Prevention Strategy of Viral Diseases in Poultry Using 1-Deoxynojirimycin

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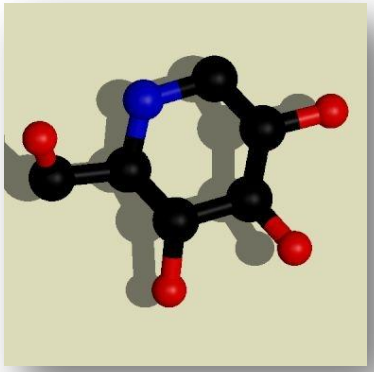
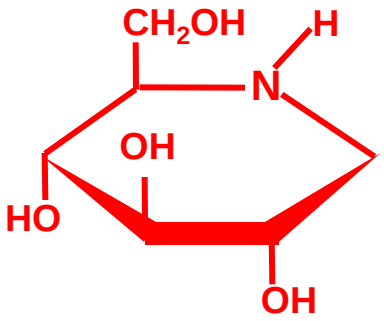
1-Deoxynojirimycin(DNJ)

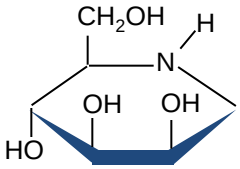
- **An alkaloid which is similar structure to glucose.**



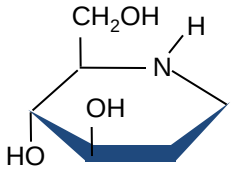
Anti-viral Alkaloid Materials



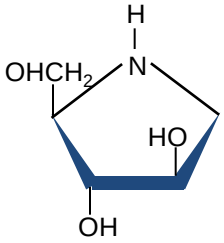
1-Deoxynojirimycin



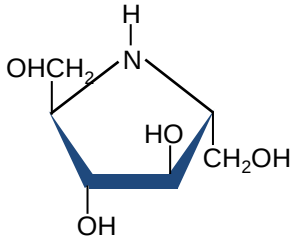
1-Deoxymannojirimycin



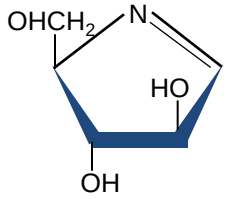
Fagomine



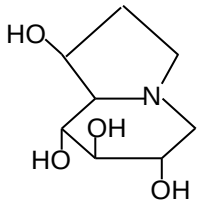
1,4-dideoxy-1,4-imino-D-arabinitol (DAB)



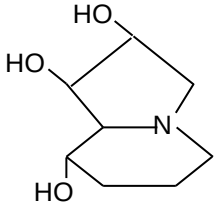
2,5-dideoxy-2,5-imino-D-mannitol(DMDP)



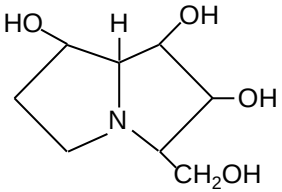
Polyhydroxypyrroline nectrisine



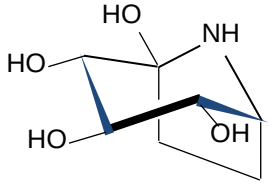
Castanospermine



Swainsonine



7a-epi-alexine(Australine)



Calystegin B_2

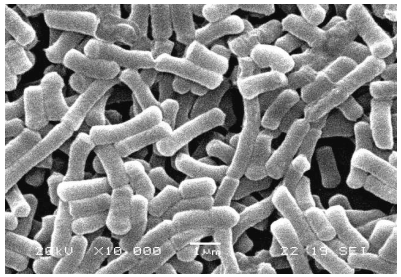
Routes to Produce 1-Deoxynojirimycin

- Extraction from plants such as the mulberry trees (root bark)

- Extraction from silkworm

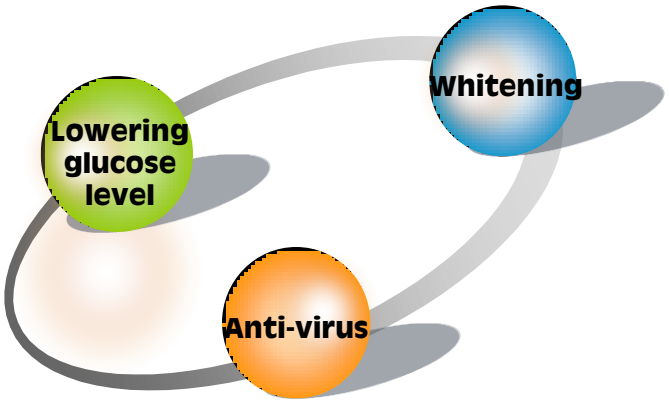
- Chemical synthesis following different synthetic strategies

- Fermentation by various *Bacillus* or *Streptomyces*.

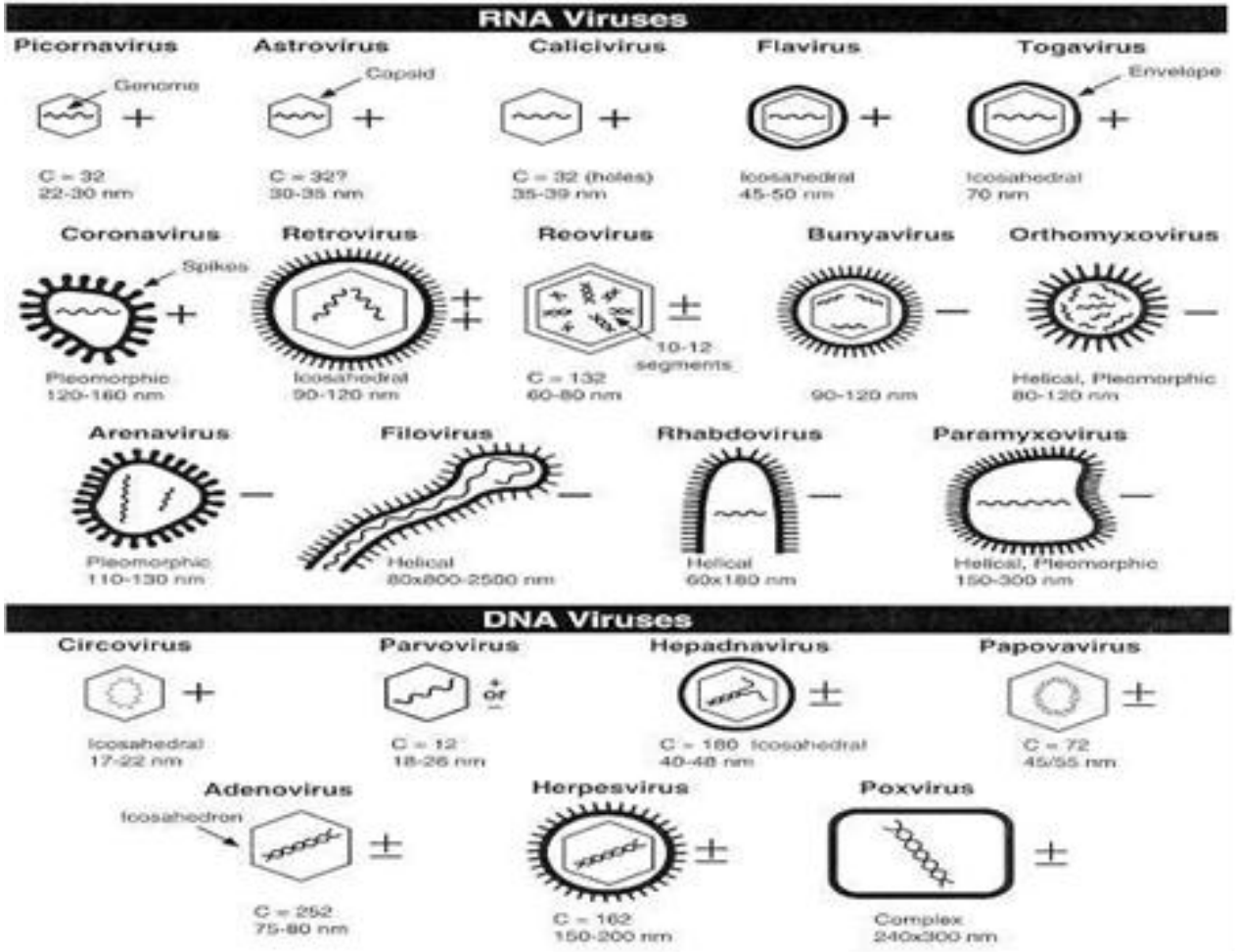


Functions of 1-Deoxynojirimycin

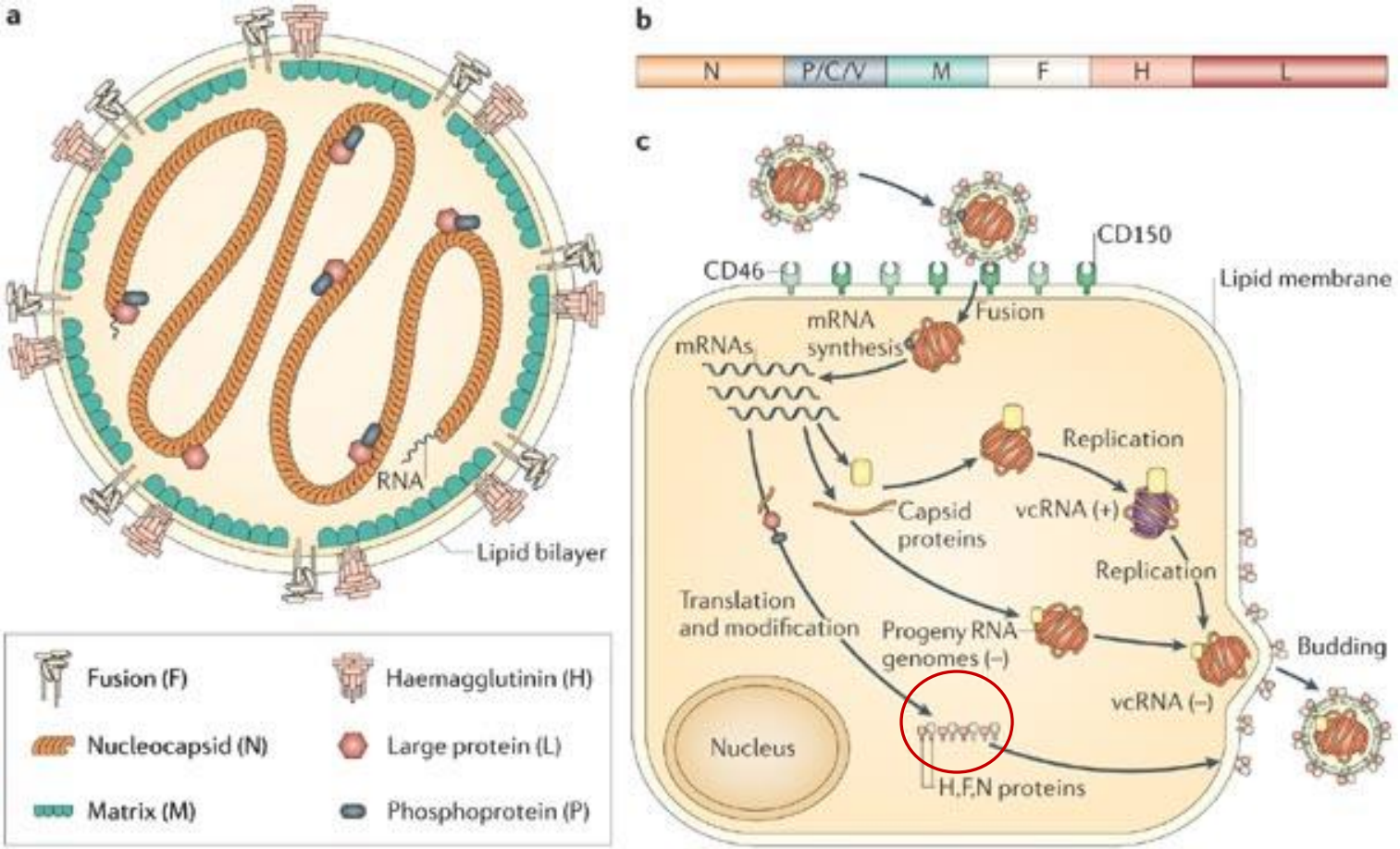
- **Inhibits virus growth** due to suppression of glycoprotein synthesis in ER lumen.
- Inhibits α-glucosidase and **delays the absorption of glucose** to the blood.
- Have a **whitening effect** due to suppression of melanin synthesis in melanocyte.
- Can be applied for foods, cosmetics and feed supplements.



