β-Glucosylation as a Part of Self-Resistance Mechanism in Methymycin/Pikromycin Producing Strain *Streptomyces venezuelae*†

Lishan Zhao,‡ Noelle J. Beyer,‡ Svetlana A. Borisova,‡,§ and Hung-wen Liu*,‡,§

Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712, and Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT: In our study of the biosynthesis of D-desosamine in *Streptomyces venezuelae*, we have cloned and sequenced the entire desosamine biosynthetic cluster. The deduced product of one of the genes, *desR*, in this cluster shows high sequence homology to β-glucosidases, which catalyze the hydrolysis of the glycosidic linkages, a function not required for the biosynthesis of desosamine. Disruption of the *desR* gene led to the accumulation of glucosylated methymycin/neomethymycin products, all of which are biologically inactive. It is thus conceivable that methymycin/neomethymycin may be produced as inert diglycosides, and the DesR protein is responsible for transforming these antibiotics from their dormant to their active forms. This hypothesis is supported by the fact that the translated *desR* gene has a leader sequence characteristic of secretory proteins, allowing it to be transported through the cell membrane and hydrolyze the modified antibiotics extracellularly to activate them. Expression of *desR* and biochemical characterization of the purified protein confirmed the catalytic function of this enzyme as a β-glycosidase capable of catalyzing the hydrolysis of glucosylated methymycin/neomethymycin produced by *S. venezuelae*. These results provide strong evidence substantiating glycosylation/deglycosylation as a likely self-resistance mechanism of *S. venezuelae*. However, further experiments have suggested that such a glycosylation/deglycosylation is only a secondary self-defense mechanism in *S. venezuelae*, whereas modification of 23S rRNA, which is the target site for methymycin and its derivatives, by PikR1 and PikR2 is a primary self-resistance mechanism. Considering that postsynthetic glycosylation is an effective means to control the biological activity of macrolide antibiotics, the availability of macrolide glycosidases, which can be used for the activation of newly formed antibiotics that have been deliberately deactivated by engineered glycosyltransferases, may be a valuable part of an overall strategy for the development of novel antibiotics using the combinatorial biosynthetic approach.

Nature continues to be an integral source of pharmaceutical drug leads, as proven by the fact that a great majority of therapeutic agents, especially many antibiotics developed thus far, are products of microorganisms and/or their derivatives (*1*). These antibiotic-producing strains have evolved multiple strategies to avoid damage by their own antimicrobial products (*2*, *3*). The three most commonly encountered self-resistance mechanisms include blocking or modifying the antibiotic target site, thereby preventing effective binding of the antibiotic product to its target; inactivating the antibiotic by enzymatic modification, rendering the possible toxicity effects of the drug harmless to the producing cells; and exporting the antibiotic agent after it is synthesized, minimizing its concentration to a level that does not interfere with the functioning of the cells.

A well-known example of the first type of mechanism is the modification of the ribosome, which is the target site for macrolide antibiotics and aminoglycoside antibiotics. Mono- or dimethylation of the N^6 amino group of a single adenine residue in the 23S rRNA has been shown to confer cellular self-protection to the producers of erythromycin (*Saccharopolyspora erythraea*) (*4*, *5*), tylosin (*Streptomyces fradiae*) (*6*, *7*), and carbomycin (*Streptomyces thermotolerans*) (*8*). A similar mechanism may also be operative in the methymycin and pikromycin producer, *Streptomyces venezuelae*, since two macrolide-linkosamine-streptogramin B (MLS_B)[1] type resistant genes, *pikR1* and *pikR2*, are found upstream of the polyketide synthase (PKS) gene (*9*). An interesting variation of this mechanism was recently found in the producer of daunorubicin, *Streptomyces peucetius*. It was speculated that the resistance protein, DrrC, binds to

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* To whom correspondence should be addressed. Fax: (512) 471-2746. E-mail: h.w.liu@mail.utexas.edu.

‡ University of Minnesota.

§ University of Texas.

[1] Abbreviations: ABC, ATP-binding cassette; Apr^R, apramycin resistant; Apr^S, apramycin sensitive; ATCC, American Type Culture Collection; BAP, blood agar plates; BSA, bovine serum albumin; FAB-MS, fast atom bombardment mass spectroscopy; FPLC, fast protein liquid chromatography; HMM, hidden Markov model; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; Kan^R, kanamycin resistant; LB, Luria−Bertani; MLS_B, macrolide-linkosamine-streptogramin B; NN, neural networks; OD_{600}, optical density at 600 nm; PCR, polymerase chain reaction; PKS, polyketide synthase; RBS, ribosome binding site; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; $Thio^R$, thiostrepton resistant; TLC, thin-layer chromatography; TSB, tryptic soy broth.

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genomic DNA regions intercalated by daunorubicin and consequently releases daunorubicin or hinders its reduction, which prevents it from damaging DNA by free radical induced nicking or from interfering with transcription and/or replication (*10*, *11*).

The second mechanism, involving modification of macrolides, has been observed in many macrolide antibiotic-producing organisms. In particular, glycosylation of macrolides to neutralize their biological activities has been detected in 15 out of a total of 32 actinomycete strains producing various polyketide antibiotics (*12*). The presence of macrolide glycosyltransferase activity in nonmacrolide producers has also been reported in a number of cases. For example, *Streptomyces vendargensis* and *Streptomyces lividans*, which themselves do not produce any macrolides, are capable of inactivating erythromycin A by glycosylating the desosamine moiety at the C-2′ position (*13*−*15*). Interestingly, *Streptomyces antibioticus*, the oleandomycin-producing strain, possesses not only the glycosyltransferase activity that inactivates oleandomycin via glycosylation but also an extracellular β-glucosidase activity capable of removing the added glucose from the modified inactive drug (*16*−*18*). It is thus speculated that *S. antibioticus* utilizes the glycosylation/deglycosylation of oleandomycin as a self-resistance mechanism (*19*).

The exportation of toxic antibiotics exhibited by the third type mechanism utilizing ATP-binding cassette (ABC) transporters and consequently conferring resistance to macrolides has been reported for *S. fradiae*, a producer of tylosin (*20*, *21*), and *S. antibioticus*, which produces oleandomycin (*22*, *23*) and a number of other antibiotic-producing organisms (*24*). It is important to note that more than one of the previous mechanisms are often utilized by the producing strain to protect itself. This is exemplified by the tylosin producer, *S. fradiae*, whose self-resistance machinery incorporates all of the previous strategies (*6*, *7*, *20*, *25*).

In our study of the biosynthesis of D-desosamine in *S. venezuelae*, which is the source of four macrolide antibiotics (methymycin (**1**), neomethymycin (**2**), pikromycin (**3**), and narbomycin (**4**) (see Figure 1)), we have cloned and sequenced the entire desosamine biosynthetic cluster (*des*) (*9*). The deduced product of one of the genes, *desR* (*26*), in this cluster shows high sequence homology to β-glucosidases, which catalyze the hydrolysis of the glycosidic linkages (*27*), a function not required for the biosynthesis of desosamine. However, since *desR* is a part of the *des* cluster, it likely has a role in the overall production of methymycin/pikromycin. It is conceivable that DesR participates in a glycosylation/deglycosylation self-resistance mechanism in *S. venezuelae*. As expected, disruption of the *desR* gene indeed led to the accumulation of deactivated glucosylated methymycin/pikromycin products, providing strong molecular evidence for the proposed self-resistance mechanism via glucosylation (*26*). Reported in this paper is a full account of the gene disruption studies of *desR* and the biochemical characterization of its encoded product, DesR. The results provide molecular and biochemical evidence substantiating the antibiotic self-defense mechanisms of *S. venezuelae*.

