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Complete Genome Sequence of the Butirosin-Producing *Bacillus vitellinus* NBRC 13296 and Its Reclassification to *Paenibacillus chitinolyticus*

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Abstract: Butirosins are naturally occurring aminoglycoside (AG) antibiotics featuring a 4,5-disubstituted 2-deoxystreptamine (2-DOS) with a (2S)-4-amino-2-hydroxybutyrate (AHBA) side chain. This side chain has been shown to confer resistance against AG modifying enzymes, leading to ongoing studies on the butirosin biosynthetic pathway and the corresponding enzymes. Butirosin is produced by *Niallia* (formerly *Bacillus*) *circulans* and *Bacillus vitellinus*, with most research focused on the former strain. To date, no whole genome analysis has been performed on *B. vitellinus*. In this study, we sequenced the complete genome of *B. vitellinus* NBRC 13296 and performed a comparative analysis of different butirosin biosynthetic gene clusters (BGCs), including those from *N. circulans*. The complete genome of *B. vitellinus* NBRC 13296 comprises a 6,331,192 base circular chromosome with a GC content of 52.68%. The annotation revealed the presence of 5,605 CDSs, 70 tRNA genes, 30 rRNA genes, and 3 ncRNA genes in NBRC 13296. The highest dDDH and ANI values between NBRC 13296 and the most closely related type strain, *Paenibacillus chitinolyticus* KCCM 41400, were 97.8% and 98.66%, respectively. Based on these genome-based comparative analyses, we propose reclassifying *B. vitellinus* NBRC 13296 as *P. chitinolyticus*. Genome mining revealed 18 gene clusters encoding the biosynthesis of diverse secondary metabolites in the genome of *B. vitellinus* NBRC 13296, indicating the enormous biosynthetic potential of this strain. The predicted structural diversity of secondary metabolites includes aminoglycoside, PKS, NRPS, PKS-NRPS hybrids, metallophores, phosphonates, terpenes, β-lactones, and RiPP peptides. We then comparatively characterized butorosin BGCs previously studied in several *N. circulans* strains. Additionally, comparative genome analysis revealed complete butirosin BGCs identified from *P. chitinolyticus* KCCM 41400, *P. chitinolyticus* NRRL B-23119, *P. chitinolyticus* NRRL B-23120, *P. chitinolyticus* B-14908, *P. chitinolyticus* YSY-3.1, *P. chitinolyticus* JMW06, *Paenibacillus* sp. GbtcB18, *Paenibacillus* sp. HGH0039, *Paenibacillus* sp. MZ04-78.2. Finally, we identified the core region consisting of BtrS, BtrN, BtrM, BtrL, BtrA, BtrB, BtrC, BtrD, BtrD, BtrE, BtrF, BtrG, BtrH, BtrI, BtrI, BtrJ, BtrK, BtrO, BtrP, and BtrV, followed by an upstream region organizing BtrQ, BtrW, BtrX, BtrY, and BtrZ in the same transcriptional direction and sequential genetic arrangement, and a downstream region organizing various proteins based on BtrT, BtrR2, BtrU, and BtrR1. Our study provided insights into the reclassification of *B. vitellinus* NBRC 13296 to *P. chitinolyticus* and suggested the need for continued studies on butirosin biosynthesis from an enzymatic perspective.

Keywords: *Bacillus vitellinus* NBRC 13296; butirosin BGC; *Paenibacillus chitinolyticus*; reclassification

1. Introduction

Aminoglycosides are an important class of antibiotics used to treat serious infections caused by several Gram-negative and Gram-positive bacteria, despite their nephrotoxic and ototoxic side

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effects. The mechanism of action of aminoglycosides involves binding to the 30S ribosomal subunit, which interferes with protein synthesis by causing errors in translation, specifically in codon reading, thereby disrupting protein synthesis [1,2]. Aminoglycosides consist of highly functionalized aminosugars and deoxysugars linked by glycosidic bonds to an aminocyclitol aglycone, with most aglycones containing a 2-deoxystreptamine (2-DOS) moiety, to which other amino sugars are attached at 4 and 5 or 4 and 6 positions of the 2-DOS carbons. Aminoglycoside producers primarily belong to the actinomycetes group of bacteria, but butirosin is one of the few aminoglycosides produced by a non-actinomycete bacterium [3,4].