Various Morphology of Animal Viruses



Newcastle Disease Virus Replication



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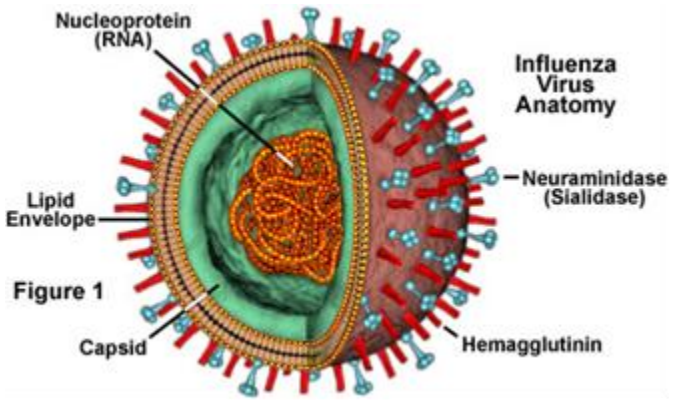
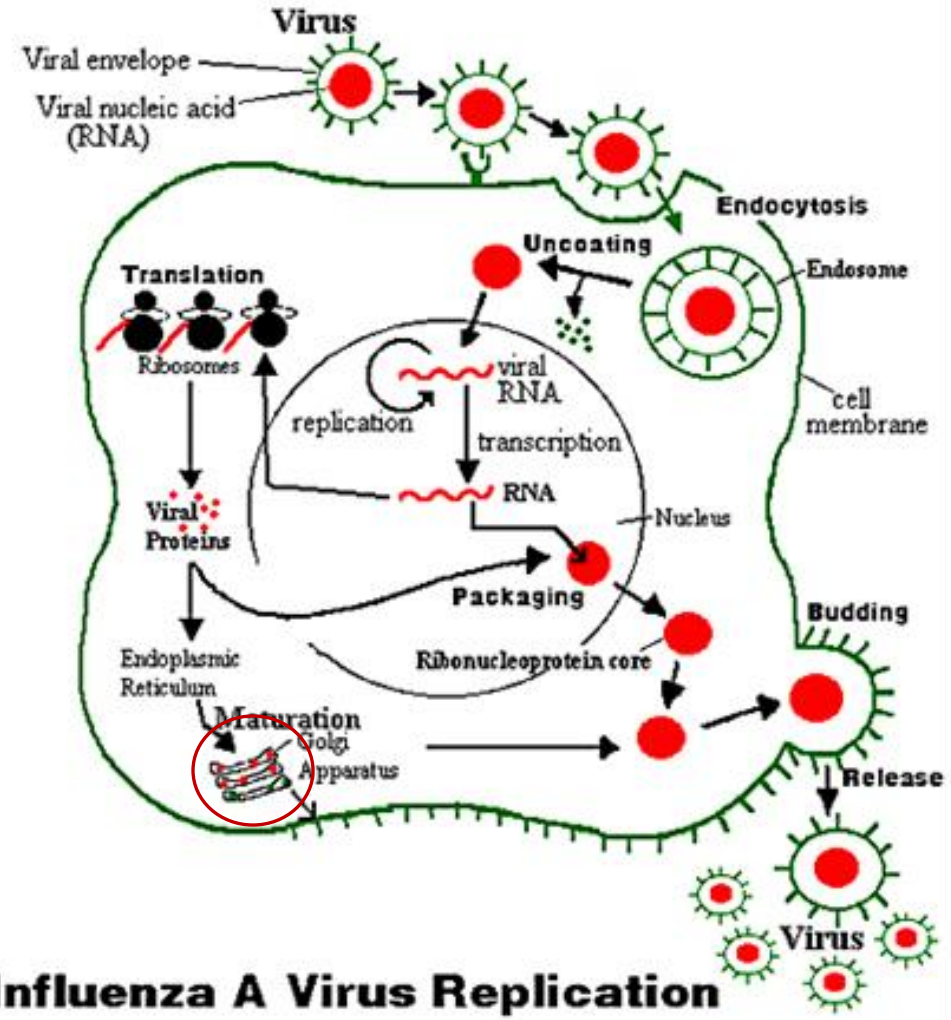
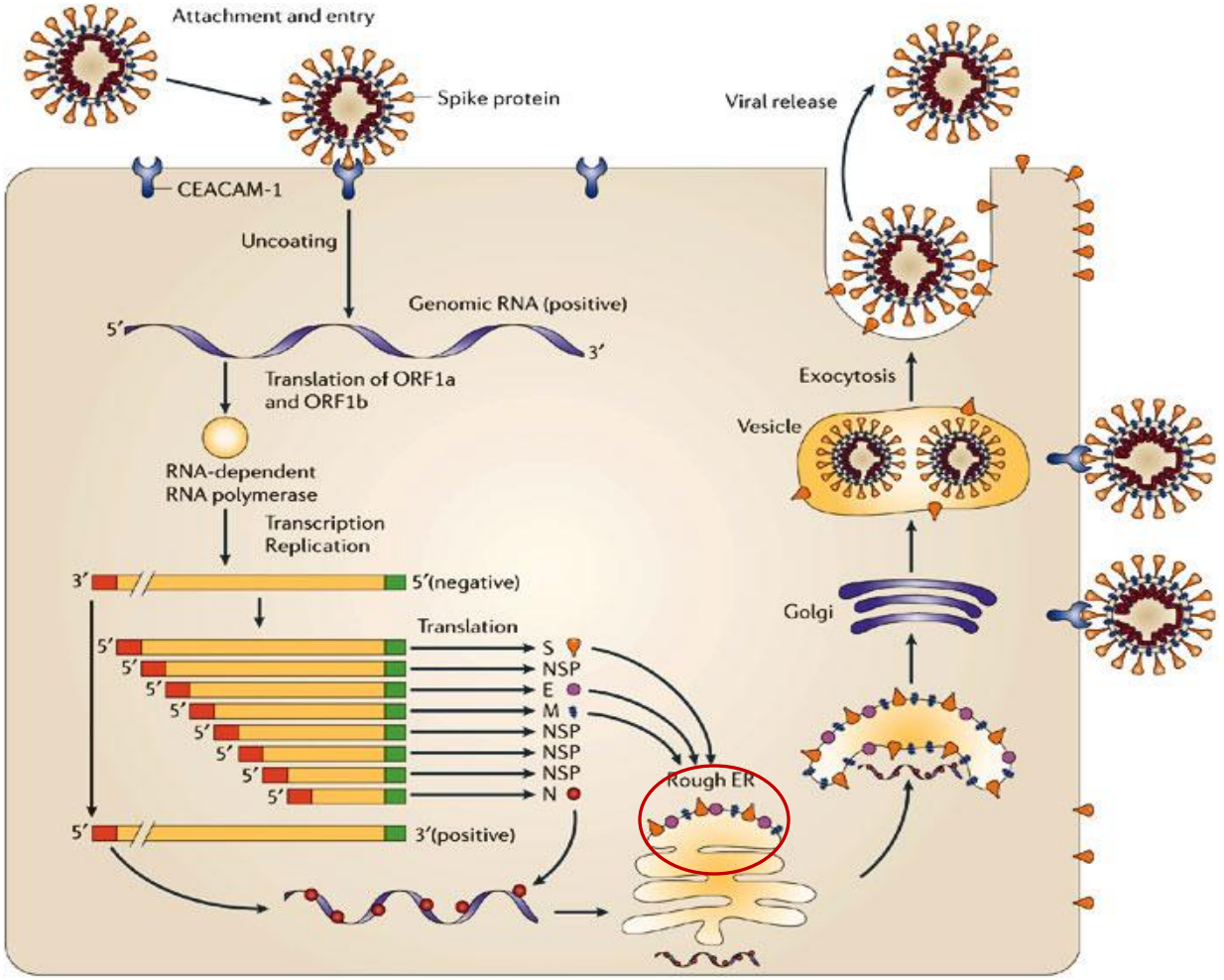
Influenza A Virus Replication