EXPERIMENTAL PROCEDURES

General. Enzymes used in the cloning experiments were obtained from Invitrogen (formerly Gibco BRL, Carlsbad, CA) or Promega (Madison, WI). The ^{32}P labeled nucleotides and the Multiprime DNA Labeling System used for DNA probe labeling during Southern blot hybridization analysis were purchased from Amersham Biosciences (Piscataway, NJ). Antibiotics and most biochemicals used in this study were products of Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The growth media components were obtained from BD Diagnostics System (Sparks, MD). HPLC analysis of enzyme assays and purification of incubation products were performed with Alltech Econosil C_{18} columns (Deerfield, IL). The native molecular mass of DesR was determined by size exclusion chromatography using an Amersham Biosciences FPLC equipped with a Superdex 200 HR 10/30 column. Protein concentrations were determined according to Bradford (*28*) using bovine serum albumin (BSA) as the standard. The NMR spectra were acquired on a Varian Unity 300 or 500 spectrometer, and chemical shifts (δ in ppm) are given relative to those for Me_4Si (for ^{1}H and ^{13}C) with coupling constants reported in hertz (Hz). Flash chromatography was performed on Lagand Chemical silica gel (230−400 mesh) by elution with the specified solvents. Analytical thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV_{254} plates (0.25 mm) (Macherey-Nagel Inc., Easton, PA).

Plasmids, Vectors, and DNA Manipulations. Plasmids pL062 and pSub20 (derivatives of pUC119 vector) and cosmids pLZ4 and pME43 (derivatives of pNJ1 vector) (*29*) containing fragments of the desosamine biosynthetic cluster

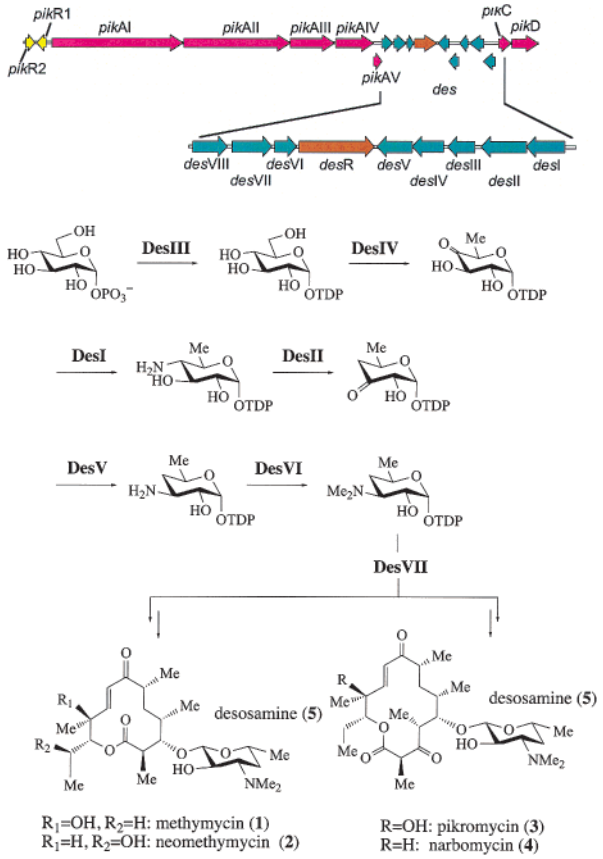

FIGURE 1: Organization of the desosamine gene cluster (*des*) and the proposed gene functions in the biosynthesis of four macrolide antibiotics, methymycin (**1**), neomethymycin (**2**), pikromycin (**3**), and narbomycin (**4**), produced by *S. venezuelae*. Arrows, each aligned along the direction of the transcription, indicate open reading frames in the cluster.

and polyketide synthase cluster (*30*) were used as templates for a polymerase chain reaction (PCR) and as the sources of appropriate DNA fragments for *desR* overexpression as well as *desR* and *pikR1*/*pikR2* disruption experiments. Plasmid pKC1139, used for the conjugal transfer of DNA to *S. venezuelae*, was a gift from Dr. Leonard Katz of Abbott Laboratories (*31*). Plasmid pDHS5000, which contains the *tsr* gene, was a gift from Prof. David Sherman of the University of Michigan. Expression vectors, pET24b(+) and pET28b(+), were products of Novagen (Madison, WI). The general methods and protocols for recombinant DNA manipulations were followed as described by Sambrook et al. (*32*), and those dealing with the *Streptomyces* strains were described by Hopwood et al. (*33*).

Bacterial Strains. Escherichia coli DH5α was used throughout the studies as a regular cloning host. *E. coli* S17−1 (*34*) was the donor strain for conjugal transfer to *S. venezuelae*. *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS were used as hosts for *desR* gene overexpression. Strains *S. venezuelae* ATCC 15439 and *S. antibioticus* ATCC 11891 were obtained from the American Type Culture Collection (Rockville, MD) as freeze-dried pellets and were revived according to the instructions from ATCC. *Streptococcus pyogenes*, used in the bioassays, was a gift from Harriet Lievan (Department of Microbiology, University of Minnesota).

Construction of Plasmid pBL1005. Plasmid pBL1005 was constructed and used for partial deletion of the *desR* gene via homologous recombination. A subclone, pSub20 (*30*), containing the *desR* gene as well as the *desVI* and a part of the *desVII* gene, was used as the starting plasmid for generating the *desR* disruption constructs. A 1.01 kb *Nco*I-*Xho*I fragment within *desR* (nucleotides 380−1390 downstream of the start codon of *desR*) was deleted and replaced with a 1.1 kb thiostrepton resistance gene (*tsr*) (*35*). From this plasmid, a 4.3 kb *Eco*RI-*Hind*III fragment containing the *tsr* gene and its downstream (380 bp of the *desR* N-terminal sequence and *desVI* gene) and upstream (1.5 kb of the C-terminal *desR* sequence) flanking sequences was cloned into pKC1139 to generate pBL1005. The pBL1005 construct was then used to transform *E. coli* S17-1.

Conjugal Transfer of Disruption Plasmids into S. venezuelae. These experiments were performed by following a literature procedure (*31*) with minor modifications. A 1 mL aliquot of *S. venezuelae* (recipient) mycelia was inoculated into 9 mL of tryptic soy broth (TSB) and grown at 29 °C for 18 h. Meanwhile, *E. coli* S17-1, which contains the disruption plasmid (donor), was streaked out on Luria−Bertani (LB) plates containing apramycin (100 μg/mL) and streptomycin (10 μg/mL) and incubated at 37 °C overnight. Subsequently, an aliquot (2 mL) of the recipient culture was reinoculated into 18 mL of TSB and allowed to grow for another 18 h. Brief sonication was carried out to homogenize the culture if cells formed clumps. Single colonies of donor cells were inoculated into 2 mL of TSB containing apramycin (100 μg/mL) and streptomycin (10 μg/mL) and allowed to grow at 37 °C overnight in an orbital shaker. Subsequently, 1 mL of the recipient culture was transferred into 9 mL of TSB for a 3 h growth. In parallel, an overnight donor culture (20 μL) was transferred into 2 mL of TSB containing appropriate antibiotics and allowed to grow at 37 °C for 3 h. Both recipient and donor cells were recovered by centrifugation and washed twice with TSB. A 2 mL aliquot of TSB was then used to resuspend recipient and donor cells separately. Three mixtures of the recipient and donor cells were prepared with ratios of 1:1, 9:1, and 1:9, respectively. Aliquots of 100 μL of the mixed cultures were spread on freshly prepared AS1 agar plates (per liter: 1 g of yeast extract, 0.2 g of L-alanine, 0.5 g of L-arginine, 5 g of soluble starch, 2.5 g of NaCl, 10 g of Na_2SO_4, 20 g of agar, pH 7.5; after autoclaving, $MgCl_2$ was added to a final concentration of 10 mM) (*36*). Three to five replicates were normally made for each mixture. In addition, two plates were inoculated with *Streptomyces* cells, and one plate was inoculated with *E. coli* cells as controls. The plates were incubated at 29 °C overnight prior to overlaying with appropriate antibiotics. For conjugation plates, 1 mL of aqueous solution of nalidixic acid (500 μg/mL), apramycin (500 μg/mL), and thiostrepton (500 μg/mL) was applied on the surface. For negative controls, one *Streptomyces* plate was overlaid with all three antibiotics, and one *E. coli* plate was overlaid with nalidixic acid (500 μg/mL). For a positive control, one *Streptomyces* plate was overlaid with nalidixic acid (500 μg/mL). The plates were incubated at 29 °C for 7−10 days.

