the blood glucose level by slowing down the release of glucose from polysaccharides or more complex carbohydrates in the small intestines.

Another pseudo-oligosaccharide, validamycin A, is one of the most successful bacterial-derived antifungal agents used to treat sheath blight disease in rice plants. It is produced by *Streptomyces hygroscopicus* spp. and has strong inhibitory activity toward trehalase enzymes (5, 6). Fungi rely on trehalose (α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-glucopyranoside) for energy storage and use trehalase to hydrolyze trehalose to two units of glucose for growth and other cellular functions. In addition to its use as a crop protectant, validamycin A is the precursor of the antidiabetic drug voglibose (7). Thus, validamycin A has been an important commodity in both the pharmaceutical and agricultural industries. In China alone, about 40,000 tons of validamycin are produced annually (8).

Following the commercial success of acarbose and validamycin A, significant efforts have been put into screening for new pseudosugar-producing microorganisms, developing synthetic strategies to produce the compounds, optimizing fermentation conditions, and engineering the biosynthesis or metabolic pathways in the producing bacteria to obtain better production yields. A number of review articles have been published to describe their discovery, biological activity, chemical synthesis, and biosynthesis (1, 9–13). Here, we focus on more recent reports on the biosynthesis, fermentation optimization, and metabolic engineering of pseudo-oligosaccharides, particularly those with significant economic value.

Pseudo-oligosaccharide natural products

Since the discovery of acarbose in the early 1970s, research on this class of natural products has continued to grow. As a result, many acarbose analogues have been identified (1). Among them are the amylostatins, the trestatins, and the oligostatins, which were isolated from the fermentation broths of *Streptomyces diastaticus* subsp. *amylostaticus* (14, 15), *Streptomyces dimorphogenes* (16), and *Streptomyces myxogenes* (17), respectively. Similar to acarbose, these compounds contain an acarviosin core residue (valienamine and 6-deoxyglucose) or its derivatives (Figure 1), which are responsible for their α -glucosidase inhibitory activity. Other acarbose analogues include the adiposins (produced by *Streptomyces calvus*) (18), salbostatin (produced by *Streptomyces albus* ATCC 21838) (19, 20), the acarviostatins (produced by *Streptomyces coelicoflavus* ZG0656) (21), and the oxirane pseudo-oligosaccharides (e.g., NS complex, W-46 complex, CK-4416, CKD-711, and CKD-711a) (1). Some of them have also been isolated as butyl or isovaleryl esters (22, 23). While the exact function(s) of pseudo-oligosaccharides in nature remains unclear, some reports have suggested that acarbose plays a role as a “carbophore”, which acts as a sugar transport vehicle by cycling in and out between intra and extracellular spaces of bacteria (Figure 2) (24, 25). Due to its strong glycosidase inhibitory activity, it may also inhibit the

degradation of starch or maltooligosaccharides by other microorganisms and provide a competitive advantage for the slow-growing acarbose-producing bacteria.

In contrast to acarbose, validamycin A contains two pseudosugars (valienamine and validamine) that form a core structure, validoxylamine A (Figure 1). The chemical structure of validoxylamine A has a high resemblance to trehalose, making it a strong competitive inhibitor for the trehalase enzymes. While the production of validamycin A and its congeners has traditionally been observed only in certain variants of *Streptomyces hygroscopicus*, recent genome mining and metabolomic studies have revealed validoxylamine A production in other bacterial genera, e.g. *Actinosynnema mirum* DSM 43827 (28).

Biosynthesis of pseudo-oligosaccharides

The C₇-pseudosugar (valienamine and/or validamine) units in acarbose, validamycin A, and some other pseudo-oligosaccharides are derived from 2-epi-5-epi-valiolone, a cyclization product of sedoheptulose 7-phosphate (a pentose phosphate pathway intermediate) (20, 29, 30). The enzyme 2-epi-5-epi-valiolone synthase (EEVS) is highly homologous to dehydroquinase synthases (DHQS) from the shikimate pathway (31, 32). Interestingly, in contrast to DHQS, which is absent in vertebrates, EEVS is present in fish, amphibians, reptiles, and birds, and is involved in the biosynthesis of the sunscreen compound gadusol (33, 34). On the other hand, not all C₇-pseudosugars are derived from 2-epi-5-epi-valiolone. In *A. mirum* DSM 43827, the valienamine and validamine moieties are derived from 2-epi-valiolone, which is also a cyclization product of sedoheptulose 7-phosphate but its formation is catalyzed by a slightly different enzyme, 2-epi-valiolone synthase (EVS) (28).