Coronavirus Replication



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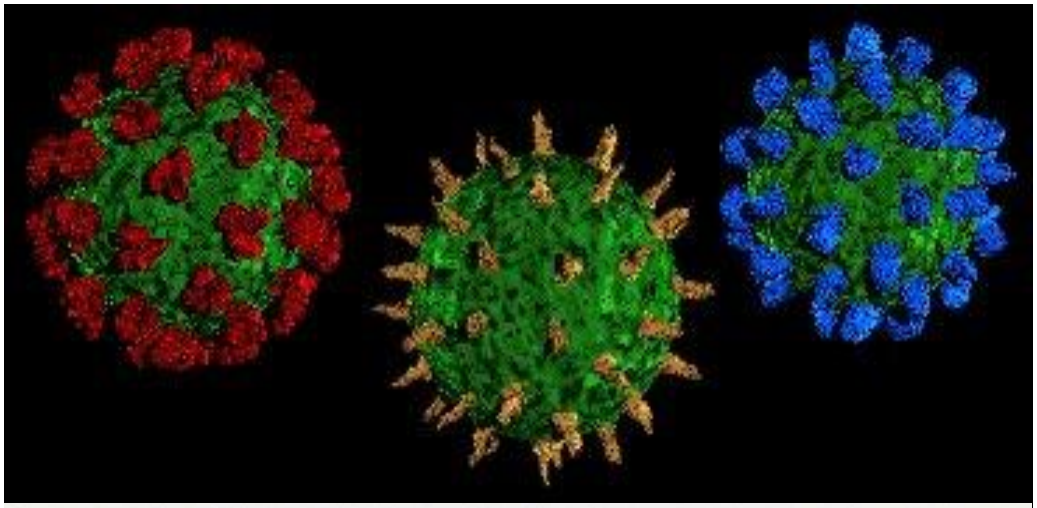
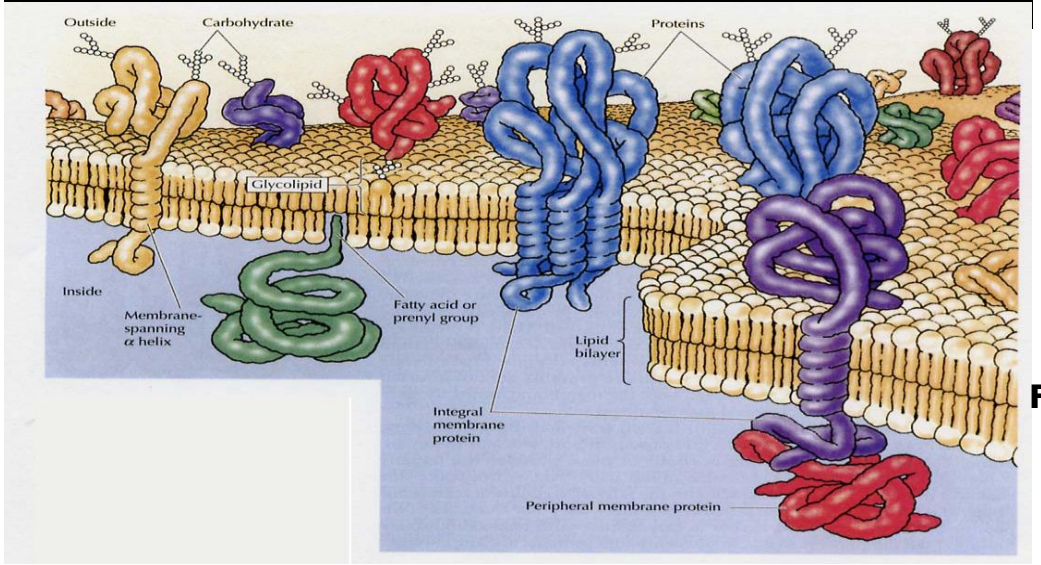
Importance of Glycoproteins of Viruses



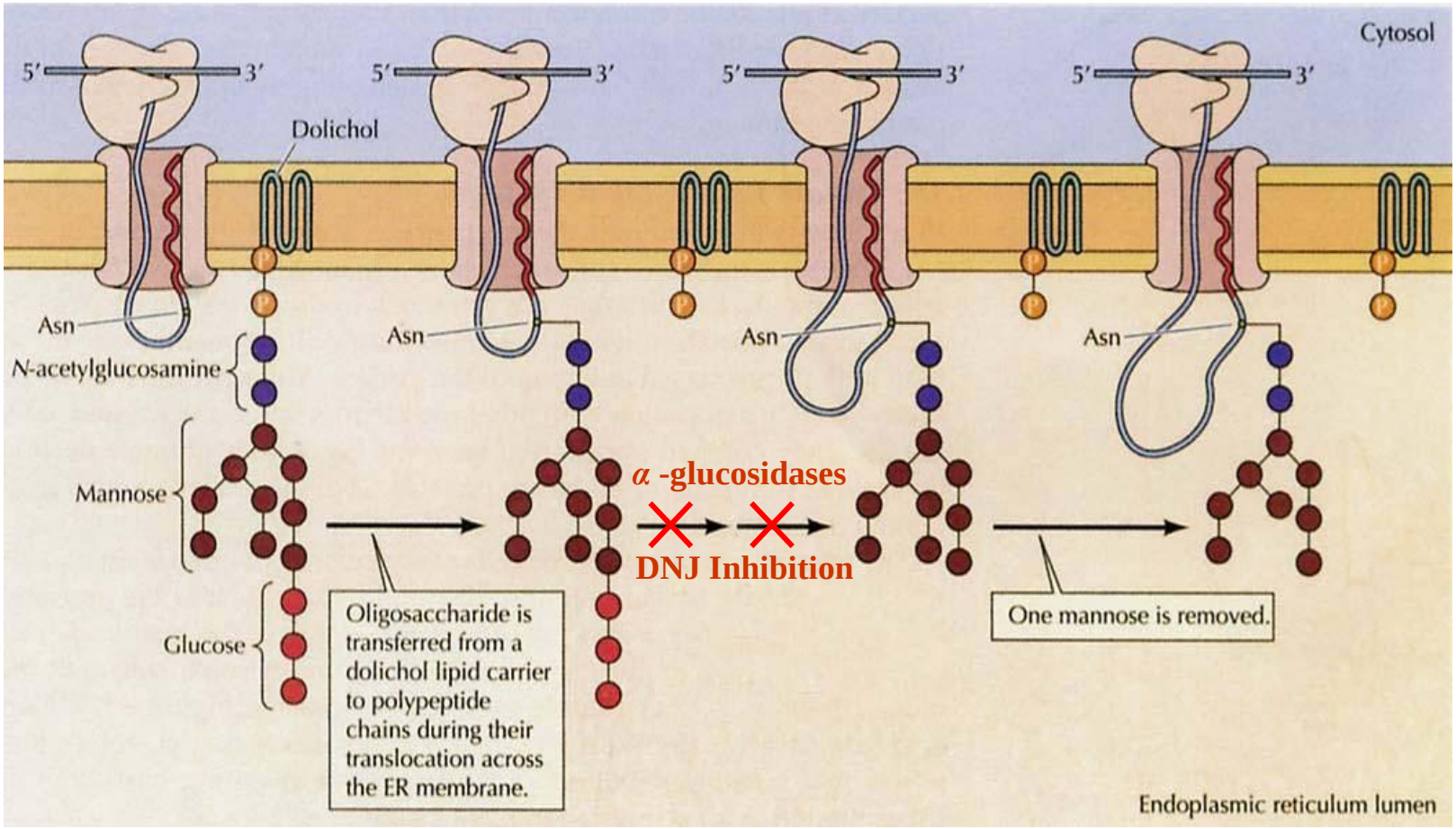


Viral Infection is not accomplished,. If the viral glycoprotein is modified and cannot interact with host cell.

Glycoprotein plays an important role in the exchange between the cells, signal transduction, as well as **binding of infection-receptor.**

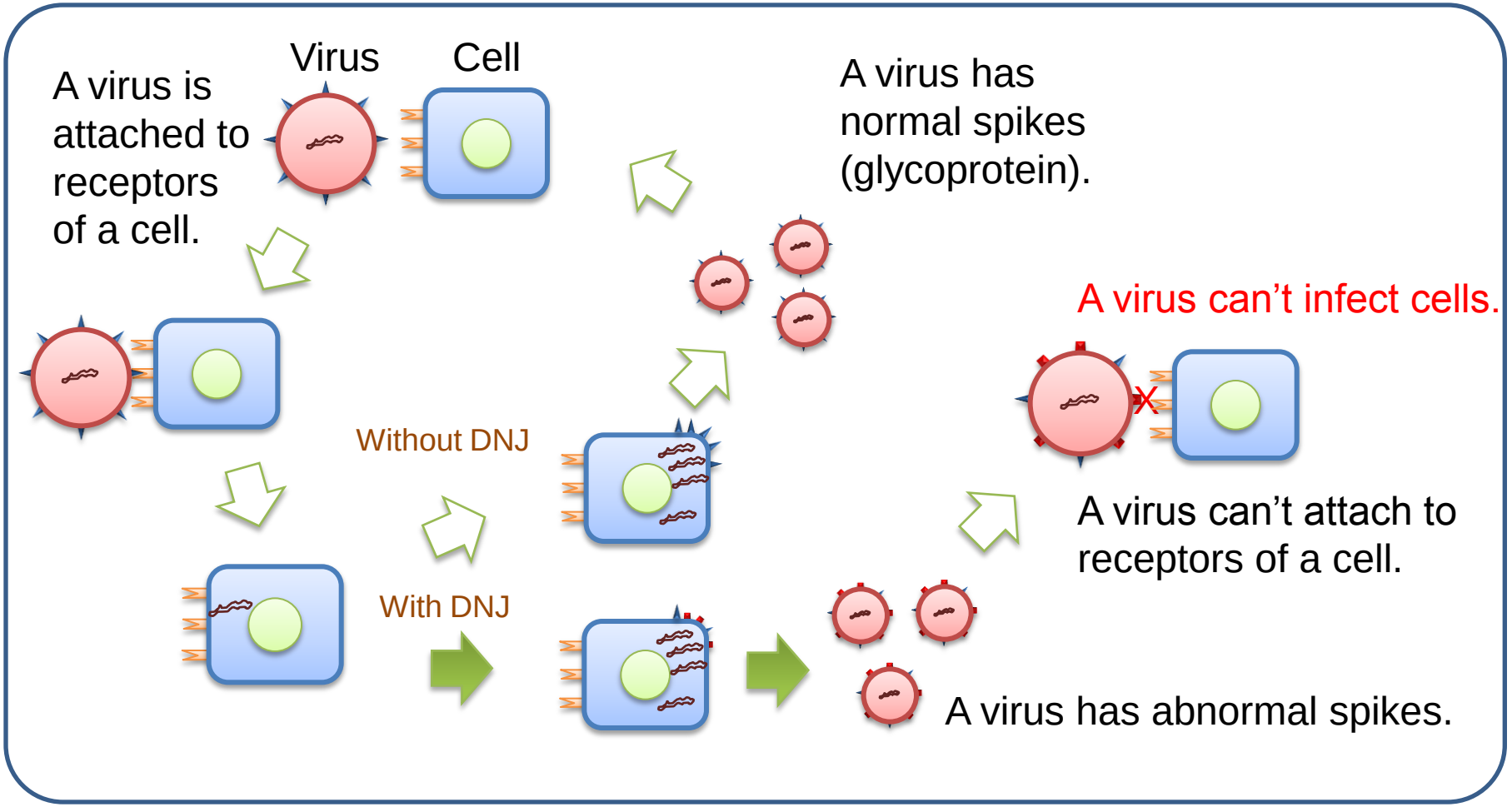
Fluid mosaic model of membrane structure

Inhibition mechanism of N-linked glycosylation in the animal cell



DNJ inhibits the viral multiplication by inhibiting the synthesis of glycoprotein.