Screening for Double-Crossover Mutant KdesR. Plasmid pBL1005 was introduced into wild-type *S. venezuelae* strain via conjugal transfer. Conjugants usually became visible on the plates after 4−5 days of incubation. Individual colonies were picked and streaked on SPA plates (per liter: 1 g of yeast extract, 1 g of beef extract, 2 g of tryptone, 10 g of glucose, several crystals of $FeSO_4$, 15 g of agar) supplemented with apramycin (100 μg/mL) and thiostrepton (50 μg/mL). Three spore-to-spore passages were carried out on SPA plates without antibiotics to facilitate homologous recombination between the disruption plasmid and the *S. venezuelae* chromosome, and the natural loss of the plasmid by *S. venezuelae*. The resulting spores were inoculated into 5 mL of SGGP medium (per liter: 4 g of peptone, 4 g of yeast extract, 4 g of casamino acids, 2 g of glycine, 0.25 g of $MgSO_4 \cdot 7\ H_2O$; after autoclaving, glucose was added to 1% w/v and KH_2PO_4 to a final concentration of 10 mM) without antibiotics for growth at 29 °C. Overnight cultures were diluted 10^{-5}−10^{-7} fold with SGGP and plated on SPA supplemented with 50 μg/mL of thiostrepton. Single colonies were then replicated on SPA plates containing 100 μg/mL apramycin. The colonies, which showed thiostrepton resistant ($Thio^R$) and apramycin sensitive (Apr^S) characteristics, were identified as double-crossover mutants KdesR. These mutants were stored as spore suspensions in 20% glycerol at −80 °C.

Purification and Characterization of Metabolites Produced by KdesR Mutants. The analysis was carried out by growing the KdesR mutant under the conditions described by Cane et al., favoring the formation of 12-membered ring macrolides (**1** and **2**) (*37*). Specifically, 5 mL of the seed medium (per liter: 20 g of glucose, 15 g of soybean flour, 5 g of $CaCO_3$, 1 g of NaCl, 0.002 g of $CoCl_2 \cdot 6H_2O$, pH adjusted to 7.2 before autoclaving) was inoculated with 10 μL of spore suspension of KdesR mutant and grown at 29 °C for 48 h. The culture was then transferred to 50 mL of the vegetative medium (per liter: 20 g of glucose, 30 g of soybean flour, 2.5 g of $CaCO_3$, 1 g of NaCl, 0.002 g of $CoCl_2 \cdot 6H_2O$, pH adjusted to 7.2 before autoclaving) and allowed to grow for another 48 h. The mycelia and cellular debris were removed

by centrifugation at 10 000*g* for 10 min. The supernatant was collected, adjusted to pH 9.5 with 10 N KOH, and extracted four times with an equal volume of chloroform. Evaporation of the organic solvent resulted in dark yellow crude products, which were analyzed by TLC developed with a solvent mixture of chloroform/methanol/25% NH_4OH (90:9.9:0.1). After staining with vanillin stain (0.75% vanilla, 1.5% H_2SO_4, in methanol), methymycin (**1**) and its derivatives showed a characteristic purple color, whereas neomethymycin (**2**) and its derivatives showed a bright blue color.

To isolate the glycosylated methymycin and neomethymycin, a large scale fermentation was carried out in which 200 mL of the seed medium was inoculated with 20 μL of the spore suspension of KdesR. This mixture, after incubating at 29 °C for 48 h, was used to inoculate 10 L of the vegetative medium (20 mL per liter). A total amount of 1.7 g of the crude products was obtained. The crude products were first purified by flash chromatography on silica gel using a gradient of 0−40% methanol in chloroform. Further purification was performed by HPLC on a C_{18} column eluted isocratically with 45% acetonitrile in 57 mM ammonium acetate buffer (pH 6.7).

^{1}H NMR (500 MHz, acetone-d_6) of **6**: 6.56 (1H, d, J = 16.0, 9-H), 6.46 (1H, d, J = 16.0, 8-H), 4.67 (1H, dd, J = 10.8, 2.0, 11-H), 4.39 (1H, d, J = 7.5, 1′-H), 4.32 (1H, d, J = 8.0, 1″-H), 3.99 (1H, dd, J = 11.5, 2.5, 6″-H), 3.72 (1H, dd, J = 11.5, 5.5, 6″-H), 3.56 (1H, m, 5′-H), 3.52 (1H, d, J = 10.0, 3-H), 3.37 (1H, t, J = 8.5, 3″-H), 3.33 (1H, m, 5″-H), 3.28 (1H, t, J = 8.5, 4″-H), 3.23 (1H, dd, J = 10.5, 7.5, 2′-H), 3.15 (1H, dd, J = 8.5, 8.0, 2″-H), 3.10 (1H, m, 2-H), 2.75 (1H, 3′-H, overlapping with H_2O peak), 2.42 (1H, m, 6-H), 2.28 (6H, s, NMe_2), 1.95 (1H, m, 12-H), 1.9 (1H, m, 5-H), 1.82 (1H, m, 4′-H), 1.50 (1H, m, 12-H), 1.44 (3H, d, J = 7.0, 2-Me), 1.4 (1H, m, 5-H), 1.34 (3H, s, 10-Me), 1.3 (1H, m, 4-H), 1.25 (1H, m, 4′-H), 1.20 (3H, d, J = 6.0, 5′-Me), 1.15 (3H, d, J = 7.0, 6-Me), 0.95 (3H, d, J = 6.0, 4-Me), 0.86 (3H, t, J = 7.5, 12-Me); ^{13}C NMR (125 MHz, acetone-d_6): 206.1 (C-7), 176.2 (C-1), 150.8 (C-9), 125.6 (C-8), 108.4 (C-1″), 104.1 (C-1′), 85.1 (C-3), 83.0 (C-2′), 78.3 (C-3″), 78.2 (C-5″), 76.8 (C-2″), 76.6 (C-11), 74.5 (C-10), 71.9 (C-4″), 69.3 (C-5′), 66.0 (C-3′), 63.8 (C-6″), 46.3 (C-6), 44.6 (C-2), 40.8 (NMe_2), 34.4 (C-5), 34.3 (C-4), 30.5 (C-4′), 21.8 (C-12), 21.5 (5′-Me), 19.4 (10-Me), 17.9 (6-Me), 17.7 (4-Me), 17.6 (2-Me), 11.3 (12-Me); high-resolution FAB-MS: calculated for $C_{31}H_{54}NO_{12}$ $(M + H)^+$ 632.3646, found 632.3686.

^{1}H NMR (500 MHz, acetone-d_6) for **7**: 6.69 (1H, dd, J = 16.0, 5.5, 9-H), 6.55 (1H, dd, J = 16.0, 1.3, 8-H), 4.71 (1H, dd, J = 9.0, 2.0, 11-H), 4.37 (1H, d, J = 7.0, 1′-H), 4.31 (1H, d, J = 8.0, 1″-H), 3.97 (1H, dd, J = 11.5, 2.5, 6″-H), 3.81 (1H, dq, J = 9.0, 6.0, 12-H), 3.72 (1H, dd, J = 11.5, 5.0, 6″-H), 3.56 (1H, m, 5′-H), 3.50 (1H, bd, J = 10.0, 3-H), 3.36 (1H, t, J = 8.5, 3″-H), 3.32 (1H, m, 5″-H), 3.30 (1H, t, J = 8.5, 4″-H), 3.23 (1H, dd, J = 10.2, 7.0, 2′-H), 3.13 (1H, dd, J = 8.5, 8.0, 2″-H), 3.09 (1H, m, 2-H), 3.08 (1H, m, 10-H), 2.77 (1H, ddd, J = 12.5, 10.2, 4.5, 3′-H), 2.41 (1H, m, 6-H), 2.28 (6H, s, NMe_2), 1.89 (1H, t, J = 13.0, 5-H), 1.83 (1H, ddd, J = 12.5, 4.5, 1.5, 4′-H), 1.41 (3H, d, J = 7.0, 2-Me), 1.3 (1H, m, 4-H), 1.25 (1H, m, 5-H), 1.2 (1H, m, 4′-H), 1.20 (3H, d, J = 6.0, 5′-Me), 1.17 (6H, d, J = 7.0, 6-Me, 10-Me), 1.12 (3H, d, J = 6.0, 12-Me), 0.96 (3H, d, J = 6.0, 4-Me); ^{13}C NMR (125 MHz, acetone-d_6): 204.1 (C-7), 175.8 (C-1), 148.2 (C-9), 126.7 (C-8), 108.3 (C-1″), 104.2 (C-1′), 85.1 (C-3), 83.0 (C-2′), 78.2 (C-3″), 78.1 (C-5″), 76.6 (C-2″), 76.4 (C-11), 71.8 (C-4″), 69.3 (C-5′), 66.1 (C-12), 66.0 (C-3′), 63.7 (C-6″), 46.2 (C-6), 44.4 (C-2), 40.8 (NMe_2), 36.4 (C-10), 34.7 (C-5), 34.0 (C-4), 29.5 (C-4′), 21.5 (5′-Me), 21.5 (12-Me), 17.9 (6-Me), 17.7 (4-Me), 17.2 (2-Me), 9.9 (10-Me); high-resolution FAB-MS: calculated for $C_{31}H_{54}NO_{12}$ $(M + H)^+$ 632.3646, found 632.3648.