The (S)-4-amino-2-hydroxybutyrate (AHBA) group attached to the C-1 amine of the 2-DOS moiety in butirosin has long been of interest because this AHBA moiety can prevent the action of several aminoglycoside-modifying enzymes (AMEs), such as aminoglycoside N-acetyltransferases (AACs), which can theoretically or practically cause aminoglycoside resistance. For example, amikacin is a semisynthetic aminoglycoside with an AHBA group attached to the C-1 of the 2-DOS aglycone of kanamycin, and is considered one of the few antibiotics that can overcome various AME mechanisms [5-7]. Butirosin was identified as a pair of epimers, butirosins A and B, from *Niallia* (formerly *Bacillus*) circulans in 1971 and was subsequently discovered in *Bacillus vitellinus* [8,9].

To date, the whole genome sequence (WGS) of *N. circulans* in the NCBI (National Center for Biotechnology Information) databases has been completed for the reference strain *N. circulans* NBRC 13626 (SAMD00046991), with additional WGS reported for *N. circulans* DC10 (SAMN18824981), isolated from textile dye-contaminated soil sediments in India, and *N. circulans* GN 3 (SAMN14847575), isolated from purple rhizosphere soil growing the cabbage *Brassica campestris* in China. However, there are no reports of complete genome information for *B. vitellinus*, one of the main producer strains of butirosin. Here, we report the complete genome sequence of *B. vitellinus* NBRC 13296 to better understand the biosynthesis of butirosin and other bioactive secondary metabolites. The genome was sequenced using long-read PacBio technologies to obtain high-quality genomic sequences [10,11].

Meanwhile, bioinformatics software such as antiSMASH, a platform for automated genome mining of secondary metabolite producers, can be used to rapidly identify, analyze, and annotate BGCs from genome-wide information and identify similar characterized gene clusters in the MIBiG repository [12,13]. Finally, comparative genomic analysis using next-generation sequencing (NGS) techniques and bioinformatics software confirmed the presence of a butirosin BGC.

Interestingly, during the BLASTP search using antiSMASH, butirosin BGCs were identified as biosynthetic enzymes in the type strain *Paenibacillus chitinolyticus* KCCM 41400 (SAMN08222605), as well as in *P. chitinolyticus* NRRL B-23119 (SAMN27675096), *P. chitinolyticus* NRRL B-23120 (SAMN27675097), *P. chitinolyticus* YSY-3.1 (SAMD00444452), *P. chitinolyticus* JMW06 (SAMN19998407), *P. chitinolyticus* B-14908 (SAMN33770086), *Paenibacillus* sp. GbtcB18 (SAMN18679176), *Paenibacillus* sp. MZ04-78.2 (SAMN28689874), *Paenibacillus* sp. HGH0039 (SAMN02596731).), *N. circulans* ATCC 21557 (AJ781030), *N. circulans* ATCC 21558 (LC571042.1), and *N. circulans* SANK 72073 (AB097196). The full genome-derived 16S rRNA gene sequences, average nucleotide identity (ANI), and digital DNA-DNA hybridization (dDDH) suggest that all the strains mentioned above should be classified as members of the same species. We propose the reclassification of *B. vitellinus* NBRC 13296 as *P. chitinolyticus* NBRC 13296 and an emended description *of P. chitinolyticus*. Finally, in this article, we aim to provide scientists with a comparative analysis of butirosin BGCs to assist in the study of the butirosin biosynthetic pathway and corresponding enzymes.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Bacillus vitellinus NBRC 13296 was obtained from the NITE Biological Research Center (NBRC, Tokyo, Japan). The cells were grown in a tryptic soy broth (TSB) medium containing 1.7% tryptone (pancreatic digest of casein), 0.3% soytone (peptic digest of soybean), 0.25% glucose, 0.5% sodium chloride, and 0.25% dipotassium phosphate (BD Biosciences, Franklin Lakes, NJ, USA) at 30°C for 24 h.

