J|A|C|S
COMMUNICATIONS

Published on Web 09/29/2006

Glycopeptide Biosynthesis: Dbv21/Orf2* from *dbv*/*tcp* Gene Clusters Are *N*-Ac-Glm Teicoplanin Pseudoaglycone Deacetylases and Orf15 from *cep* Gene Cluster Is a Glc-1-P Thymidyltransferase

Jin-Yuan Ho,[†] Yu-Ting Huang,[‡] Chang-Jer Wu,[‡,§] Yi-Shan Li,[‡] Ming-Daw Tsai,[†,||] and Tsung-Lin Li*[,‡,§]

Genomics Research Center, Academia Sinica, Taipei, Department of Food Science and Center for Marine Bioscience and Biotechnology, National Taiwan Ocean University, Keelung 202, Taiwan, Republic of China, and Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

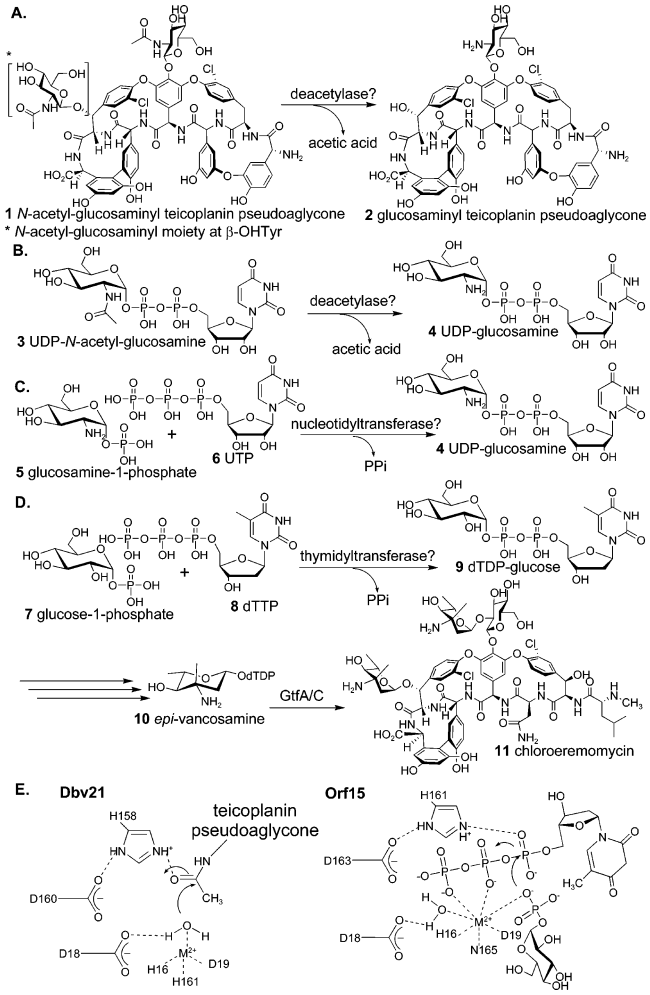
Received July 6, 2006; E-mail: tlli@mail.ntou.edu.tw

There has recently been considerable progress in elucidating the biosynthetic steps of glycopeptide antibiotics.[1] However, many important questions remain to be answered. Here we report that three homologous (>65% identical) proteins Dbv21, Orf2* and Orf15, previously assigned as hypothetical proteins in the biosynthesis of A40926, teicoplanin, and chloroeremomycin, respectively, are novel deacetylases (Dbv21 and Orf2*) and thymidyltransferase (Orf15).