The biosynthetic gene clusters for acarbose (acarviosatins) have so far been identified in three different strains of bacteria, *Actinoplanes* sp. SE50/110 (the *acb* cluster), *Streptomyces glaucescens* GLA.O (the *gac* cluster), and *S. coelicoflavus* ZG0656 (the *sct* cluster), with some genetic distinctions between the *acb* cluster and the *gac/sct* clusters, predominantly in the early steps of the pathways (Figure 3A-3C) (11, 26, 35). The lack of genes homologous to *acbL* and *acbN* in the *gac* cluster also suggests that the pathways to the pseudosugar moiety in *Actinoplanes* sp. SE50/110 and *S. glaucescens* GLA.O are different. However, besides the activity of GacC and GacK (26, 34), no enzymes in the Gac pathway have been biochemically characterized, leaving the exact pathway to acarbose in this organism unclear.

In *Actinoplanes* sp. SE50/110, 2-epi-5-epi-valiolone is phosphorylated by the ATP-dependent kinase AcbM, followed by epimerization at C2 by the epimerase AcbO to yield 5-epi-valiolone 7-phosphate (Figure 4) (38). It has been suggested that the product is subsequently reduced by the cyclitol dehydrogenase AcbL to give 5-epi-valiolol 7-phosphate (11), but no report describing the detailed characterization of this enzyme has been published. What lies further downstream of the pathway is largely unknown at the moment, leaving speculation as to how this high-value pseudo-oligosaccharide is synthesized in nature.

While acarbose and validamycin A share a pseudosugar (valienamine) moiety in their structures, the pathways leading to valienamine in these compounds are somewhat different. In contrast to what is seen in the acarbose pathway, in the validamycin producer *S. hygroscopicus*, 2-*epi*-5-*epi*-valiolone is first epimerized by the epimerase enzyme ValD to give 5-*epi*-valiolone (39), and then undergoes dehydration to give valienone (Figure 4) (40). So far, the enzyme that is responsible for this dehydration reaction has not been identified. Phosphorylation of valienone by the kinase ValC gives valienone 7-phosphate (41). Interestingly, in *A. mirum*, valienone 7-phosphate is formed through a different route involving 2-*epi*-valiolone (42) (Figure 4). Valienone 7-phosphate serves as a common precursor for both the unsaturated (valienamine) and the saturated (validamine) pseudosugar units (30), where the saturated pseudosugar is derived from the unsaturated one by the zinc-dependent dehydrogenase ValN (43).

One of the most intriguing questions related to the biosynthesis of pseudo-oligosaccharides asks how the pseudosugar is attached to the acceptor to form a C-N bond in those compounds. Detailed bioinformatic and biochemical studies on the validamycin pathway revealed that a glycosyltransferase-like protein (VldE/ValL) is responsible for the formation of the nonglycosidic C-N bond in validoxylamine A using GDP-valienol as a donor pseudosugar and validamine 7-phosphate as an acceptor pseudosugar (Figure 5) (44, 45). The product, validoxylamine A 7'-phosphate, is then converted to validoxylamine A by the phosphatase VldH. These reactions are homologous to those of trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (TPP) enzymes in trehalose biosynthesis. X-ray crystal structures of VldE/ValL and OtsA, a TPS from *Escherichia coli*, show high similarity between the two proteins (46, 47). Equally interesting is the presence of a nucleotidyltransferase that can convert valienol 1-phosphate to GDP-valienol. The enzyme, VldB/ValB, is a sugar 1-phosphate nucleotidyltransferase homologue similar to the glucose 1-phosphate adenyltransferase GlgC (48). Finally, the transformation of validoxylamine A to validamycin A is catalyzed by the glycosyltransferase ValG (49).