*Bioassay for Antibiotic Activities of **6** and **7**.* The antibiotic activity of the diglycosides was assayed on the basis of their ability to inhibit *S. pyogenes'* growth (*38*). A frozen culture of *S. pyogenes* was streaked out on blood agar plates (BAP; per 500 mL: 20 g of Tryptic Soy Agar and 475 mL of deionized water, autoclaved, cooled to 45 °C, followed by the addition of 5% defibrinated sheep blood (Micropure Medical, Inc., White Bare, MN), which was prewarmed to 35 °C) and grown overnight at 37 °C. A single colony was then used to inoculate 5 mL of the Todd−Hewitt broth for growth in a shaker incubator at 37 °C. Overnight cultures were diluted with Todd−Hewitt medium to a McFarland 0.5 standard (0.05 mL of 1% aqueous $BaCl_2$, 9.95 mL of 1% sulfuric acid) or an OD_{600} of 0.085. A 500 μL aliquot of dilute culture was plated on a Mueller−Hinton agar plate with a sterile cotton swab. Aliquots of 20 μL of **6** or **7** samples (1.6 mM in MeOH) were separately applied to presterilized filter paper disks (4 mm in diameter) and allowed to dry. The disks were then placed on the surface of the Mueller−Hinton plate. The plate was incubated at 37 °C overnight and examined for zones of inhibition.

Cloning of Truncated desR into Vector pET28b(+). A truncated version of the *desR* gene was amplified by PCR using the following primers: forward primer 5′-GCG*CATATG*GCTCC**T**GGCGCCGCCGACACG-3′, containing an *Nde*I restriction site (in italics) and the codons for seven amino acid residues (*38*−*44*) of DesR with one base modified (in bold) to disrupt extensive hydrogen bonding resulting from the high G−C content of the gene; and reverse primer 5′-GCGC*GAATTC*TCACCAGACGTTGACCG-3′, containing an *Eco*RI restriction site (in italics). The PCR-amplified DNA fragment was cloned into the *Nde*I/*Eco*RI sites of the vector pET28b(+) to give pDesR-3. This plasmid was used to transform *E. coli* BL21(DE3)pLysS.

DesR Purification from E. coli BL21(DE3)pLysS/pDesR-3 Strain. An overnight culture of *E. coli* BL21pLysS(DE3)/pDesR-3 was used to inoculate four 1 L cultures (0.1% v/v) of LB medium containing kanamycin (50 μg/mL). The cultures were incubated at 37 °C until the OD_{600} reached 0.25. The temperature of incubation was lowered to 20 °C, and incubation continued until the OD_{600} reached 0.40. The cultures were then induced with 0.3 mM IPTG, and incubation at 20 °C continued for 16 h. The cells were harvested by centrifugation (5000*g*, 15 min). Cell pellets were resuspended in 35 mL of lysis buffer (50 mM sodium phosphate, 150 mM NaCl, 10 mM imidazole, pH 8.0), and the cells were disrupted by sonication in six 1 min bursts with a 1 min cooling period between each blast. Cell debris was removed by centrifugation (35 000*g*, 30 min), and the supernatant was mixed by slow agitation with 2 mL of packed Ni−NTA agarose resin (Qiagen, Valencia, CA) for 1 h at 4 °C. The slurry was poured into a capped column

and washed with 60 mL of lysis buffer. It was followed by washing with 30 mL of washing buffer that contained 30 mM imidazole. Elution of nonbinding proteins was monitored at A_{280}, and once the absorbance reached the background level, the target protein was eluted with elution buffer containing 250 mM imidazole and 15% glycerol. The desired fractions, as detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), were pooled and dialyzed against 3 L of 50 mM sodium phosphate buffer (pH 7.5) containing 15% glycerol. The purified enzyme was stored at −80 °C.

Electrophoresis. Electrophoresis was carried out in the discontinuous buffer system of Laemmli (*39*), and the separating gel and stacking gel were 12 and 4% polyacrylamide, respectively. Prior to electrophoresis, protein samples were heated in 62.5 mM Tris·HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.0025% bromophenol blue. Electrophoresis of the denatured samples was run in 25 mM Tris·HCl, 192 mM glycine, and 0.1% SDS (pH 8.3). Gels were stained with Coomassie blue and destained with acetic acid/ethanol/water (3:4:33 by volume).

Molecular Mass Determination. The native molecular mass of the DesR protein was determined by gel filtration performed on an Amersham FPLC Superdex 200 HR 10/30 column eluted with 50 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl at a flow rate of 0.8 mL/min. Protein standards included β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

DesR Enzyme Assay. DesR activity was analyzed by a discontinuous HPLC assay using an analytical Econosil C-18 column (4.6 × 250 mm), which was eluted isocratically with 35% acetonitrile in ammonium acetate buffer (57 mM, pH 6.8) at a flow rate of 1 mL/min. The wavelength of the detector was set at 235 nm. The retention times for the substrate and products under these conditions were as follows: 5.7 min for glucosylated methymycin (**6**), 8.7 min for methymycin (**1**), 4.6 min for glucosylated neomethymycin (**7**), and 7.8 min for neomethymycin (**2**). The substrate and product ratios were calculated from the integration of the corresponding peaks from the HPLC chromatogram. A typical reaction contained 10 μM glucosylated methymycin (**6**) or neomethymycin (**7**) and 40 ng of DesR (concentration determined using standard Bradford assay) (*28*) in 250 μL of 50 mM potassium phosphate (pH 7.0). The solution was incubated at room temperature for 5 min, followed by heating at 85 °C for 3 min to inactivate the enzyme.

Determination of Kinetic Parameters. Assays were carried out as described previously. The kinetic measurements were analyzed by fitting the data to the Michaelis−Menten equation using the KaleidaGraph software.

Construction of Plasmid pOleD. Plasmid pOleD was constructed for expression of the *oleD* gene in *S. venezuelae* KdesR mutant. The 1.3 kb DNA fragment containing *oleD* was PCR amplified using primers 5′-GCCG*GAATTC*GCAACGTCTCGTCTACC-3′ (forward) and 5′-CGCG*TCTAGA*TCACAAAGCGGATCTC-3′ (reverse) and genomic DNA of *S. antibioticus* digested with *Eco*RI as a template. The PCR product was digested with *Eco*RI and *Xba*I (restriction sites are shown in italics in the sequences of the primers) and cloned into the *Eco*RI/*Xba*I sites of vector pDHS617 (*40*), containing the apramycin resistance marker, to give plasmid pOleD. This plasmid was used to transform *E. coli* S17-1 and then introduced into the KdesR-11 mutant.

Construction of S. venezuelae KdesR Strain Complemented with oleD. Plasmid pOleD was introduced into *S. venezuelae* KdesR-11 strain via conjugal transfer as described previously. Selected colonies of *S. venezuelae* oleD/KdesR mutant were propagated on SPA plates containing apramycin and thiostrepton, and spore suspensions were prepared for storage.

*Large Scale Preparation of Compounds **6** and **7** Produced by S. venezuelae oleD/KdesR.* To isolate compounds **6** and **7** in large quantities for the DesR assay, the recombinant *S. venezuelae* oleD/KdesR strain was used as the producer. The isolation and purification for **6** and **7** were carried out as described previously.