Abnormal Infection and Inhibition of Propagation of Virus



Inhibition of the Release of Viral Particles by DNJ

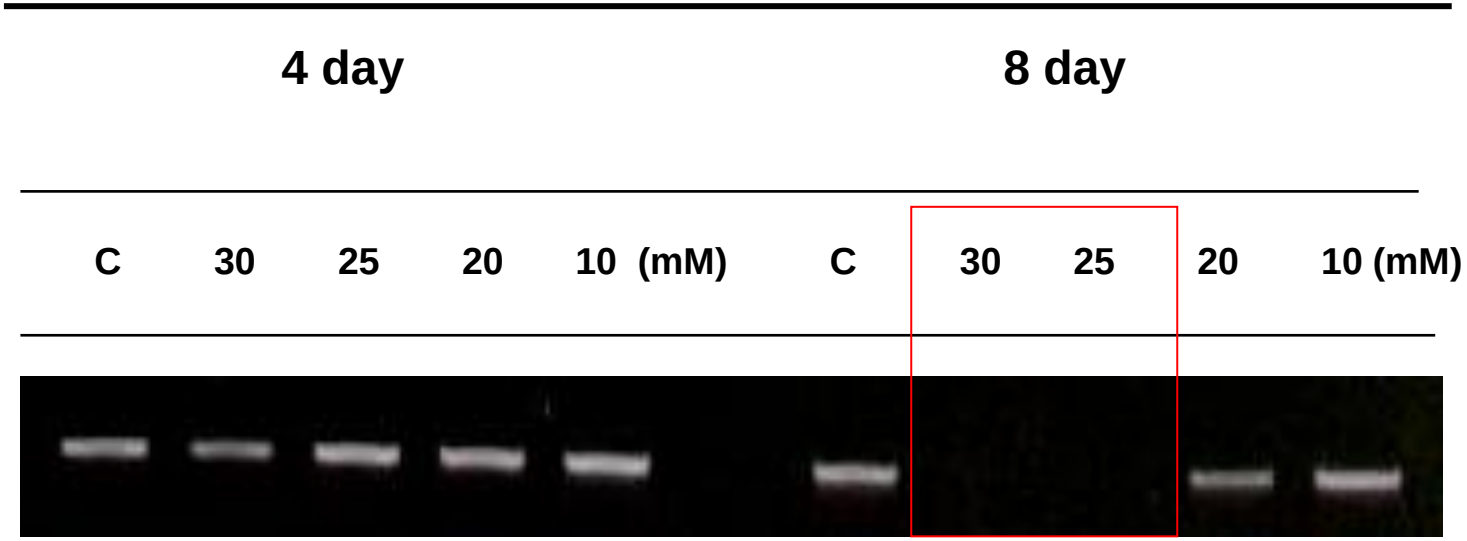
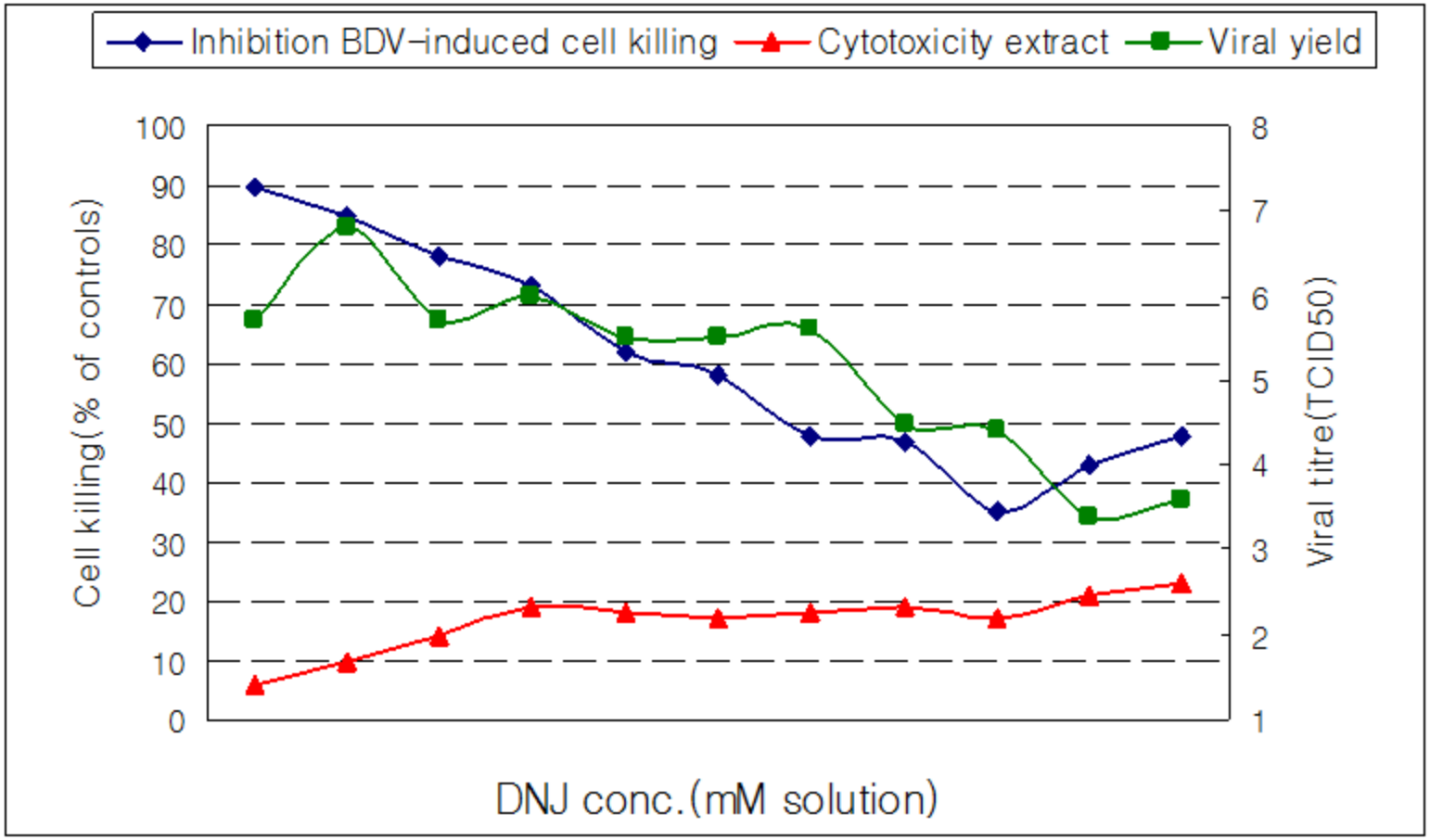
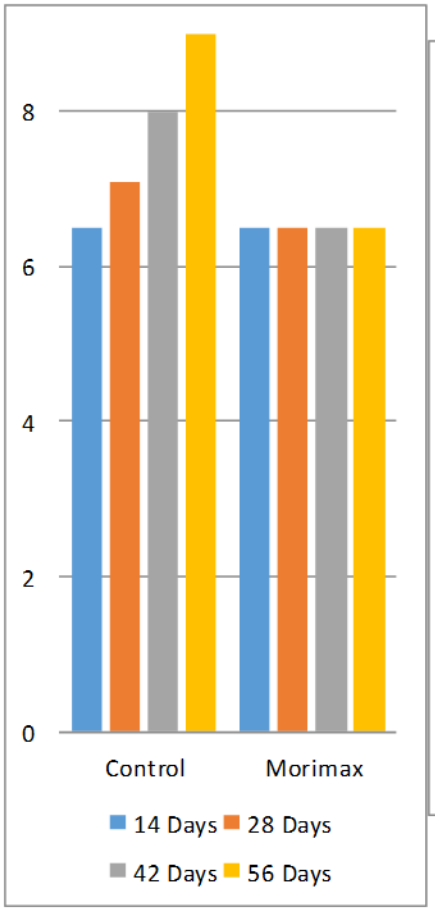


Figure . DNJ inhibits the release of HBV viral particles in the HepG2.2.15 cells. (Kim et al., 2003)**.**