Effects of medium and growth condition on pseudo-oligosaccharide production

Most pseudo-oligosaccharides are produced as a mixture of compounds varying in the length of the oligosaccharides and the type of anomeric bonding (1→4 or 1→1) (1). For example, the acarbose-producing *Actinoplanes* spp. strains also produce a number of acarbose analogues (e.g., acarviosyl-glucose, acarviosyl-maltotriose, and acarviosyl-maltotetraose) and a significant amount of component C, an acarbose byproduct in which the terminal maltose (α -D-glucopyranosyl-(1→4)- α -D-glucopyranoside) unit has been converted to trehalose (α -D-glucopyranosyl-(1→1)- α -D-glucopyranoside) (Figure 4). The conversion of acarbose into component C is catalyzed by an intramolecular glycosyltransferase (also known as maltooligosyltrehalose synthase, TreY) that in addition to converting acarbose into component C, can also convert maltose into trehalose and maltooligosaccharides into maltooligosyl trehaloses (50).

As the production of byproducts is undesirable and cost-ineffective, efforts to increase acarbose production and eliminate side pathways have been actively pursued. The production of pseudo-oligosaccharides of various lengths by acarbose-producing bacteria appears to be dictated by the carbon source available in the production broth (27, 51, 52). A high concentration of maltose in the medium has been found to be necessary to achieve efficient production of acarbose (51, 52), and the use of other carbohydrates as carbon sources, such as glucose and galactose, can negatively affect acarbose production (27). *Actinoplanes* sp. SE50/110 grown in a minimal medium supplemented with glucose produced acarviosyl-glucose as the major product with acarbose as a minor product, whereas those supplemented with galactose mainly produced acarbose, albeit in low yield (~5% of that in maltose-containing media) (27).

A genome-wide transcriptomic analysis of *Actinoplanes* sp. SE50/110 between cells grown in maltose- or glucose-containing media showed that the acarbose biosynthetic genes were highly expressed in maltose-containing media and almost silent in the glucose-containing medium (53). This lack of expression of the acarbose genes, many of which are necessary for the formation of the acarviosyl moiety, is surprising and inconsistent with the high production of acarviosyl-glucose in the glucose-containing medium (27). Although one could argue that the determination of the production yield is an integral measurement over a period of culturing time whereas gene transcription only reflects a snapshot of the cells (53), it is not clear if the timing of the gene expression would differ significantly between cultures containing mannose and glucose.

The transcriptomic study also revealed low expression levels of *acbHFG* which are proposed to code for an acarbose importer in the carbophore model (Figure 2), in all media (53). This is consistent with the results of binding assays and crystallographic studies that show AcbH exclusively binds D-galactose and not acarbose (54), which suggests that AcbH is not likely to be involved in acarbose transport. Interestingly, similar studies with GacH, a homologue of AcbH from *S. glaucescens* GLA.O, showed that it does bind acarbose as well as other oligosaccharides, but the pseudosugar moiety partially protrudes from the GacH surface (25). This suggests that GacH is not specific for acarbose or its derivatives. On the other hand, analysis by 2D protein gel electrophoresis of the cytosolic and extracellular proteome of *Actinoplanes* sp. SE50/110 grown in a maltose minimal medium revealed that some of the acarbose gene cluster proteins (AcbD, AcbE, and AcbZ) were found both inside and outside of the cells, which is consistent with the proposed roles of these proteins in the context of the carbophore model (Figure 2) (55).

Increased osmolality in the production broths also has a positive effect on acarbose production (51). Some acarbose producers, e.g., *Actinoplanes utahensis* ZJB-08196, have been reported to be osmosis resistant, with a broad osmolality optimum between 309 mOsm/kg and 719 mOsm/kg (52). However, as component C is derived from acarbose, its production also increases with the increase of osmolality. Interestingly, valienamine was found to be a potent inhibitor of this conversion, resulting in a >90% reduction in component C formation (50, 51). The addition of validamine to the culture broths of *A. utahensis* ZJB-08196 also increased acarbose production from 3.5 g/L to 5 g/L and reduced the production of component C byproduct from 289 mg/L to 107 mg/L (52). Moreover, the

addition of validamine to the fermentation medium prior to inoculation followed by two supplementations with a mixture of glucose (6 g/L), maltose (14 g/L), and soybean flour (9 g/L) at 72 h and 96 h of cultivation further increased the production of acarbose to 6.6 g/L while maintaining low production of component C at 212 mg/L at 168 h of cultivation. Besides the carbon source and osmolality, pH and dissolved oxygen have also been shown to have an effect on acarbose production (56).