2.2. Genome De Novo Sequencing, Assembly, and Annotation

The genomic DNA of *B. vitellinus* was extracted and purified using the Nanobind DNA extraction kit, which produces HiFi reads on the PacBio systems (Pacific Biosciences, Menlo Park, CA, USA). The gDNA obtained from the extraction was first sheared using the g-TUBE (Covaris) following the manufacturer's protocol. SMRTbell libraries were created using the PacBio SMRTbell Express template preparation kit (v1.0). SMRTbell templates were annealed using the PacBio DNA/Polymerase Binding Kit P6. The PacBio DNA Sequencing Kit 4.0 and 8 SMRT cells were used for sequencing. SMRT cells (Pacific Biosciences, Menlo Park, CA, USA) using C4 chemistry and 240 min movies were captured for each SMRT cell using the PacBio Sequel II (Pacific Biosciences) sequencing platform. These sequences were assembled de novo using the CANU ver. 2.2 workflow. The assembly was circularized by Circulator (v. 1.5.5) and rearranged to start at the dnaA gene. [14]. Regarding gene prediction and annotation, protein-coding, tRNA, rRNA genes, and repeat regions were predicted using the NCBI PGAP (Prokaryotic Genome Annotation Process). Gene annotation was performed using RefSeq and the HMM library (TIGRFAM, Pfam, PRK HMMs) database [15, 16].

2.3. Phylogenetic Analysis

Phylogenetic trees were constructed based on the complete 16S rRNA gene sequence derived from the whole genome information of *B. vitellinus* NBRC 13296. The evolutionary tree based on 16S rRNA sequences was constructed with the MEGA 11 package [17], applying neighbor-joining and maximum-likelihood algorithms after multiple alignments of the sequence data by the ClustalW program. This process primarily used BlastN homology searches and referenced BacDive, the worldwide largest database for standardized bacterial information, for type strains. The confidence of the tree topologies was assessed by 1000 bootstrap replicates.

2.4. Comparative Genomic Studies and Whole Genome Relatedness

Digital DNA-DNA hybridization (dDDH) values of the B. vitellinus NBRC 13296 genome and its neighbors were calculated using the Genome-to-Genome Distance Calculator (GGDC 4.0) within the Type (Strain) Genome Server (TYGS) of the Leibniz Institute DSMZ [18]. The average nucleotide identity (ANI) values between *B. vitellinus* NBRC 13296 and its nearest neighbors were computed using the ANI calculator of Ezbiocloud, an online tool used to compare two prokaryotic genome sequences [19].

2.5. Secondary Metabolite and Butirosin BGCs Analysis

To discover BGCs involved in the production of secondary metabolites, including butirosin, various computational programs such as Known ClusterBlast, ClusterBlast, SubClusterBlast, ActiveSiteFinder, and Cluster PFam analysis were employed. The PRISM 4 and BAGEL 4 tools were implemented with default settings. Anti-SMASH 7.0 facilitated the identification, annotation, and analysis of secondary metabolite BGCs across the genome [12]. RiPPs and bacteriocins were mined by BAGEL 4 [20], while PRISM 4 version 4.4.5 was used for the comprehensive analysis of secondary metabolite structures and biological activities [21]. These sophisticated computational tools provide accurate predictions of microbial secondary metabolite encoding potential and putative structures.

3. Results and Discussion

3.1. Sequencing, Assembly, Phylogenetic Analysis, and Genomic Characteristics

In whole-genome sequencing based on the PacBio Sequel IIe platform, the complete genome sequence of *B. vitellinus* NBRC 13296 was composed of 1 contig with a total length of 6,331,192 bps and an average G + C content of 50.98%. A total of 5,731 genes were identified in its genome, including

5,605 annotated protein-coding genes, 92 tRNA, 30 rRNA, 3 ncRNA, and 1 tmRNA genes. Ten copies of 16SrRNAs were identified in the genome of *B. vitellinus* NBRC 13296, and variations at seven positions were also identified by alignment between the 16S rRNA sequences, totaling 1,544 bp, using ClustalW. No plasmid was detected. In the 16S rRNA gene sequence analysis, *B. vitellinus* NBRC 13296 showed sequentially 99.66%, 95.71%, 95.60%, and 94.96% similarities to the type strains *Paenibacillus chitinolyticus* HSCC 596 (NR_040854.1), *Paenibacillus gansuensis* B518 (NR_043219.1), *Paenibacillus lutrae* N10 (NR_173496.1), and *Paenibacillus favisporus* GMP01 (NR_029071.1), respectively (Table S1). The phylogenetic tree constructed from the BLASTN, BacDive, and EzBioCloud 16S database [22] using maximum-likelihood and neighbor-joining methods in the MEGA 11 application with 1000 bootstrap values showed *B. vitellinus* NBRC 13296 forming a clade with *P. chitinolyticus* as shown in Figure 1 and Figure S1.