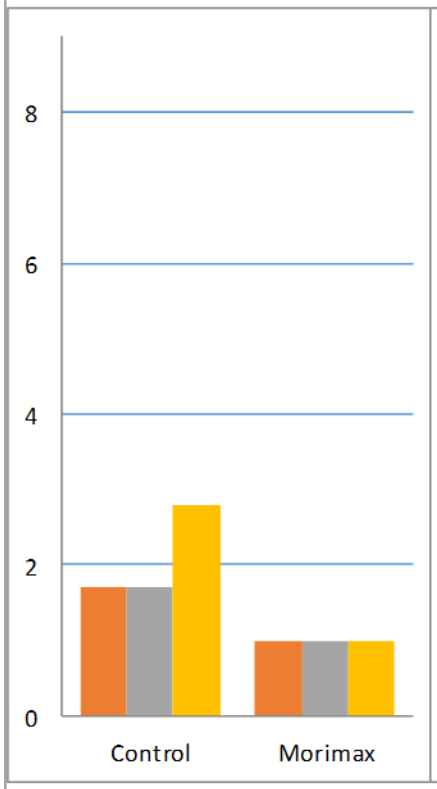
Inhibition of BVD Virus by DNJ



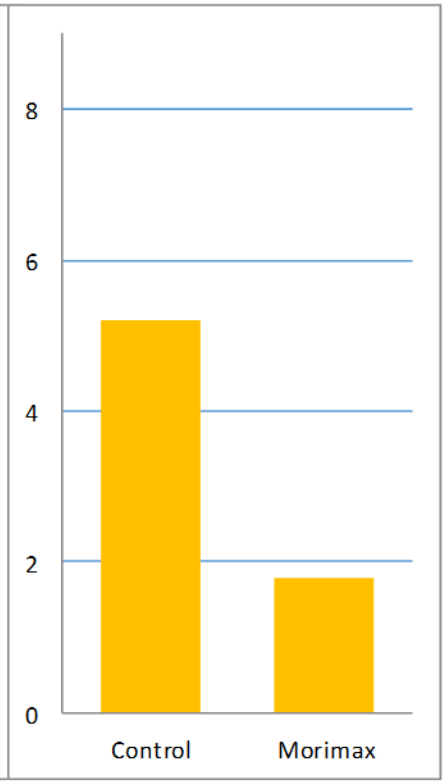
Antiviral test by DNJ



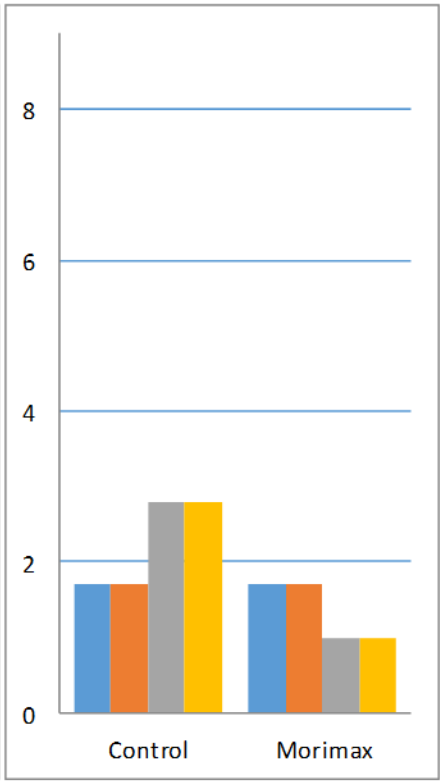
PRRSV in serum



PCV2 in serum

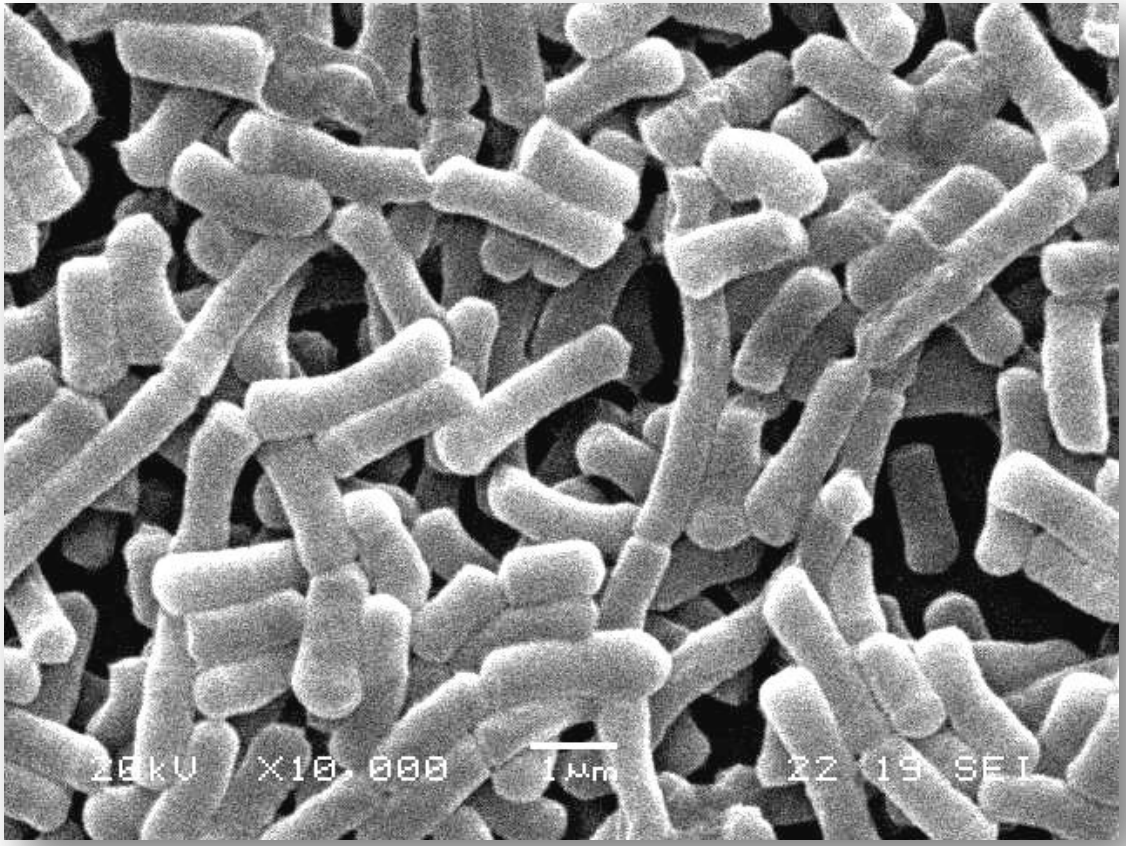


PCV2 in lymph node

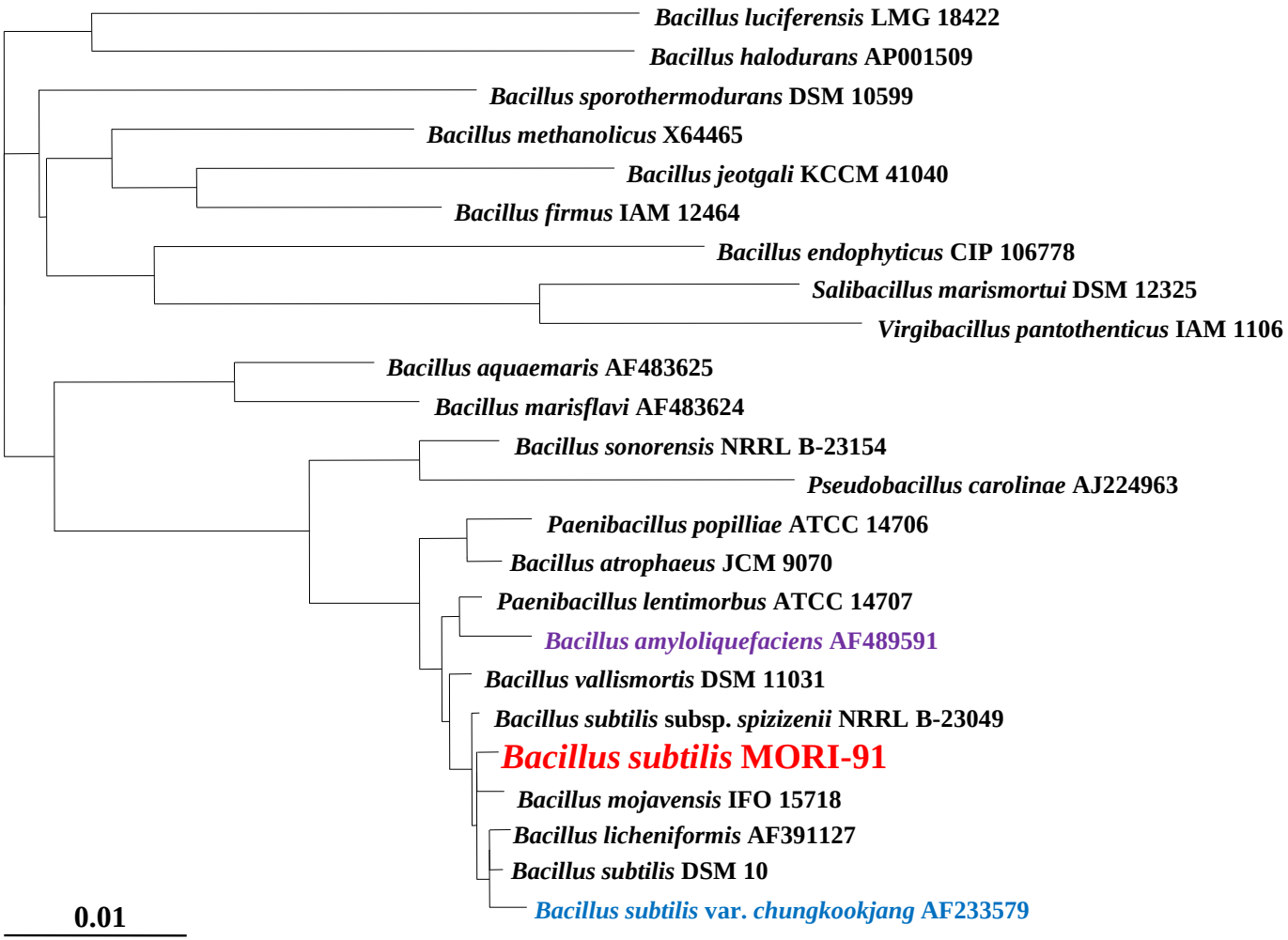


PEDV in feces

SEM Picture of DNJ Producing *B. subtilis* MORI-91



Dendrogram of *B. subtilis* MORI-91 Established on the Basis of 16S rRNA



HPLC Analysis of *B. subtilis* MORI-91

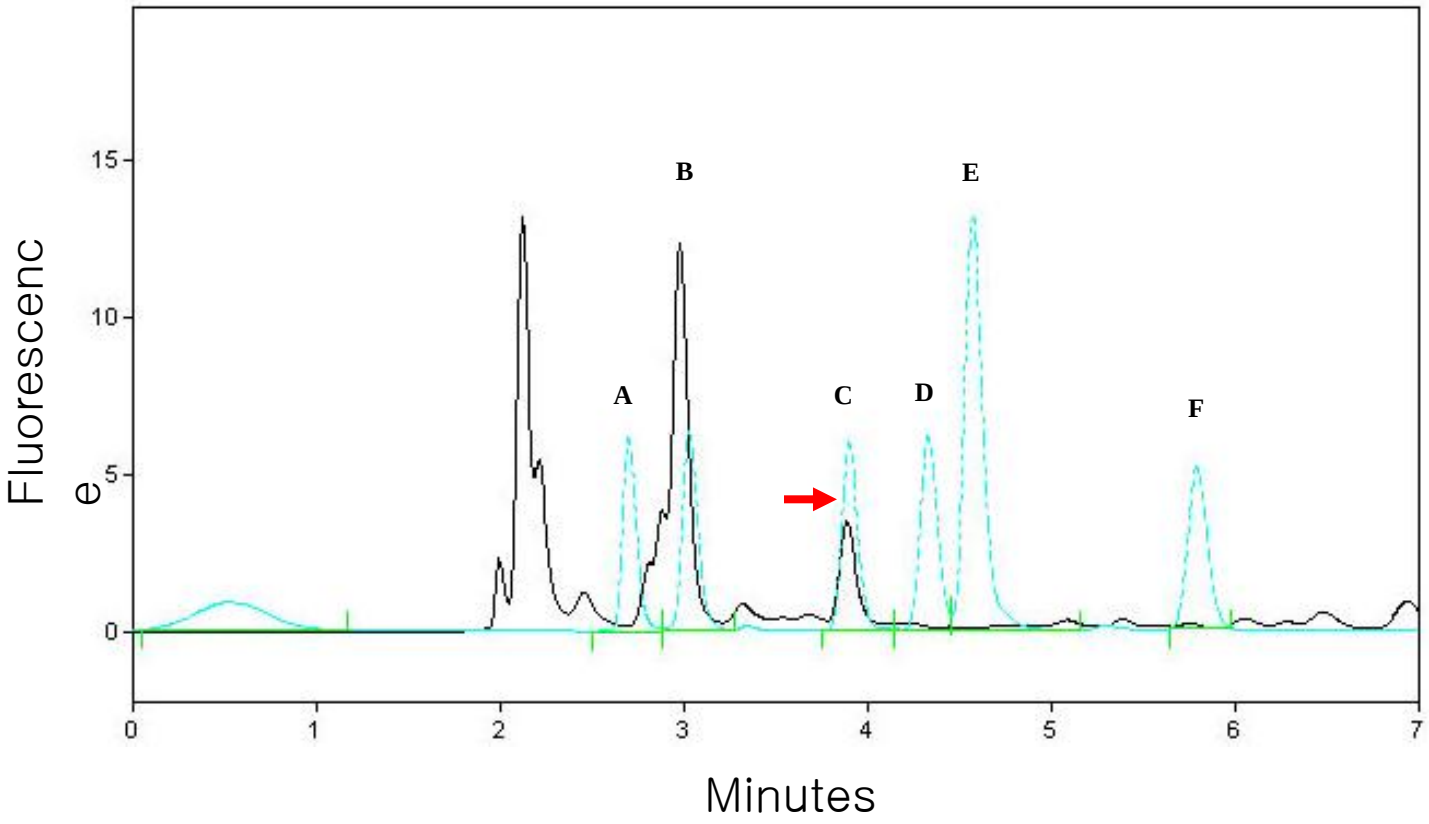
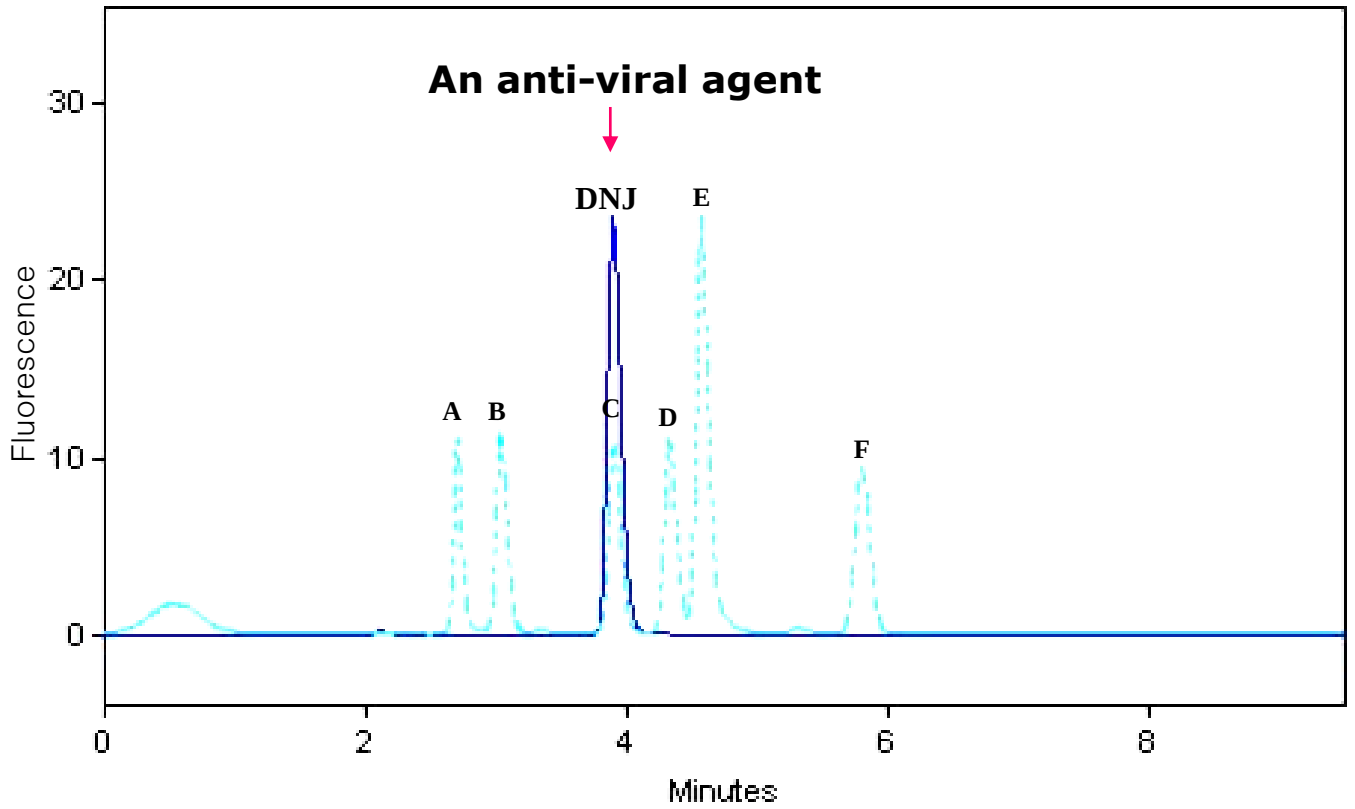


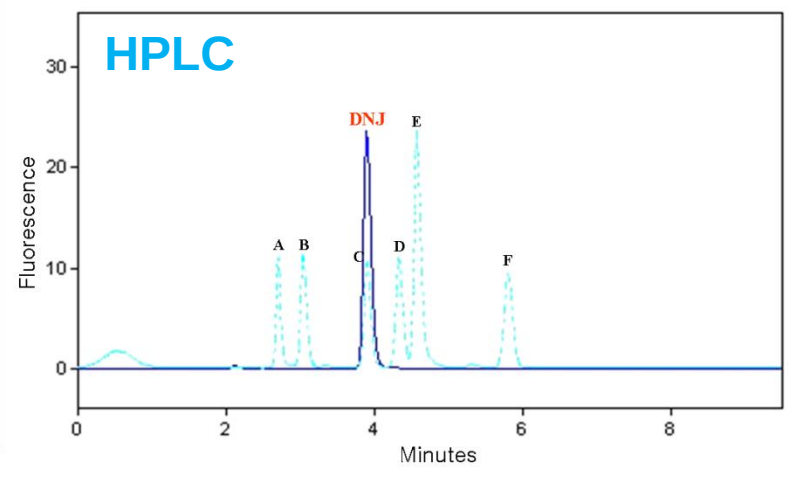