We considered possible deacetylase activities for these hypothetical proteins because of a recent report that the gene products of *orf*10* in *tcp* and *dbv*9 in *dbv* were characterized as glycosyltransferases capable of transferring either Glm (**4**) or *N*-Ac-Glm moiety (**3**) (from the corresponding UDP-sugar) onto the aglycone of teicoplanin and A40926, respectively.[2] If *N*-Ac-Glm moiety is incorporated, then the resulting pseudoaglycone (**1**) needs to be deacetylated (Scheme 1A). Alternatively, these gene products may catalyze deacetylation of UDP-*N*-Ac-Glm to provide substrate UDP-Glm for the glycosyltransferases (Scheme 1B).[3] As a third possibility, these proteins may function as uridyltransferases to catalyze the formation of UDP-Glm directly from Glm-1-P (**5**) and UTP (**6**) (Scheme 1C). The latter is analogous to the reaction catalyzed by the recently discovered novel nucleotidyltransferase BtrD from the butirocin biosynthetic gene cluster.[4] Our gene analyses suggested a number of distant *btr*D homologues, *orf*2*, *dbv*21, *orf*15, and *orf*2 from *tcp*, *dbv*, *cep*, and *bal* gene clusters, respectively.[2,5]

Purified *N*-terminal His_6-tagged recombinant Dbv21 and Orf2* were first assayed for the uridyltransferase activity corresponding to that of BtrD (Scheme 1C). No activity was observed when Glc-, Glm-, *N*-Ac-Glm-, Man-, or Gal-1-P was incubated with Dbv21/Orf2* in the presence of NTP/dNTP and divalent metals. Dbv21 and Orf2* were then tested for deacetylation activity with UDP-*N*-Ac-Glm (Scheme 1B). Likewise, no activity was detected in the presence of divalent metal ions. To test whether deacetylation occurs at the stage of pseudoaglycone (Scheme 1A), the substrate *N*-Ac-Glm-teicoplanin pseudoaglycone was enzymatically prepared by Dbv9/Orf10* in the presence of the teicoplanin aglycone and UDP-*N*-Ac-Glm.[2] A40926 aglycone was replaced with teicoplanin aglycone because of their structural similarity and the limited availability of A40926. In addition, the product standard, glucosaminyl-teicoplanin pseudoaglycone, was also prepared by Dbv9 but with UDP-Glm instead. That the yield of the Glm-teicoplanin pseudoaglycone was found much lower than that of the *N*-Ac-Glm counterpart may suggest that UDP-*N*-Ac-Glm is a better substrate at this stage. When *N*-Ac-Glm-teicoplanin pseudoaglycone was

Scheme 1



incubated with/without Dbv21 at 25 °C overnight, the peak of *N*-Ac-Glm-teicoplanin pseudoaglycone (12.1 min; Figure 1 (**1**)) on the LC trace of the reaction added with Dbv21 significantly decreased and a new peak at 10.1 min emerged, which also coeluted with the synthetic standard (Figure 1 (**2**)). Further LC/MS analysis verified that the new product is Glm-teicoplanin pseudoaglycone on the basis of molecular weight and the characteristic dichlorine isotope pattern (Figure 1 (**2**)). The synthetic standard showed the same pattern. Orf2* was also tested. The results were in line with that of Dbv21. Thus Dbv21 and Orf2* were verified as a new class of the deacetylase superfamily.

When discerning the structure of chloroeremomycin (**11**, Scheme 1D), we found no comparable deacetylase role for Orf15; we thus

[†] Genomics Research Center.
[‡] Department of Food Science, National Taiwan Ocean University.
[§] Center for Marine Bioscience and Biotechnology, National Taiwan Ocean University.
[||] The Ohio State University.

10.1021/ja0644834 CCC: $33.50

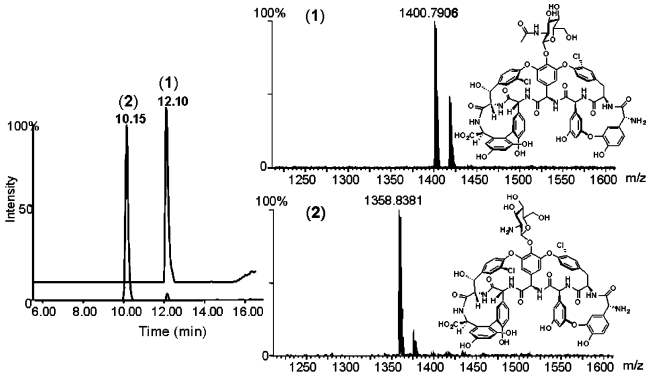


Figure 1. LC traces and mass spectra of enzymatic assays of Dbv21/Orf2*. *N*-Ac-Glm-teicoplanin pseudoaglycone (1) substrate retained at 12.1 min with the molecular weight of 1400.79 (M + 1), while the product Glm-teicoplanin pseudoaglycone (2) appeared at 10.15 min with the molecular weight of 1358.83 (M + 1).