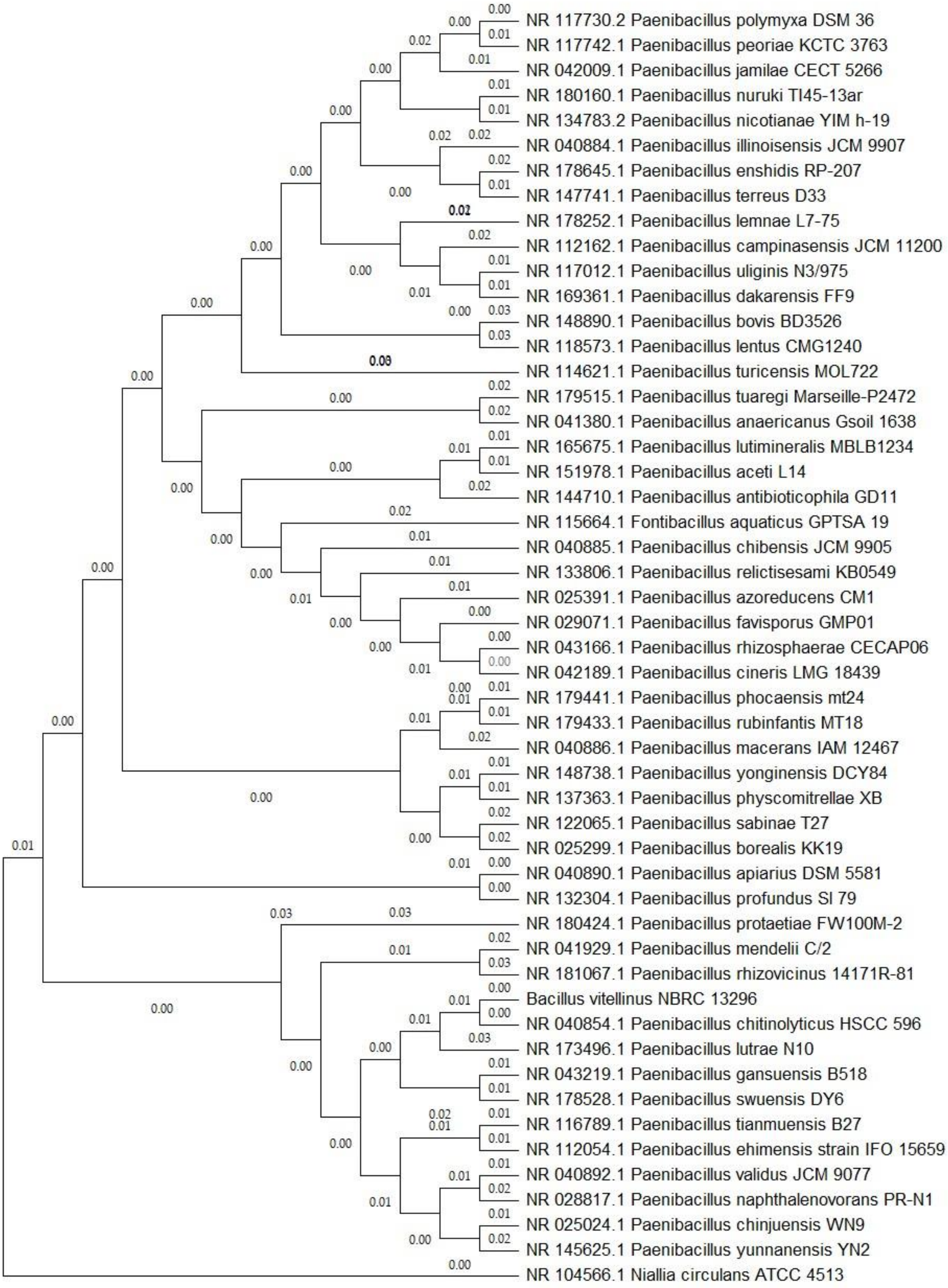

Figure 1. Neighbor-joining phylogenetic tree based on the complete 16S rRNA gene sequence extracted from the assembled genome. The relationship between closely related species of type cultures from genus *Paenibacillus* are demonstrated. *Niallia circulans* ATCC 4513 was used as an out group. The analysis, performed using MEGA 11, demonstrates that *P. chitinolyticus* HSCC 596T is its closest neighbor.