Construction of Disruption Plasmid pPikR-K. Plasmid pPikR-K was constructed and used to replace the *pikR1* and *pikR2* genes with a neomycin resistance gene (which also confers resistance to kanamycin) in the genome of *S. venezuelae* to eliminate the PikR1 and PikR2 activities. Genes *pikR1* and *pikR2* are clustered and located upstream of the PKS cluster (*9*). The two sequence fragments flanking *pikR1* and *pikR2* genes were amplified by PCR using primers 5′-CGGACAC*GAATTC*CTCGGAC-3′ and 5′-GCGC*TCTAGA*ATACGGTGACCCTC-TCC-3′ for the *pikR1* end (1.13 kb long) and 5′-CGCG*AAGCTT*TGCAGCATGTTCCTGTCC-3′ and 5′-GGTA*CTGCAG*GAAGTCGTGG-3′ for the *pikR2* end (1.27 kb long). These primers contain the recognition sites (in italics) for the restriction enzymes *Eco*RI, *Xba*I, *Hind*III, and *Pst*I, respectively. Cosmid pME43 (*30*), which contains *pikR1* and *pikR2* genes as well as a part of polyketide synthase genes, *pikA*, was used as a template for the PCR amplification. To amplify the fragment containing the neomycin resistance gene (1.38 kb long) from plasmid pFD666 (*41*), the following primers were used: 5′-CGCG*TCTAGA*TACCTACAGCGTGAGC-3′ and 5′-CGCG*CTGCAG*CCACGAATTAGCC-3′ (containing the restriction sites for *Xba*I and *Pst*I, respectively). The PCR products were digested with the corresponding restriction enzymes and ligated into the *Eco*RI and *Hind*III sites of pUC119 to give plasmid pPikR-d. The product of the four-way ligation, pPikR-d, was then digested with *Eco*RI and *Hind*III enzymes, and the resulting 3.74 kb fragment was cloned into pKC1139 affording the disruption plasmid pPikR-K, which conferred resistance to both apramycin and kanamycin. Following the same procedure for the construction of the KdesR mutants, this plasmid was used in homologous recombination with chromosomal DNA of the KdesR-11 mutant to create the triple disruption mutant of *S. venezuelae*, KdesR/KpikR1,R2.

Screening of the Triple KdesR/KpikR1,R2 Mutant of S. venezuelae. Conjugal transfer of pPikR-K into *S. venezuelae* KdesR-11 and screening for double-crossover mutants were carried out as described previously with the following exceptions. First, kanamycin antibiotic (50 μg/mL) was used, in addition to apramycin and streptomycin, during the growth of donor *E. coli* and for screening and propagation of the mutants. Second, the *S. venezuelae* KdesR-11 recipient strain was used in place of the wild-type strain. Finally, thiostrepton (20 μg/mL) was used for the propagation of recipient and the resulting mutant cultures. Conjugation plates were flooded with 1 mL of aqueous solution of nalidixic acid (500

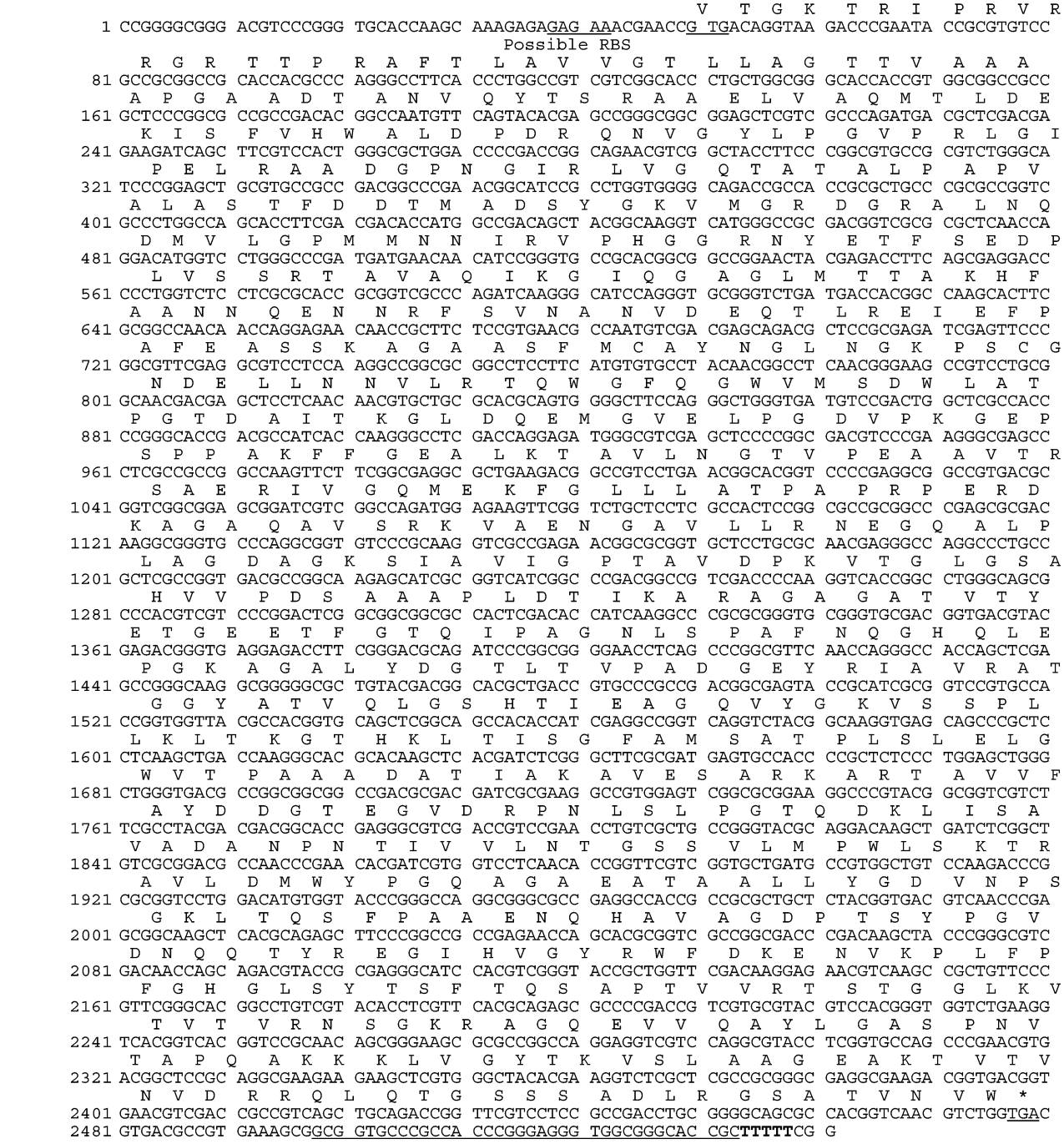

FIGURE 2: Sequences of *desR* gene of *S. venezuelae* and its deduced product DesR.

μg/mL), apramycin (500 μg/mL), kanamycin (500 μg/mL), and thiostrepton (500 μg/mL) per plate. Throughout screening, individual colonies exhibiting Thio^R, kanamycin resistant (Kan^R), and Apr^S characteristics were identified as double-crossover mutants KdesR/KpikR1,R2.

RESULTS AND DISCUSSION

Nucleotide Sequence of the desR Gene. Positioned downstream of *desVI*, *desR* is the last open reading frame of a possible single transcript that also contains the *pikA*, *desVIII*, *desVII*, and *desVI* genes (Figure 1) (*9*). The 2427 nucleotides long *desR* encodes an 809 amino acid protein (GenBank accession number AF 079762), with GTG being the start codon (Figure 2). A sequence of GAGAA, 8 bp upstream of GTG, may serve as a potential ribosome binding site (RBS) for the translation of *desR*. Interestingly, there exists a 36-bp GC-rich dyad symmetry with a 4-bp loop (underlined in Figure 2) located 18 bp downstream of the *desR* termination codon TGA. This sequence is likely to serve as a transcription termination signal because consensus transcription termination sites generally have uridine-rich tracks following the hairpin structure in mRNA (*42*). A track of five T residues is found immediately downstream of the dyad symmetries.

DesR Is Likely a Secretory Protein. Signal peptides control the entry of virtually all proteins to the secretory pathway, both in eukaryotes and prokaryotes (*43*−*46*). These peptides comprise the N-terminal part of the amino acid chain and are cleaved off, while the protein is translocated through the membrane. The common structural features of signal pep-

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                257                                                  304

DesR    nnVLRtqWGF qGwVMSDWla tpGTd.aitk GLDqEmgvEl Pgdvpkgep

Cbg-1   tkVLReeWGF dGvVMSDWfG shStaetinA GLDlEmPg..