The use of D-glucose, D-xylose, and L-arabinose as carbon sources in *S. hygroscopicus* 5008 cultures was found to improve the production of validamycin A in a concentration-dependent manner, where D-xylose appears to be the most efficient carbon source (57). Remarkably, increased validamycin A and decreased validoxylamine A production were also observed when a high concentration of UDP-glucose was used to supplement the fermentation broth of *S. hygroscopicus* TL01 (58). It remains unclear, however, how UDP-glucose could penetrate or be transported into the cells, or whether it would merely be used by an extracellular glycosyltransferase to transform validoxylamine A to validamycin A.

In addition to the carbon source, which plays a direct role in pseudo-oligosaccharide biosynthesis, nitrogen concentration (59), temperature (60, 61), alkaline pH shock (62), and intracellular reactive oxygen species (ROS) (63–65) can affect validamycin production in *S. hygroscopicus* 5008. In contrast to the normal growth and sporulation temperature of actinomycetes, the optimal temperature for validamycin A production is 35–37 °C. When grown at this temperature, transcription levels of all eight necessary structural genes, which are organized in three operons (*valABC*, *valKLMN* and *valG*), increased. Similarly, the activity of the glucose 6-phosphate dehydrogenase (G6PDH) of the pentose-phosphate pathway was also elevated (60). In fact, the expression of 7.5% of coding sequences in the *S. hygroscopicus* 5008 genome, including the validamycin biosynthetic genes, increased at 37 °C. This thermo-controlled transcription appears to be regulated by a SARP-family regulatory gene (66).

A sudden change of pH can also increase validamycin production. An alkaline pH shock using an NaOH solution (pH 8) at 20 h of incubation was found to increase validamycin A production by 27% (62). This change in pH also increased the sugar consumption rate and the cell weight after 96 h of incubation. Under these conditions, the transcription level of most validamycin A biosynthetic genes also increased, except for the glycosyltransferase gene *valG*. Furthermore, the addition of ethanol (63), hydrogen peroxide (64), or liquid paraffin (65), as well as a temperature shift (61), appears to increase the medium dissolved oxygen and/or the intracellular oxidative stress level. Intracellular ROS was found to act as the molecular signal for the transcription of validamycin A biosynthetic genes.

Metabolic engineering of pseudo-oligosaccharides

Due to the lack of a suitable genetic tool to manipulate the acarbose biosynthetic pathway in *Actinoplanes* sp. SE50/110, earlier studies were limited to isotopic incorporation experiments (29, 67–71) and biochemical characterizations of the putative enzymes in the pathway (31, 38, 72, 73). However, the identification of other acarbose producers (26, 74) as well as the recent development of a genetic method to engineer *Actinoplanes* sp. SE50/110

strains have opened up new opportunities for the metabolic engineering of acarbose in these bacteria (75). For instance, the formation of the byproduct component C can now be eliminated by inactivating the maltotriose synthase gene (*treY*) in *Actinoplanes* sp. SE50/110, which slightly increases the overall yield of acarbose from 2.7 g/L to 3 g/L (75).

In contrast to *Actinoplanes* sp. SE50/110, the validamycin A producer *S. hygroscopicus* 5008 is more susceptible to genetic manipulations. This has greatly aided the elucidation of the validamycin biosynthetic pathway through genetic approaches (32, 37, 41, 48, 49). In addition, metabolic engineering in *S. hygroscopicus* 5008 has led to the production of two new pseudo-oligosaccharides, 1,1'-bis-valienamine and validienamycin (43).

More recent metabolic engineering studies have been directed toward improving the production yield of validamycin A. This includes amplification of the validamycin gene cluster in *S. hygroscopicus* 5008 (76), elimination of endogenous linear plasmids in the producing strain (77), and modulation of the regulatory systems for validamycin biosynthesis (78–80). Using the *zouA*-mediated amplification system (81, 82), mutant strains containing multiple (3–5) copies of the validamycin gene cluster have been generated. However, despite having multiple copies of the gene cluster, validamycin synthesis increased only moderately (~34%) compared to that in the wild type strain. Also, the downside of this approach is that the copy number of the gene cluster gradually decreased in the absence of selection pressure, and was completely lost after sporulation (76).