Comparative genomic analysis revealed that the Digital DNA-DNA hybridization (dDDH) values between *B. vitellinus* NBRC 13296 and the closely related strain *P. chitinolyticus* KCCM 41400 were far above the 70% threshold value for species evaluation [23]. The average nucleotide identity (ANI) values were also within the threshold range (94–96%) for species identification [24]. The dDDH and ANI values based on genome sequences between *B. vitellinus* NBRC 13296 and *P. chitinolyticus* KCCM 41400 were 97.8% and 98.66%, respectively, providing strong evidence that these strains may belong to the same taxonomic species.

3.2. Prediction of Secondary Metabolite Biosynthetic Gene Clusters (BGCs)

Using the standard cluster rule-based approach in antiSMASH analysis, a variety of natural product classes were identified in *B. vitellinus* NBRC 13296. Approximately 12.4% of the *B. vitellinus* NBRC 13296 genome is dedicated to secondary metabolism. The secondary metabolite gene cluster are concentrated in the center of the chromosome, spanning regions 5 to 16 (Figure S2). In total, 18 putative biosynthetic gene clusters were identified (Table 1). Interestingly, region 15 contained two-copies of an opine-like-metallophore gene cluster, consisting of opine metallophore biosynthesis dehydrogenase, nicotianamine synthase family protein, and nickel/cobalt ABC transporter substrate-binding protein/permease (Figure S3). Thus, a total of 19 BGCs were identified through antiSMASH analysis. Next, we used BAGEL to analyze the genome sequence of NBRC 13296 and found a total of 2 BGCs for different NRPS/LAP and sactipeptides. The former cluster identified by BAGEL overlaps with region 1 of antiSMASH, while the latter sactipeptides cluster is novel (Table S2 and S3). Using the PRISM algorithm, a total of 11 clusters were identified. Ten of these clusters overlapped with the AntiSMASH results, but an additional polyketide cluster, consisting of beta-ketoacyl-ACP synthase II, acyl carrier protein, ACP S-malonyltransferase, etc., was identified in cluster 10 (Figure S3 and Table S4). Based on the BGCs identified through the three approaches, we were ultimately able to identify 22 BGCs, including 19 from antiSMASH and an additional one each from BAGEL 4 and PRISM 4.4.5.

Table 1. Putative gene clusters coding for secondary metabolites in *B. vitellinus* NBRC 13296.

Region	Type	From	To	Most similar known cluster	Similarity
Region 1	NRPS, LAP	305619	356240		
Region 2	RRE-containing	747520	767798		
Region 3	RiPP-like	851869	864055		
Region 4	Crocagin, HR-T2PKS	945752	1014754		
Region 5	TransAT-PKS, NRPS	2461872	2549008		
Region 6	TransAT-PKS, NRPS	2563881	2640650	Pelgipeptin	25%
Region 7	Cyclic-lactone-autoinducer	2734390	2754992		
Region 8	Terpene	2821778	2843727		
Region 9	Betalactone	3032661	3062918		
Region 10	TransAT-PKS, NRPS	3175761	3243813		
Region 11	Phosphonate	3441175	3454343		
Region 12	Amglyccycl	3489373	3517357	Butirosin A/B	84%
Region 13	NRPS	3540249	3611046	Octapeptin C4	29%
Region 14	NRP-metallophore, NRPS	3614412	3666638	Bacillibactin	100%
Region 15	Opine-like-metallophore	3687196	3729044	Bacillopaline	100%
Region 16	T3PKS	3837645	3878793		
Region 17	Proteusin	5176800	5197111		
Region 18	TransAT-PKS-like, NRPS	5276428	5336092		

* "similarity" refers to the ratio of homologous genes in the query and hit clusters. As defined by antiSMASH, homologous genes were selected based on high sequence identity (>30%) and short BLAST alignments (>25%).