Figure. HPLC analysis of MORI-91 cultured broth. Several alkaloid compounds are shown on the same chromatogram in order to compare with cultured broth. A; Gal-DNJ, B; Glc-DAB, C; DNJ, D; 3-epi-fagomine, E; fagomine/ DAB, F; Calystegine B_2.

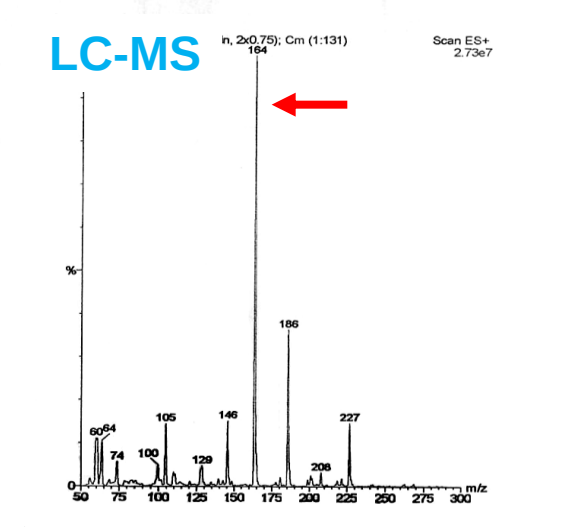
Purification and Analysis of Anti-viral Agents from *B. subtilis* MORI 91

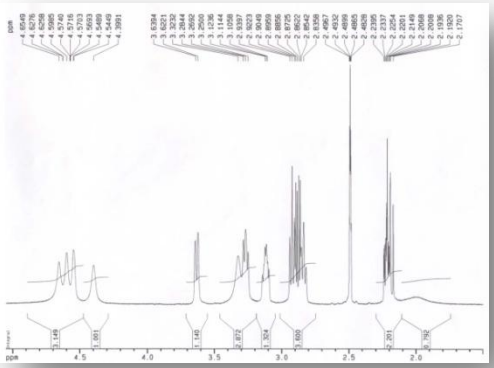


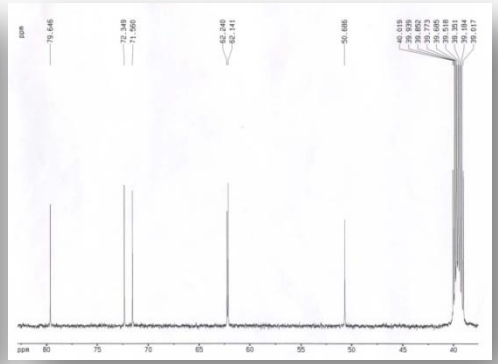
Chromatogram of HPLC of Pool A-5(DNJ) from ***Bacillus subtilis*** MORI-91.

DNJ Analysis





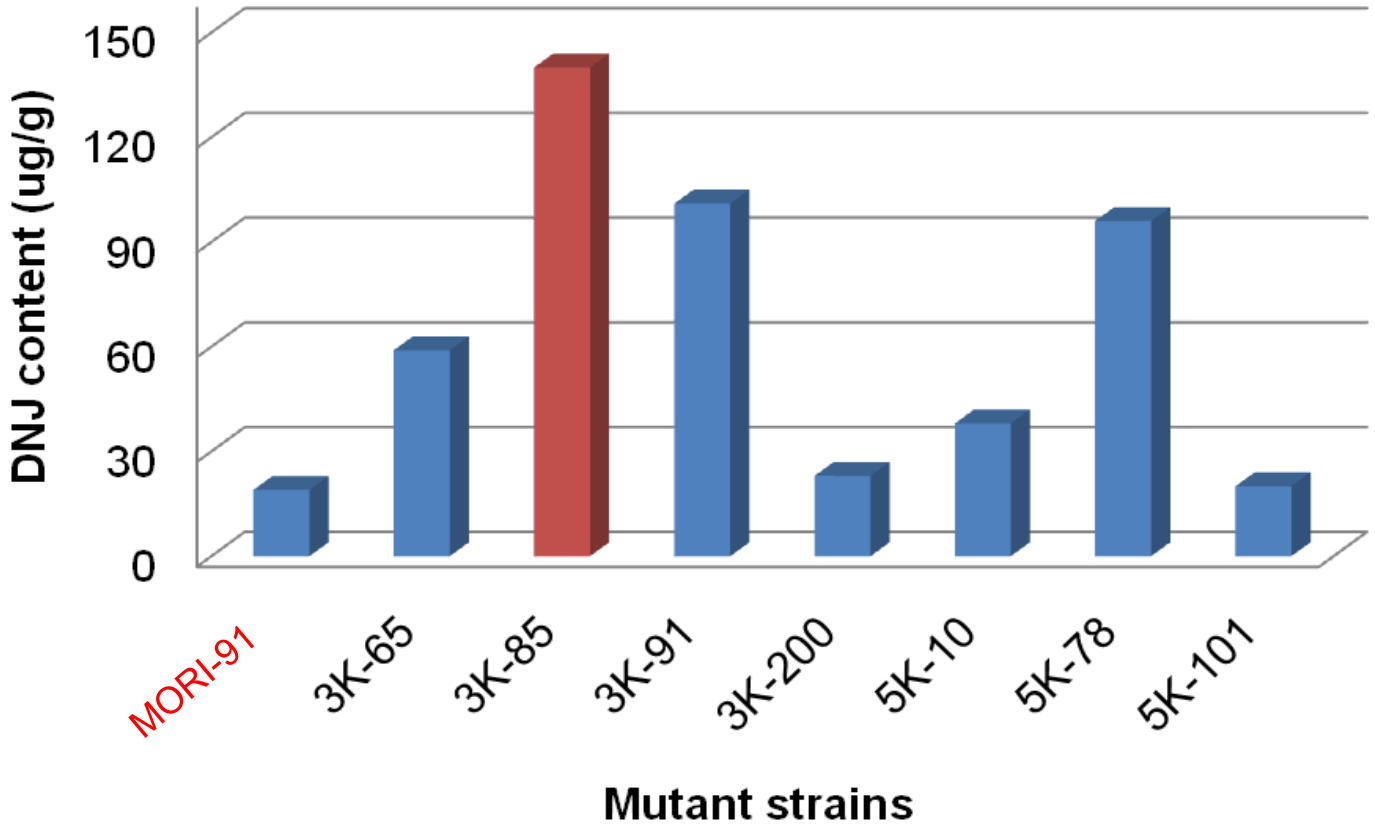




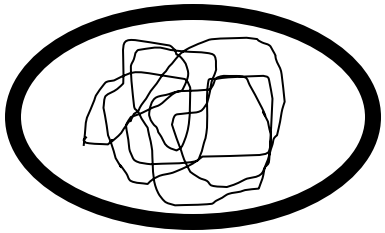
^{1}H- NMR

^{13}C- NMR

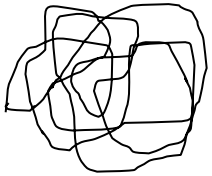
Comparison of DNJ Production Among Various Mutant Strains