The 10,383,684-bp genome of *S. hygroscopicus* 5008 consists of a linear chromosome, a 164.57-kb linear plasmid (pSHJG1), and a 73.28-kb circular plasmid (66). Interestingly, eliminating the linear plasmid pSHJG1 in *S. hygroscopicus* 5008 increased the production of validamycin A up to 43%, or 12.5% in the high-yielding TL01 strain (from 19.16 ± 1.93 to 21.56 ± 2.25 g/L) (77). However, mutants lacking the linear plasmid grow more slowly, indicating the presence of genes that support cellular growth in pSHJG1.

The addition of exogenous 1,4-butyrolactone has also been shown to stimulate A-factor-like cascades and increase validamycin biosynthesis by 30% in *S. hygroscopicus* 5008 (78). However, a high concentration of this compound (10 mM) is necessary to enhance the biosynthetic pathway. Although γ -butyrolactones (e.g., 2-isocaprolyl-3 β -hydroxymethyl- γ -butyrolactone or A-factor) have been known to play an important role in the regulation of sporulation and secondary metabolism in *Streptomyces* spp., 1,4-butyrolactone is not likely to be a natural inducer of validamycin biosynthesis. Nevertheless, a significant increase in the transcription of the pleiotropic regulatory genes *adpA-H* (transcriptional activator genes) and the validamycin biosynthetic genes were observed upon the addition of this compound.

Bioinformatic studies have revealed that there are three pairs of *afsA/arpA* homologues in the *S. hygroscopicus* 5008 genome (79). AfsA catalyzes the biosynthesis of A-factor and ArpA (A-factor receptor protein) is an *adpA-H* transcriptional repressor (83, 84). Deletion of the *afsA* homologues (*shbA1*, *shbA2*, and *shbA3*) significantly decreased the biosynthesis of validamycin (61–90%), whereas deletion of the *arpA* homologues *shbR1* and *shbR3* (but not

shbR2) increased validamycin production by 26% and 20%, respectively (79). When deleted together, the mutant gave a 55% increase in validamycin production. Among these *arpA* homologues, only *shbR3* appears to encode the receptor for the exogenous 1,4-butyrolactone (78).

A global regulator in nitrogen metabolism (GlnR) has been found to be involved in validamycin biosynthesis through its specific association with the *valK-valA* intergenic promoter region (80). Internal deletion of *glnR* in *S. hygroscopicus* 5008 increased the transcription of certain validamycin biosynthetic genes, but it reduced validamycin production by 80%. It also impaired aerial hyphae formation and sporulation. It appears that GlnR has two binding sites in the *valK-valA*-intergenic promoter region (Figure 6). Site-specific mutagenesis of GlnR binding site I enhanced validamycin A production by 2.5-fold, whereas mutation of GlnR binding site II resulted in a >50% reduction in validamycin A production. It is proposed that GlnR serves as both an inhibitor and an activator by binding sites I and II, respectively (Figure 6) (80).

Conclusion

Due to their high resemblance to carbohydrates, pseudo-oligosaccharides show significant biological activities. In nature, they may play a role as carbophores, which shuttle sugars in and out between intra and extracellular spaces of bacteria, and/or are used for niche protection by inhibiting carbohydrate-degrading enzymes of other organisms. Based on this second function, acarbose has been developed as an antidiabetic drug, whereas validamycin A has been used widely as a crop protectant. Due to this significant economic value, recent efforts have been focused on understanding their biosyntheses, optimizing their fermentation conditions, and engineering their metabolic pathways to obtain better production yields.

The pseudo-oligosaccharides are uniquely assembled via a number of different metabolic pathways. The existence of multiple pathways to the same pseudosugar unit in various bacteria is fascinating and may indicate that it is a privileged chemical scaffold in living organisms. The biosynthetic pathways involve many unusual enzymes such as cyclitol kinases, aminotransferases, oxidoreductases, cyclitol nucleotidyltransferases, and many others. While these enzymes are similar to those commonly found in carbohydrate biosynthesis, they have obtained new capabilities that recognize non-sugar compounds as substrates. For instance, the pseudoglycosyltransferase VldE/VaL can catalyze the formation of a non-glycosidic C-N bond in validamycin biosynthesis. While the X-ray crystal structures and detailed biochemical studies of this enzyme have been reported, the mechanism underlying the C-N bond formation remains unclear.