3.3. Comparative Characterization of the Butorosin BGCs

Enzymatic studies of the butirosin biosynthetic pathway have led to remarkable scientific advances in *Niallia circulans*, with BGCs for *N. circulans* SANK 72073, *N. circulans* ATCC 21557, and *N. circulans* ATCC 21558 [4, 25, 26] identified in the NCBI database. First, we obtained a large gene cluster from BtrA to BtrZ based on the butirosin BGC of *N. circulans* SANK 72073 and the BGC of *N. circulans* ATCC 21558, extended by genomic walking. We then identified additional genes in the upstream and downstream regions of the genome of *B. vitellinus* NBRC 13296, in addition to the tentative butirosin BGCs in region 12 analyzed by antiSMASH. As shown in Figure 2 and Table S5, *B. vitellinus* NBRC 13296 and the butirosin BGC from *N. circulans* share 67.81% to 92.87% high homology. Interestingly, during a BLASTP search for region 12 derived by antiSMASH, putative butirosine BGCs were identified in the type strain *P. chitinolyticus* KCCM 41400 as well as in *P. chitinolyticus* NRRL B-23119, *P. chitinolyticus* NRRL B-23120, *P. chitinolyticus* B-14908, *P. chitinolyticus* YSY-3.1, *P. chitinolyticus* JMW06, *Paenibacillus* sp. GbtcB18, *Paenibacillus* sp. HGH0039, *Paenibacillus* sp. MZ04-78.2. Next, we analyzed the genetic similarities and differences between *B. vitellinus* NBRC 13296, *N, circulans*, and the putative butirosin BGCs identified in *Paenibacillus* sp. The results showed that all BGCs had the following genes in common: BtrT, BtrS, BtrN, BtrM, BtrL, BtrA, BtrB, BtrC, BtrD, BtrD, BtrE, BtrF, BtrG, BtrH, BtrI, BtrI, BtrJ, BtrK, BtrO, BtrP, BtrV were located in the same transcriptional orientation, followed by BtrQ, BtrW, BtrX, BtrY, and BtrZ genes upstream of these core regions in the same transcriptional orientation and consecutive order. In contrast, the enzymes BtrT, BtrR2, BtrU, and BtrR1 are commonly found downstream of the core region, but beyond that, the genes are arranged in a strain-specific manner. Specifically, *B. vitellinus* NBRC 13296, *P. chitinolyticus* KCCM 41400, *P. chitinolyticus* NRRL B-23119, *P. chitinolyticus* NRRL B-23120, and *Paenibacillus* sp. GbtcB18 B18 show the same genetic organization from BtrT2 to BtrT7, while *P. chitinolyticus* YSY-3.1 is similar only up to BtrT2, and the remaining strains lack some of the BtrT3 to BtrT7 genes. Furthermore, *Paenibacillus* sp. MZ04-78.2 contained five completely unrelated ORFs instead of the BtrT, BtrR2, BtrU, and BtrR1 genes. Finally, we sought to deduce the minimal unit of the butirosin BGC. Many classes of secondary metabolite BGCs, such as aminoglycosides, type I polyketides, type II polyketides, indolocarbazoles, and enediynes, are often accompanied by regulatory and resistance genes [27-31]. Therefore, it is likely that these genes are also present in the butirosin BGC. Based on these findings, it is highly likely that the regulatory genes BtrR1 and BurR2 are included in the butirosin BGC, as are all or part of the transporter genes BtrT2, BtrW, BtrX, and BtrY, which may act as resistance genes. However, further studies, such as heterologous expression or enzymatic approaches, are required.