BglS    ndVLkgaWGy rGwVMSDWGG TpSwecaL.A GLDqEcgaqi davlwqs

Cgb     seVLkrdWGF rGyVMSDWGa ThStvaaans GLDqqsgqef d....ks

BglZ    tdVLRneWGF eGiVvSDWGa vnervkgLeA GLDlEmPssf gigdqk.

A3              GF zGfVMSDWaa hhagvsgalA GLDmgsmpg

Cons    --VLR--WGF -G-VMSDW-- --S------A GLD-E----- -------
```

FIGURE 3: Comparison of conserved active site residues in DesR and its homologues with those identified in A3 of *Aspergillus wentii*. The sequences were aligned using Pileup of GCG program. GenBank accession numbers: *A. tumefaciens*, P27034; *M. tuberculosis*, Z97050; *C. gilvus*, D14068; *C. stercorarium*, Z94045; and *A. wentii*, O29090. Capital letters indicate consensus residues. Numbering refers to that of the DesR amino acid sequence.

tides from various proteins include a positively charged n-region, a hydrophobic h-region, and a neutral but polar c-region (*47*). Small and neutral amino acids, usually alanine or glycine, are typically found in the −1 and −3 positions relative to the cleavage site. A SignalP program (http://www.cbs.dtu.dk/) allows the analysis and prediction of the probability of signal peptides. When the sequence of the first 50 amino acids of DesR was subjected to SignalP analysis, a signal peptide sequence was predicted with a very high probability number by both the neural networks (NN) (*48*) and hidden Markov models (HMM) (*49*). The SignalP-NN model predicts a cleavage site between positions 37 and 38 (AAA-AP), while SignalP-HMM predicts a cleavage site between 42 and 43 (GAA-DT). In both cases, there are either alanine or glycine at the −1 and −3 positions, which is typical for a signal peptide cleavage site. Despite the discrepancy in pinpointing the cleavage site, the fact that DesR contains a possible signal peptide sequence strongly suggests that it is an extracellular protein secreted by *S. venezuelae*.

Similarity of DesR to Other β-Glucosidases. Sequence alignment with known genes in the databank led to several characterized fungal and bacterial β-glucosidases having high sequence homology to DesR. These include Cbg1 from *Agrobacterium tumefaciens* (45% similarity, 39% identity) (*50*), BglS from *Mycobacterium tuberculosis* (46% similarity, 39% identity) (*51*), which also has a possible signal peptide sequence, Cgb from C*ellvibrio gilvus* (43% similarity, 39% identity) (*52*), and BglZ from *Clostridium stercorarium* (45% similarity, 38% identity) (*53*). These enzymes, as well as DesR, belong to the family 3 of glycoside hydrolases along with many other β-glucosidases, xylosidases, and hexosaminidases (Coutinho, P. M., and Henrissat, B. (1999). Carbohydrate-Active Enzymes server at http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). This class of enzymes catalyzes the hydrolysis of the glycosidic linkages in aryl and alkyl β-glucosides and cellobiose (*27, 54*). A double displacement mechanism involving the general acid/base catalyzed formation and hydrolysis of a glycosyl−enzyme intermediate via an oxocarbenium ion-like transition state has been proposed for this group of enzymes (*27, 54*). The alignment of DesR and its homologues with the active site sequence of β-glucosidase A3 from *Aspergillus wentii* is shown in Figure 3. The Asp shown in bold face in the A3

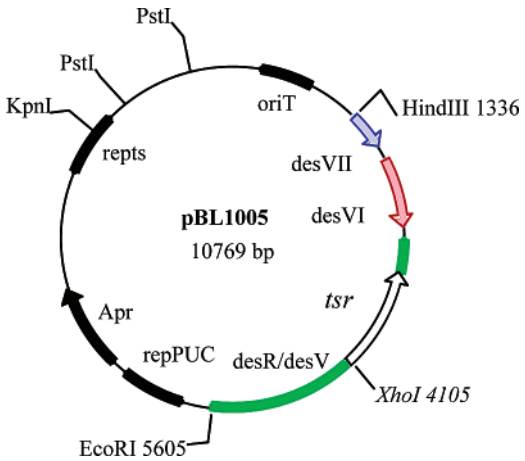

FIGURE 4: Map of plasmid pBL1005 used for partial deletion/replacement of *desR* in *S. venezuelae*.

sequencehas been identified as the active site nucleophile in the A3 catalyzed reaction (*55*). This Asp residue is preserved in DesR and its homologues and is expected to play an analogous role. A second conserved motif, Gly-Leu-Asp, has also been shown to be important for the activity of β-glucosidases (*56*).

It should be noted that the sequences of two other macrolide β-glucosidase genes, *eryBI* from the erythromycin producer *Sac. erythraea* and *oleR* from the oleandomycin biosynthetic gene cluster in *S. antibioticus*, became available during the course of this study. Both show great similarity to DesR (61% similarity and 55% identity for EryBI and 66% similarity and 60% identity for OleR). The *eryBI* gene was originally proposed to be involved in the biosynthesis of mycarose in the erythromycin pathway (*57*). However, this hypothesis was refuted because the insertional inactivation of *eryBI* produced a wild-type phenotype (*58*). In view of its high sequence homology to DesR, the encoded EryBI protein is most likely a β-glucosidase serving a similar role as DesR. The *oleR* had been expressed in *S. lividans*, and the function of the encoded protein was confirmed to be a β-glucosidase capable of converting glucosylated oleandomycin to oleandomycin (*19*). The OleR protein possibly corresponds to the glycosidase activity previously detected in the culture of *S. antibioticus* (*16, 18*).

Inactivation of desR via Partial Gene Deletion. If DesR does play the proposed role in the self-resistance mechanism

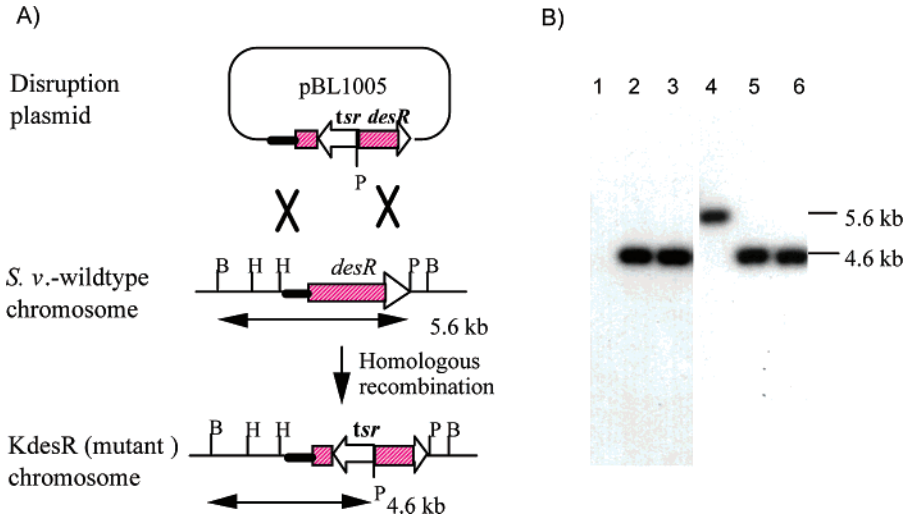

FIGURE 5: Deletional disruption of *S. venezuelae desR*. (A) Generation of *desR*-disrupted KdesR mutants via homologous recombination of disruption plasmid pBL1005 and *S. venezuelae* wild-type chromosome. The endonuclease restriction sites are abbreviated as follows: B, *Bam*HI; H, *Hinc*II; and P, *Pst*I. (B) Southern blot analysis of *Bam*HI/*Pst*I digested genomic DNA of *S. venezuelae* wild-type and KdesR mutants. Samples of lanes 1 (wild-type), 2 (KdesR-11), and 3 (KdesR-21) were probed by the 1.1 kb *tsr* gene, while those in lanes 4 (wild-type), 5 (KdesR-11), and 6 (KdesR-21) were probed by a 1.1 kb *Hinc*II fragment.

of *S. venezuelae*, disruption of the *desR* gene should prevent the hydrolysis of glucosylated macrolides, resulting in the accumulation of the diglycoside intermediates. A plasmid pBL1005 was constructed to generate *S. venezuelae* mutants with a partially deleted *desR*. As depicted in Figure 4, an *Nco*I-*Xho*I fragment of *desR* that encodes amino acid residues 127−463 of DesR, including its active site Asp residue, was deleted and replaced with the thiostrepton resistance gene *tsr*. Plasmid pBL1005 was introduced into the wild-type *S. venezuelae* via conjugal transfer, and homologous recombination between pBL1005 and *S. venezuelae* chromosome, as illustrated in Figure 5A, led to the partial deletion of *desR*. Two of the double-crossover mutants, KdesR-11 and KdesR-21, were chosen for further analysis by Southern blot hybridization to verify the change on the mutant chromosome. As shown in Figure 5, when *Bam*HI-*Pst*I digested chromosomal DNA prepared from the two mutants KdesR-11 and KdesR-21 was hybridized with ^{32}P-labeled *tsr*, a 4.6 kb *Bam*HI-*Pst*I band was detected, whereas no such a band was discernible in the sample derived from the wild-type strain. As expected, using a 1.1 kb *Hinc*II fragment as the probe, the same 4.6 kb band was detected in the mutant sample, while a 5.6 kb band became visible in the wild-type DNA. These results confirmed that the chromosomal DNA in the mutant strains had been successfully altered and should no longer have the DesR activity. These two mutants were then grown in methymycin/neomethymycin production medium, and their metabolites were extracted and analyzed by TLC. The major products produced by KdesR-11 and KdesR-21 were still methymycin (**1**) and neomethymycin (**2**). However, two new spots with greater polarities were also detected by TLC. The total yield of these new compounds was estimated to be approximately 5% of the combined yield of methymycin and neomethymycin.