While the carbon source is one of the most important factors that dictates the production of pseudo-oligosaccharides, other conditions such as osmolality, pH, temperature, and oxidative stress can also affect their production yields. In addition, the use of small molecules to modulate biosynthetic gene expression and/or byproduct reduction has resulted in increased overall yields of acarbose and validamycin A. Modulation of the regulatory systems in validamycin biosynthesis has also been achieved through metabolic engineering with some meaningful success. This area holds great promise and needs further

investigation, particularly with the acarbose-producing bacteria. Also, there remain many gaps in our knowledge of the biosynthesis of these high-value natural products. For example, with the exception of the first few steps, the acarbose pathway is largely unknown at the moment. This is also the case with other pseudo-oligosaccharides. Therefore, further studies are needed to uncover the modes of formation of this promising class of natural products and how they are regulated.

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Figure 1.
Chemical structures of select natural pseudo-oligosaccharides. $m = 0\sim1$; $n = 0\sim2$; $p = 0\sim3$; $q = 1\sim3$; $r = 0\sim8$; $s = 0\sim5$; $t = 1\sim5$.

Figure 2. Carbon sources for acarbose biosynthesis and proposed acarbose carbophore cycle in the acarbose producers *Actinoplanes* sp. and *Streptomyces glaucescens* GLA.O. In this model, acarbose is produced in the cell as acarbose 7-phosphate and exported by the AcbWXY (GacWXY) ABC transport system as free acarbose. Outside the cell, the free unbound acarbose is used as an acceptor molecule for the uptake of glucose or oligosaccharides catalyzed by the glycosyltransferase AcbD. The resulting pseudooligosaccharides are transported into the cell through the AcbHFG importer, presumably in complex with an ATPase subunit (MsiK). In the cell, glucose units are then released and shuttled into metabolism. The free acarbose is rephosphorylated and reexported for a new round of carbophore cycle. Dashed arrow indicates suboptimal conversion. (Modified from (25–27))

Figure 3. Biosynthetic gene clusters for acarbose and validamycin A in various bacteria. (A)-(C) Acarbose/acarviostatin gene clusters in *Actinoplanes* sp. SE50/110 (11), *S. glaucescens* GLA.O (26), and *S. coelicoflavus* (35). (D)-(F) Validamycin/validoxylamine gene clusters in *S. hygroscopicus* subsp. *limoneus* KCCM 11405 (36), *S. hygroscopicus* subsp. *jinggangensis* 5008 (37), and *A. mirum* DSM 43827 (28). Yellow arrows, acarbose structural genes; dark brown arrows, transport proteins; blue arrows, glycosidase/glycosyltransferase genes; green arrows, acarbose 7-kinase; orange arrows, validamycin structural genes, red arrows, validamycin tailoring genes; black arrows, unknown/putative/other genes; asterisk, EVS (instead of EEVS); double asterisk, a bifunctional cyclitol kinase/epimerase.

Figure 4. Biosynthetic pathways to acarbose and validamycin A. Although both acarbose and validamycin A contain a valienamine moiety, their biosynthetic pathways are different.

Figure 5.

The biosynthetic pathway to validamycin A is highly similar to that to trehalose. **(A)** Biosynthetic pathway to trehalose. NT, nucleotidyltransferase. OtsA, a trehalose 6-phosphate synthase. OtsB, a trehalose 6-phosphate phosphatase. **(B)** Biosynthetic pathway to validamycin A. VldB, a cyclitol nucleotidyltransferase. VldE, a pseudoglycosyltransferase similar to OtsA. VldH, a phosphatase similar to OtsB. ValG or VldK, a glycosyltransferase.

Figure 6. Proposed regulatory model for GlnR roles in validamycin biosynthesis in *S. hygroscopicus* 5008. It is speculated that GlnR first binds to site II and then to site I. The binding of GlnR to site II positively enhances the expression of *val* genes and validamycin A production. When the concentration of GlnR reaches a critical level, it would bind to site I. This would block the interaction between the two bound AdpA dimers, resulting in a decrease in *val* gene expression and validamycin A production. When the GlnR binding site I is mutated, the production of validamycin A increased. (Modified from (80))