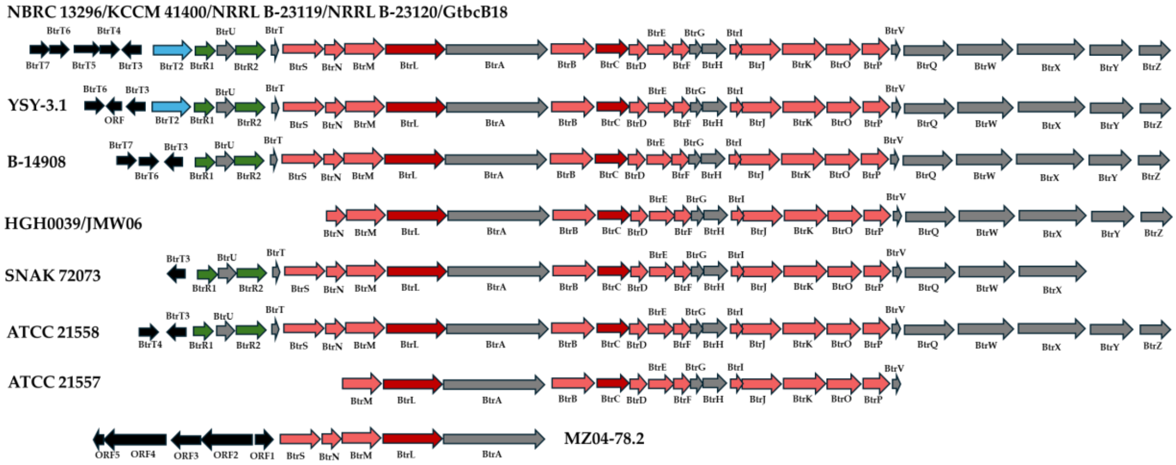

Figure 2. Comparison of butirosin BGCs encoded in the genomes of *Bacillus vitellinus* NBRC 13296, *Paenibacillus chitinolyticus* KCCM 41400T (SAMN08222605), *P. chitinolyticus* NRRL B-23119 (SAMN27675096), *P. chitinolyticus* NRRL B-23120 (SAMN27675097), *P. chitinolyticus* YSY-3.1 (SAMD00444452), *P. chitinolyticus* JMW06 (SAMN19998407), *P. chitinolyticus* B-14908 (SAMN33770086), *Paenibacillus* sp. GbtcB18 (SAMN18679176), *Paenibacillus* sp. MZ04-78.2 (SAMN28689874), and *Paenibacillus* sp. HGH0039 (SAMN02596731). The individually reported *N. circulans* ATCC 21557 (AJ781030), *N. circulans* ATCC 21558 (LC571042.1), and *N. circulans* SANK 72073 (AB097196) are also compared.

4. Conclusions

Over the past three decades, the 2-deoxystreptamine (2-DOS) amonoglycoside antibiotic butirosin has been intensively studied in *N. circulans* to develop semisynthetic antibiotics using the (2S)-4-amino-2-hydroxybutyrate (AHBA) side chain, and nearly all biosynthetic enzymes and BGCs have been characterized. The present study was initiated by the identification of the whole genome of another butirosin-producer *B. vitellinus* NBRC 13296, to pursue a new approach to the butirosin biosynthetic pathway. Through complete genome analysis of *B. vitellinus* NBRC 13296, we compared its dDDH and ANI values with *P. chitinolyticus*, the most closely related species based on 16SrRNA phylogenetic analysis, including *N. circulans*. Based on these genome-based comparative analyses, we propose to reclassify *B. vitellinus* NBRC 13296 to *P. chitinolyticus*. In this study, genome mining using AntiSMASH, BAGEL 4, and PRISM programs revealed 22 gene clusters encoding the biosynthesis of diverse secondary metabolites in the genome of *B. vitellinus* NBRC 13296. Finally, we comparatively characterized butirosin BGCs from *N. circulans* and *P. chitinolyticus*, revealing the minimal region of the butirosin BGC from BtrA to BtrZ and highlighting the need for continued study of butirosin biosynthesis.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, K.A.H. and C.G.H.; methodology, K.A.H; bioinformatic analyses, K.A.H.; writing—original draft preparation, C.G.H.; writing—review and editing, C.G.H.; supervision, K.H.B. S.Y.K., and C.G.H.; project administration, W.J.C.; funding acquisition, W.J.C. All the authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The whole-genome of B. vitellinus NBRC 13296 has been deposited at the NCBI genome database under the accession number CP*****. The assembly reported in the paper is associated with NCBI BioProject: ******** and BioSample: *******. The authors confirm that all the data needed to support the study is represented within the article and supplementary files.

Conflicts of Interest: The authors declare no conflict of interest.

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