Procedure for Investigation of the Genes for DNJ biosynthesis



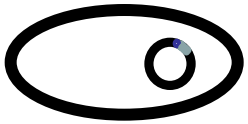
Extraction of gDNA



Digestion of gDNA with *Bam*HI

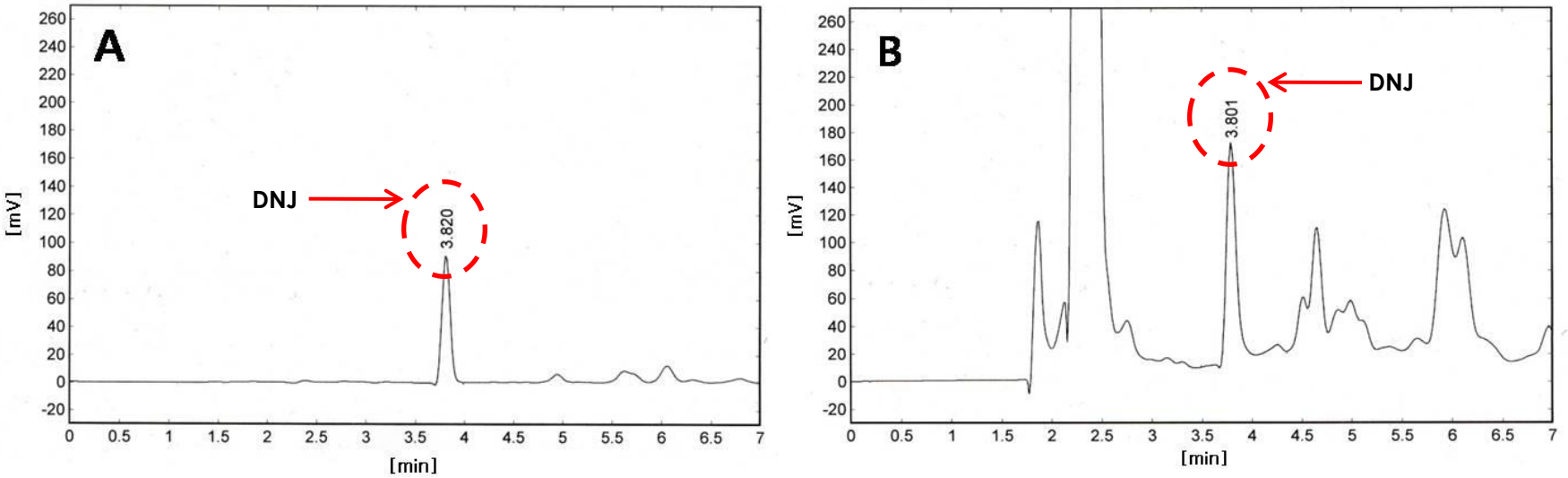
Ligation

Transfection



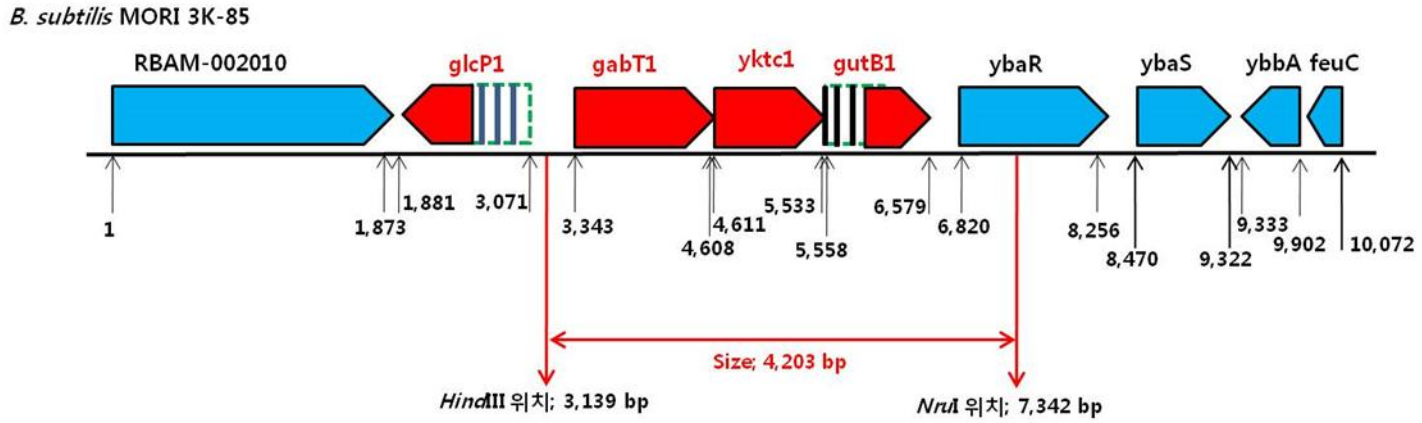
α-glucosidase inhibition assay

HPLC Chromatogram of Standard DNJ and the Culture Medium of Clone 36-4



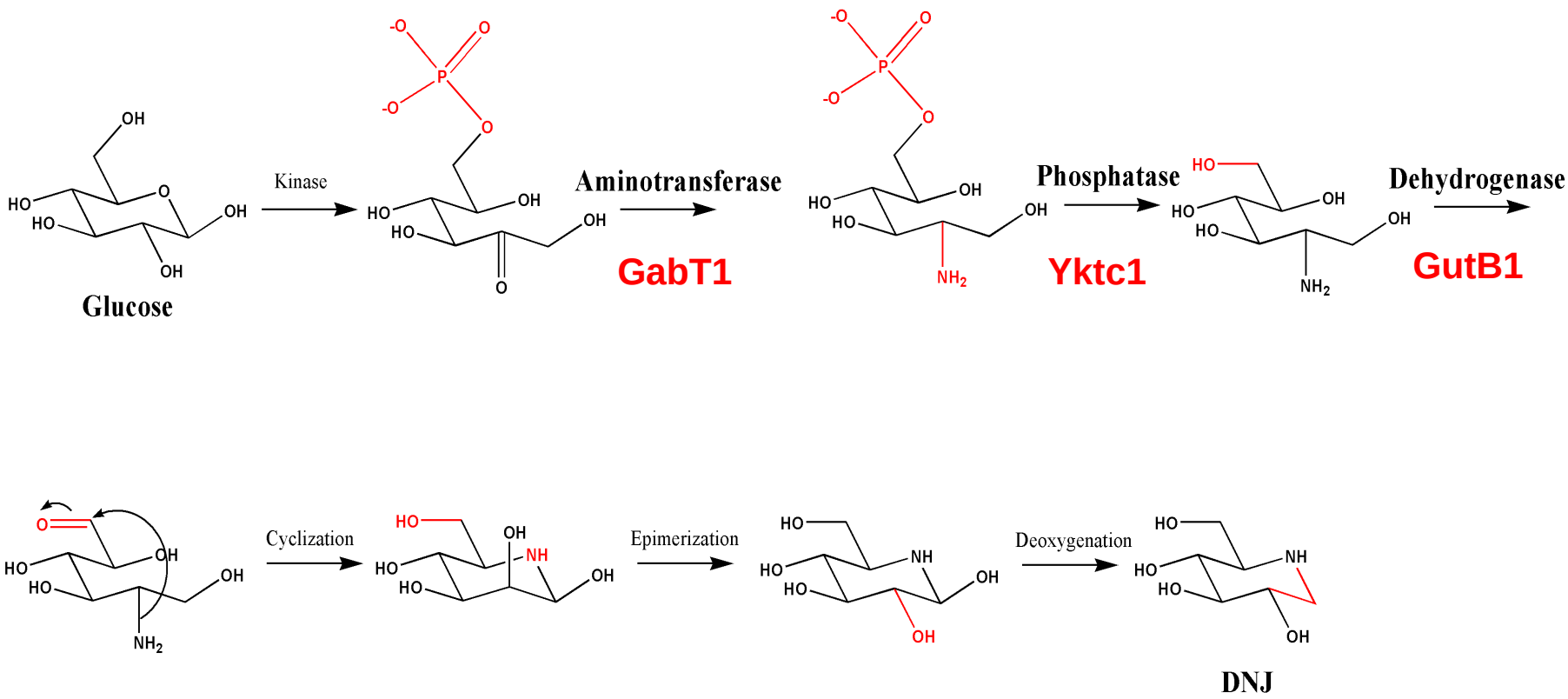
A; standard DNJ, B; the culture medium of selected clone 36-4