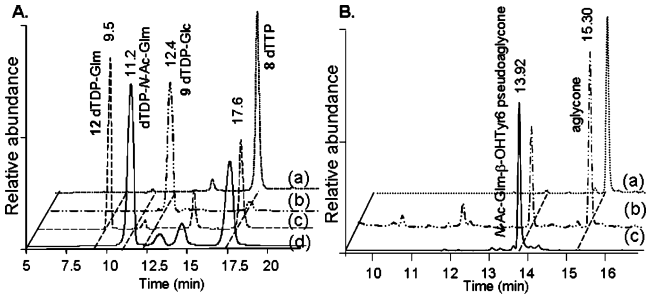


Figure 2. LC traces of enzymatic assays of Orf15 and Dbv21/Orf2*. (panel A) Products of Orf15 were identified to be dTDP-Glc at 12.4 min (b), dTDP-Glm at 9.5 min (c) and dTDP-*N*-Ac-Glm at 11.2 min (d), in which dTTP control at 17.6 min (a). (panel B) Trace (a) is aglycone control, trace b is the assay added wtih Orf1, in which peak at 13.9 min is the reaction product *N*-Ac-Glm-β-OHTyr$_6$ teicoplanin pseudoaglycone, and trace c is the assay of Dbv21 added with the product of assay b.

considered it for the BtrD-like activity (Scheme 1C). The *epi*-vancosamine moiety (**10**) of chloroeremomycin has been shown to be biosynthesized via a five-step reaction sequence starting with dTDP-Glc (**9**), which led us to hypothesize that Orf15 may act as a Glc-1-P thymidyltransferase to provide dTDP-Glc for the unusual amino-sugar biosynthesis (Scheme 1D).[6] To test this idea, an array of sugar-1-phosphates as above was individually incubated with the purified recombinant Orf15 in the presence of NTP/dNTP and divalent metals. Orf15 was found to catalyze only the formation of dTDP-Glc/-Glm/-*N*-Ac-Glm but nothing else (Figure 2 A). The stringent use of dTTP and Glc-1-P/Glm-1-P/*N*-Ac-Glm-1-P is distinct from that of BtrD, which can utilize the same sugar-1-phosphates but in conjunction with either UTP or dTTP. Considering if there is a possible functionality relationship between Orf15 and the newly identified thymidyltransferase (a bifunctional nucleotidyl-transferases from archaeal bacterium, *Pyrococcus furiosus*),[7] the same condition except an extra addition of acetyl-CoA was assayed, but no corresponding acylated product detected. In light of the result and the lack of sequence similarity, Orf15 is thereby characterized as a novel thymidyltransferase (with $K_M = 133\ \mu M$ and $k_{cat} = 0.01\ s^{-1}$, measured in the presence of Glc-1-P) involved in the chloroeremomycin biosynthesis. Experimental procedures are provided in Supporting Information.

It is extremely interesting to learn that the two homologues have 65% protein sequence identity (72% similarity) but play completely different roles, perhaps only by subtle changes in their active sites. The proteins resemble an uncharacterized LmbE-like protein by domain search. Sequence and structural alignments in particular with the MshB structure suggested that a number of highly conserved residues may play pivotal roles toward the diversity.[8] The detailed catalytic mechanisms and structural information are currently being pursued, but our working models are proposed in Scheme 1E. The different reactions may be a result of a Lewis acid activated H_2O and a metal ion stabilized Glc-1-P, respectively. Considering the molecular recognition, one may reason that UDP-*N*-Ac-Glm does not fit into the active site of Dbv21/Orf2* because of electrostatic repulsion by the multiple charged diphosphate moiety and that the aglycone without the *N*-Ac Glm group could be away from the recognition owing to the bulkier structure. On the other hand Dbv21/Orf2* are highly specific and displayed no activity to another *N*-Ac-Glm teicoplanin pseudoaglycone (Scheme 1A, *N*-Ac-Glm moiety at β-OHTyr) (Figure 2B), or to the monosugar *N*-Ac-Glm, suggesting that the aglycone moiety is involved in the recognition.