Characterization of the New Products Produced by S. venezuelae KdesR Mutants. To facilitate the characterization of the new metabolites produced by the KdesR mutants, a 10 L culture was grown from which compounds **6** (2.0 mg) and **7** (4.0 mg) were isolated and identified as 2′-glucosylated methymycin and neomethymycin, respectively. Purification of these compounds proved to be challenging due to their similar chromatographic properties, resulting in low recovery yields. The glycosidic linkage at C-2′ in **6** and **7** was assigned to have a β-configuration based on the coupling constant (d, J = 8.0 Hz) of the anomeric hydrogen (1″-H) of the appended glucose and the magnitude of the downfield shift (11.8 ppm) of C-2′ of desosamine (*59*). An activity-based disk diffusion assay was used to examine the antimicrobial activity of **6** and **7**. When methymycin or neomethymycin was applied to the disk and placed on a lawn of *S. pyogenes*, a clear inhibition zone was observed. However, when **6** and **7** were used, no inhibition zone was discernible. Evidently, β-glucosylation at the C-2′ of desosamine in methymycin and neomethymycin renders these antibiotics inactive.

Overexpression of desR and Purification of the Recombinant Protein. To fully characterize the function of the DesR protein, the *desR* gene was cloned into the expression vectors, pET24b(+) and pET28b(+), to produce His-tagged fusion proteins in *E. coli*. Unfortunately, no soluble DesR protein was found in either the cytoplasmic or the periplasmic fractions of the cells (data not shown). It was speculated that the leading sequence of DesR might be the cause of precipitation of the expressed proteins. Thus, a truncated *desR* gene lacking the sequence encoding the first 37 amino acid residues of DesR was constructed and cloned into pET28b(+) vector, and the resulting plasmid, pDesR-3, was used to transform *E. coli* BL21pLysS(DE3) competent cells. The induction of *E. coli* BL21pLysS(DE3)/pDesR-3 with IPTG resulted in expression of the truncated DesR. While the majority of the expressed protein was again inclusion bodies, more soluble protein was obtained by lowering the incubation temperature to 20 °C prior to induction with IPTG and continued incubation at 20 °C for 16 h after induction. The desired protein was purified to near homogeneity by a Ni-NTA column (Figure 6), and its identity was confirmed by N-terminal amino acid sequencing. Judging from a M_r of 84.1 kDa, estimated by gel filtration, and a calculated mass of 83 130 Da based on the translated sequence, this truncated DesR was determined to exist as a monomer. The electronic absorption spectrum of the purified enzyme shows no

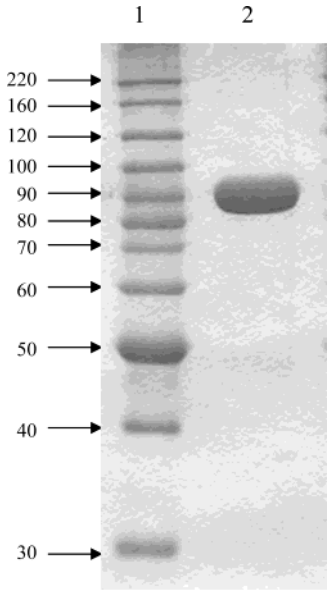

FIGURE 6: SDS−PAGE gel showing DesR purified from *E. coli* BL21pLysS(DE3)/pDesR-3. Lane 1, molecular weight markers and lane 2, purified DesR.

absorption above 300 nm. The enzyme appears to be stable to endure several freeze/thaw cycles before precipitating out of solution and requires no additional stabilizing factors, such as BSA or glycerol, during dilution for kinetic studies.

Catalytic Properties of DesR. The capability of the recombinant DesR to hydrolyze the C-2′ β-linked glucose from **6** and **7** was studied by HPLC analysis of the enzyme incubation mixture at appropriate time intervals. It was found that the rate of substrate depletion correlated well with the rate of product accumulation. Maximum activity of DesR was observed at pH 7.0 in 50 mM potassium phosphate buffer. The enzyme exhibited a much sharper reduction in activity under alkaline conditions than that observed under acidic conditions. The kinetic parameters were determined by fitting the data points to the Michaelis−Menten equation (Figure 7). For glucosylated methymycin, the K_m value was determined to be 4.1 ± 0.1 μM, and the V_{max} value was determined to be 0.585 ± 0.004 μmol min^{-1} mg^{-1}. For glucosylated neomethymycin, the K_m and V_{max} values were determined to be 43 ± 2 μM and 16 ± 3 μmol min^{-1} mg^{-1}, respectively. These results firmly established that DesR is a macrolide β-glucosidase. The fact that prevention of the in vivo production of DesR leads to the accumulation of biologically inactive glucosylated macrolides, which can be reactivated by the action of DesR, is consistent with a glycosylation/deglycosylation self-resistance model similar to that found in the oleandomycin case (*19*).

S. venezuelae Does Not Have a Second Copy of desR Gene. If the proposed glycosylation/deglycosylation model were the primary self-resistance mechanism for *S. venezuelae*, one would expect **6** and **7** be the major products produced by the KdesR mutants. However, contrary to this prediction, both compounds existed only as the minor components. It is generally believed that certain housekeeping metabolic genes may be duplicated and evolved into drug resistance genes by evolutionary selection (*60*). If this happens, the parent gene is expected to have at least partial activity found in the evolved resistance gene. Hence, it is possible that a second copy of *desR* may exist in *S.*

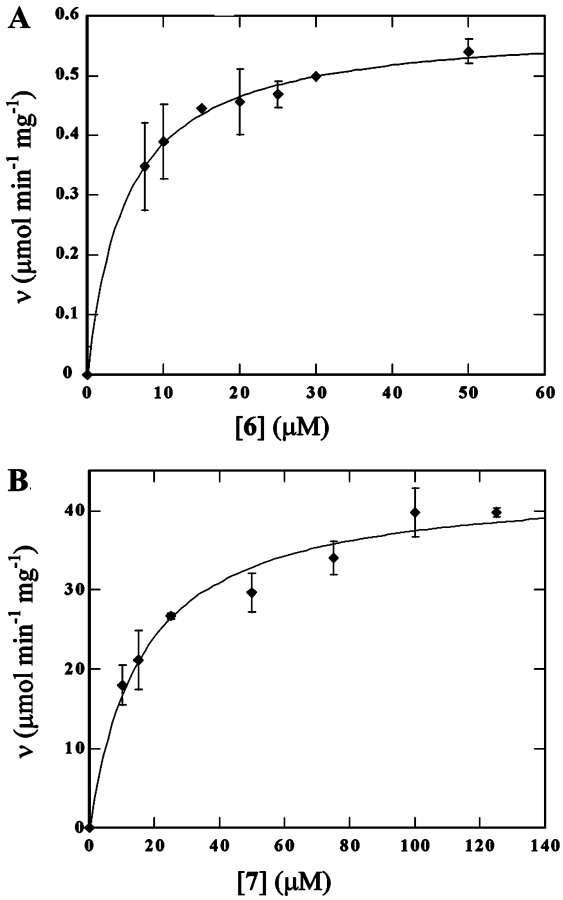

FIGURE 7: Kinetic data for the activity assay of DesR. The assays were performed as described in the Experimental Procedures. The lines through the points represent the Michaelis−Menten equation calculated by the fitting program for (A) glucosylated methymycin (**6**) and (B) glucosylated neomethymycin (**7**).

venezuelae capable of acting as a β-glucosidase whose activity leads to the low yield of glucosylated methymycin and neomethymycin. To test this hypothesis, the genomic DNA of *S. venezuelae* was digested with *Bam*HI and *Bam*HI/*Pst*I and probed with ^{32}P-labeled *desR* by Southern blot hybridization. Under mild stringency, only a single 5.6 kb *Bam*HI/*Pst*I band and a single 6.0 kb *Bam*HI band were observed, both of which correspond to the DNA fragments that contain the *desR* gene. Because no other bands could be detected, the existence of a second copy of *desR* or other *desR* homologues that may hydrolyze the diglycosides produced in the *desR* disrupted mutants appeared to be unlikely.