Why would the bacterium develop this deacylation/reacylation mechanism? We hypothesize that it is result of a possible protective-group chemistry in addition to substrate salvage. The reasoning is that the subsequent glucosaminyl pseudoaglycone acylase has been shown previously to react with the free amine group of UDP-Glm.[2] It is possible that knocking the acylase out of the producing strain may render glucosaminyl glycopeptide, which can be further organochemically or enzymatically transformed to produce novel analogues for pharmacokinetic/pharmacodynamic studies. In conclusion, we showed that three highly homologous hypothetical proteins from the biosynthetic clusters of three clinically important glycopeptide antibiotics are novel enzymes with different activities in the biosynthetic pathways of these antibiotics. In addition, detailed structure−function analyses of these enzymes can also expand our knowledge in the two important classes of enzymes − deacetylases and thymidyltransferases.

Acknowledgment. T.L.L. and M.D.T. thank the National Science Council and Academia Sinica for financial support.

Note Added after ASAP Publication. After this paper was published ASAP on September 29, 2006, corrections were made in Scheme 1B−D and the table of contents graphic. The corrected version was published ASAP on October 4, 2006.

Supporting Information Available: Experimental procedures, chromatograms, spectra, and sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C. T. *Chem. Rev.* **2005**, *105*, 425−448.
(2) (a) Li, T. L.; Huang, F.; Haydock, S. F.; Mironenko, T.; Leadlay, P. F.; Spencer, J. B. *Chem. Biol.* **2004**, *11*, 107−19. (b) Kruger, R. G.; Lu. W.; Oberthur, M.; Tao, J.; Kahne, D.; Walsh, C. T. *Chem. Biol.* **2005**, *12*, 131−140.
(3) (a) *N*-acetylation normally occurs before uridyltransferation in the in vivo synthesis of UDP-*N*-acetylglucosamine that is a readily available primary metabolite in cytoplasm. (b) Milewski, S.; Gabriel, I.; Olchowy, J. *Yeast* **2006**, *23*, 1−14.
(4) Kudo, F.; Kawabe, K.; Kuriki, H.; Eguchi, T.; Kakinuma, K. *J. Am. Chem. Soc.* **2005**, *127*, 1711−1718.
(5) (a) Sosio, M.; Kloosterman, H.; Bianchi, A.; de Vreugd, P.; Dijkhuizen, L.; Donadio, S. *Microbiology* **2004**, *150*, 95−102. (b) Sosio, M.; Stinchi, S.; Beltrametti, F.; Lazzarini, A.; Donadio, S. *Chem. Biol.* **2003**, *10*, 541−549. (c) van Wageningen, A. M.; Kirkpatrick, P. N.; Williams, D. H.; Harris, B. R.; Kershaw, J. K.; Lennard, N. J.; Jones, M.; Jones, S. J.; Solenberg, P. J. *Chem. Biol.* **1998**, *5*, 155−162. (d) Pelzer, S.; Süssmuth, R.; Heckmann, D.; Recktenwald, J.; Huber, P.; Jung, G.; Wohlleben, W. *Antimicrob. Agents Chemother.* **1999**, *43*, 1565−1573.
(6) Chen, H.; Thomas, M. G.; Hubbard, B. K.; Losey, H. C.; Walsh, C. T.; Burkart, M. D. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11942−11947.
(7) Mizanur, R. M.; Jaipuri, F. A.; Pohl, N. L. *J. Am. Chem. Soc.* **2005**, *127*, 836−837.
(8) Hernick, M.; Gennadios, H. A.; Whittington, D. A.; Rusche, K. M.; Christianson, D. W.; Fierke, C. A. *J. Biol. Chem.* **2005**, *280*, 16969−16978.

JA0644834