Increasing Production of Glucosylated Macrolides by Expression of oleD in KdesR-11 Mutant. Since the poor yield of glucosylated macrolides produced by the *desR* disrupted mutants may simply be due to the low level of expression of the enzyme catalyzing the glucosylation reaction in *S. venezuelae*, the yield of the glucosylated macrolides may be increased if the responsible glycosyltransferase can be induced or an exogenous macrolide glycosyltransferase is introduced into the *desR* disrupted mutants. Given that the identity of the corresponding glycosyltansferase remained elusive, an alternative candidate for this experiment is OleD that, along with OleI, is one of the two macrolide glycosyltransferases involved in a self-resistance mechanism in the oleandomycin producing *S. antibioticus* (*19*). The *oleI* gene

is located within the oleandomycin biosynthetic cluster, and the encoded OleI is specific for oleandomycin. In contrast, *oleD* is not part of the oleandomycin cluster, and its translated product, OleD, is capable of glycosylating a number of macrolide antibiotics including oleandomycin, erythromycin, carbomycin, and tylosin (*19*). To express the *oleD* gene in *S. venezuelae*, it was amplified by PCR from the genomic DNA of *S. antibioticus* and cloned into pDHS617 at *Eco*RI/*Xba*I sites to give plasmid pOleD. The vector pDHS617 is derived from pOJ446 (*31*), and a promoter sequence from the methymycin/pikromycin cluster (*9*) was incorporated to enable the expression of foreign genes in *S. venezuelae* (*40*). The plasmid pOleD was introduced into KdesR-11 by conjugal transfer using *E. coli* S17-1, and pOleD containing strains were selected on the basis of their Thio^R and apramycin resistant (Apr^R) phenotypes. Examination of macrolide production using TLC showed that the amount of glucosylated methymycin and neomethymycin made by the oleD/KdesR strain was approximately eight times more than that made by KdesR mutants. Up to 40% of methymycin and neomethymycin produced are now C-2′ glucosylated.

Deletion of pikR1 and pikR2. Since glucosylated methymycin and neomethymycin remained the minor products in the fermentation broth of the oleD/KdesR mutant, glycosylation of the macrolide products may not be the primary self-resistance mechanism in *S. venezuelae*. Instead, the two macrolide-linkosamide-streptogramin B type resistant genes, *pikR1* and *pikR2*, found upstream of the PKS (Figure 1) may provide the major cellular self-protection for *S. venezuelae*. Disruption of both genes might force the bacterium to rely on the secondary self-protection mechanism, if it could survive at all. Thus, it is conceivable that a triple mutant in which *pikR1*, *pikR2*, and *desR* genes are disrupted or deleted may lead to the accumulation of glycosylated macrolides as the major products. On the basis of this premise, a disruption plasmid, pPikR-K, was constructed in which most of the *pikR1* and *pikR2* sequences were deleted and replaced by the neomycin resistance gene. The plasmid pPikR-K was introduced into the KdesR-11 mutant, followed by the screening of double-crossover mutants. However, despite extensive efforts to obtain such mutants with a phenotype of Apr^S, Thio^R, and Kan^R, all attempts failed. This observation suggests that methylation of an adenine residue in the 23S RNA mediated by PikR1/PikR2 likely confers primary cellular self-protection to *S. venezuelae*, and *S. venezuelae* cannot survive without *pikR1* and *pikR2*.

CONCLUSIONS

The high homology of DesR to known bacterial and fungal β-glucosidases and its competence to hydrolyze β-linked glucose at C-2′ of methymycin/neomethymycin strongly support the assignment of DesR as a β-glucosidase. The occurrence of *desR* within the desosamine gene cluster and the accumulation of inactive **6** and **7** upon disruption of *desR* provide strong molecular evidence suggesting that a self-resistance mechanism via glucosylation may be operative in *S. venezuelae*. It is conceivable that methymycin and neomethymycin are produced in part as the inert diglycosides, and the *desR* encoded macrolide β-glucosidase then transforms methymycin/neomethymycin from their dormant state to their active form. The likelihood that DesR has a signal peptide sequence characteristic of secretary proteins further

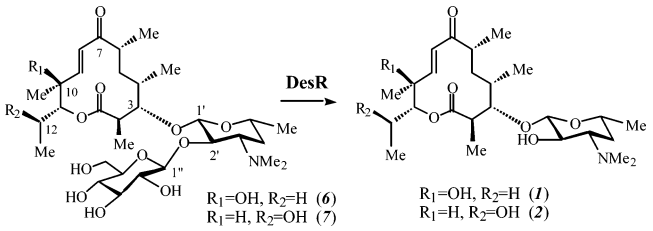

FIGURE 8: Reaction catalyzed by DesR.

implies that the modified antibiotics (**6** and **7**) may be transported out of the cell and hydrolyzed by DesR extracellularly (Figure 8). However, glycosylation/deglycosylation may not be the primary self-defense mechanism operated in *S. venezuelae* since the glucosylated methymycin/neomethymycin are produced in significantly less quantity than their unmodified counterparts in the KdesR mutants. Instead, *pikR1* and *pikR2*, the two rRNA methyltransferase genes found upstream of the PKS genes, may confer the primary self-resistance. This is consistent with the well-known fact that antibiotic-producing organisms generally have more than one defensive option (*2*).

The three known macrolide β-glucosidases (DesR, OleR, and EryBI) have high sequence similarity and presumably catalyze the same type of reaction. However, the importance of their intended roles to their respective organisms varies. For the oleandomycin producer, *S. antibioticus*, which has not evolved to possess oleandomycin-resistant ribosomes (*61*), glycosylation of oleandomycin, combined with an efficient secretion system, appears to be the primary self-protection mechanism, and OleR, acting as a β-glucosidase, assumes a critical function to reactivate the antibiotic (*19*). In *Sac. erythraea* and *S. venezuelae*, methylation the 23S rRNA of ribosomes via rRNA methyltransferases is likely the primary self-resistance mechanism. Hence, glycosylation and deglycosylation involving DesR in *S. venezuelae* may only play a secondary self-protection role. Whether a similar mode of self-protection involving EryBI is operative in *Sac. erythraea* remains to be investigated. It is possible that both DesR and EryBI originally served a similarly significant role as OleR in their respective organisms. However, their function became less important or was even lost through evolution when the producer organisms acquired a more effective survival mechanism (i.e., target site modification by a constitutive ribosomal methyltransferase).

Nevertheless, glycosylation holds promise as a tool to regulate and/or minimize the potential toxicity associated with new macrolide antibiotics produced by genetically engineered microorganisms. Thus, the identification of *desR* as a β-glucosidase gene is important since this gene may be used as a probe to search for possible homologues in other antibiotic biosynthetic pathways. The availability of DesR and a few other macrolide β-glycosidases, which can be used for the activation of newly formed antibiotics that have been deliberately deactivated by engineered glycosyltransferases, may be a valuable part of an overall strategy for the development of novel antibiotics using the combinatorial biosynthetic approach.

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