Sequence Analysis of the Inserted DNA



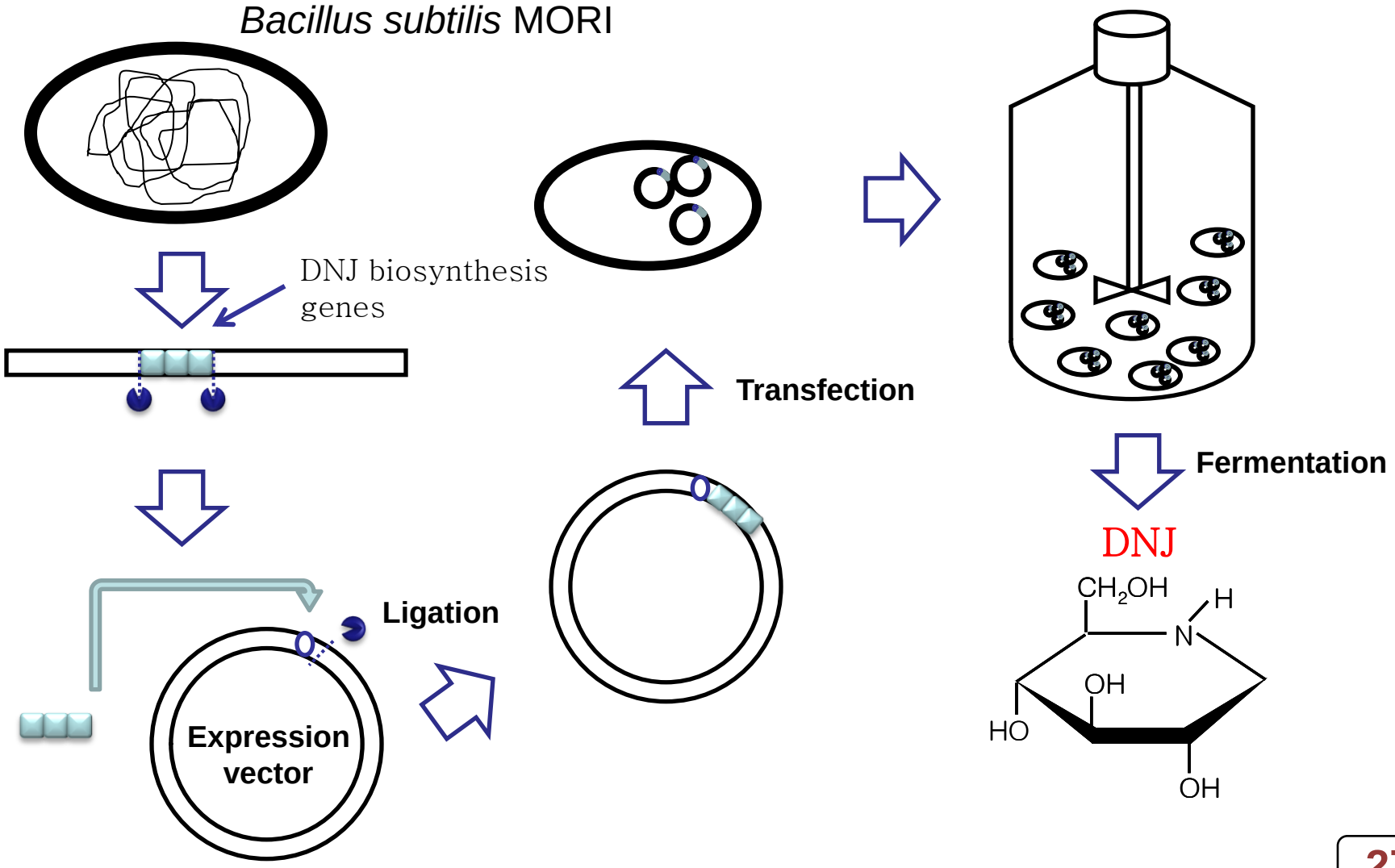
ORF	Size (aa)	Protein name	Molecular function	Biological process
glcP1	404	GlcP1	Transmembrane transport	Unknown
gabT1	425	GabT1	4-Aminobutyrate transaminase activity and pyridoxal phosphate binding	Unknown
yktc1	316	Yktc1	Inositol or phosphatidylinositol phosphatase activity	Unknown
gutB1	348	GutB1	Oxidoreductase activity and zinc ion binding	Oxidation reduction

Biosynthetic Pathway of 1-Deoxynojirimycin



Biosynthetic scheme of DNJ in *B. subtilis* MORI 3K-85

Concept for Mass Production of DNJ



The World's First Paper about DNJ Biosynthesis Genes

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Identification of the Genes Involved in 1-Deoxynojirimycin Synthesis in *Bacillus subtilis* MORI 3K-85

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1-Deoxynojirimycin (DNJ), a D-glucose analogue with a nitrogen atom substituting for the ring oxygen, is a strong inhibitor of intestinal α-glucosidase. DNJ has several promising biological activities, including its antidiabetic, antitumor, and antiviral activities. Nevertheless, only limited amounts of DNJ are available because it can only be extracted from some higher plants, including the mulberry tree, or purified from the culture broth of several types of soil bacteria, such as *Streptomyces* sp. and *Bacillus* sp. In our previous study, a DNJ-producing bacterium, *Bacillus subtilis* MORI, was isolated from the traditional Korean fermented food Chungkookjang. In the present study, we report the identification of the DNJ biosynthetic genes in *B. subtilis* MORI 3K-85 strain, a DNJ-overproducing derivate of the *B. subtilis* MORI strain generated by γ-irradiation. The genomic DNA library of *B. subtilis* MORI 3K-85 was constructed in *Escherichia coli*, and clones showing α-glucosidase inhibition activity were selected. After DNA sequencing and a series of subcloning, we were able to identify a putative operon which consists of *gabT1*, *yktc1*, and *gutB1* genes predicted to encode putative transaminase, phosphatase, and oxidoreductase, respectively. When a recombinant plasmid containing this operon sequence was transformed into an *E. coli* strain, the resulting transformant was able to produce DNJ into the culture medium. Our results indicate that the *gabT1*, *yktc1*, and *gutB1* genes are involved in the DNJ biosynthetic pathway in *B. subtilis* MORI, suggesting the possibility of employing these genes to establish a large-scale microbial DNJ overproduction system through genetic engineering and process optimization.

Keywords: *Bacillus subtilis* MORI 3K-85, genomic DNA library screening, 1-deoxynojirimycin (DNJ), α-glucosidase inhibitor, gene cloning

1-Deoxynojirimycin (DNJ) is a polyhydroxylated piperidine alkaloid. These alkaloids can be considered as analogues of glucose in which the ring oxygen has been replaced by nitrogen. DNJ inhibits α-glucosidase, which hydrolyzes α-glucose residues within an oligosaccharide chain. α-Glucosidases are involved in a wide range of important biological processes. Therefore, the possibility of modifying or blocking these processes using DNJ as a glucosidase inhibitor has gained an increasing amount of interest related to cell biological and therapeutic applications, especially in relation to viral infections and diabetes (Asano *et al.*, 2000; Watson *et al.*, 2001).

DNJ has been shown to inhibit α-glucosidases I and II, which are involved in the *N*-linked glycosylation of secretory proteins (Asano *et al.*, 2000; Dwek *et al.*, 2002). *N*-linked oligosaccharides play important roles in the fate and functions of glycoproteins (Asano *et al.*, 2000; Dwek *et al.*, 2002). For example, *N*-glycosylation can assist in the folding of glycoproteins. Thus, prevention of the *N*-glycosylation process by an α-glucosidase inhibitor will cause some proteins to be misfolded and retained within the endoplasmic reticulum (ER). Because proper folding of key viral envelope glycoproteins are critical for the life cycle of viruses, such as the human immunodeficiency virus (HIV), hepatitis B virus (HBV), and bovine viral diarrhea virus (BVDV), DNJ has been regarded as a promising antiviral agent (Gruters *et al.*, 1987; Fleet *et al.*, 1988; Karpas *et al.*, 1988; Mehta *et al.*, 1998; Asano *et al.*, 2000; Watson *et al.*, 2001; Dwek *et al.*, 2002; Jacob *et al.*, 2007).

DNJ is also a potent inhibitor of various mammalian digestive α-glucosidases, such as sucrase, maltase and isomaltase, all of which are involved in the digestion of disaccharides in mammals. These enzymes are expressed on the surface of the epithelial cells of the brush border in the small intestine. Thus, α-glucosidase inhibitors such as DNJ can be used therapeutically in the oral treatment of the non-insulin-dependent (type II) diabetes mellitus (Yoshikuni *et al.*, 1988; Asano *et al.*, 1994, 2000; Watson *et al.*, 2001; Jang and Rhee, 2004; Cho *et al.*, 2008; Hwang *et al.*, 2008; Kong *et al.*, 2008; Schedel, 2008). In addition, it has been suggested that DNJ can be developed as a more efficient antidiabetic by chemical deriva-

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Identification of a Gene Cluster that Initiates Azasugar Biosynthesis in *Bacillus amyloliquefaciens*

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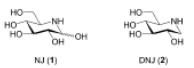
Azasugars feature a ring nitrogen atom instead of an oxygen atom, and are also known as iminosugars, iminocyclitols, or more formally as polyhydroxy derivatives of piperidines or pyrrolidines.[1] The first azasugar to be characterized was nojirimycin (NJ, 1) isolated in 1966 from *Streptomyces* cultures.[2,3] Its derivative, 1-deoxynojirimycin (DNJ, 2) was initially obtained



through chemical synthesis and later isolated from diverse natural sources.[4,5] These include some *Bacilli* species, in addition to various plants, such as *Albus* (mulberry).[6] Azasugars and their derivatives are potent inhibitors of glycosidases, N-glycohydrolases, phosphorylases, and glycosyltransferases.[7] This activity is typically attributed to the basic ring nitrogen atom, which when protonated may serve as a charge-mimic of glycosyl oxocarbenium ions, or related transition states. Azasugars and their analogues are of interest for a number of applications,[8] including the treatment of type 2 diabetes (miglitol) and lysosomal storage diseases (miglustat).[9] Though the naturally occurring parent microbial azasugars are available through fermentation, synthetic routes to their derivatives typically have the overheads of protection and deprotection. Various synthetic strategies have been and continue to be reported for this class of natural product.[5,10] Despite this work, the biosynthetic pathway of azasugars remained unknown with the exception of some insightful results obtained from feeding experiments with labeled precursors, performed nearly 20 years ago.

In the early 1990s, Hardick and co-workers fed stable-isotope-labeled glucose to *Bacillus atrophaeus* and *Streptomyces subrutilus* to establish glucose as a precursor of DNJ.[11] This is in contrast to the biosynthesis of castanospermine and swainsonine, inhibitory polyhydroxy alkaloids with an amino acid biosynthetic origin.[12] Furthermore, it was shown that the carbon skeleton of glucose undergoes inversion, as evidenced by [1-^{13}C]glucose having produced DNJ labeled at C6 (Scheme 1). This implied that DNJ was formed via a C2-*N*-C6 cyclization reaction, operating in the following way. Glucose could readily be drawn out of glycolysis as a fructosyl species, which upon transamination would yield a 2-aminomannitol (3). Oxidation of the C6 hydroxyl group would yield a 6-oxo species that would reasonably be expected to rapidly cyclize to mannojirimycin (4). Epimerization at the new C-2 (former C-5 of glucose) would produce nojirimycin, yielding 1-deoxynojirimycin after loss of 1-OH and reduction. This proposal, consistent with the results of the labeling studies is presented in Scheme 1. We sought evidence for this proposed pathway, both in terms of the chemical intermediates and the identity of the genes coding for the biosynthetic enzymes. Only recently has this become practical, with the reports of sequenced genomes for the azasugar producing *Bacillus amyloliquefaciens* and *B. atrophaeus*.[13,14]

In this communication we identify three enzymes implicated in the first steps of biosynthesis of DNJ in *Bacillus amyloliquefaciens*. We searched for candidate genes by focusing on those coding for enzymes that could catalyze all or some of the reactions required to convert a fructosyl species to DNJ. While a priori there was no reason to require it, we sought genes which were clustered, and also passed the following criteria. We centered on aminotransferases and redox enzymes and secondarily, gave higher priority when one or more of the gene products exhibited specificity for carbohydrates or carbohydrate-like molecules. A cluster of three genes was identified, designated[15] *gabT1*, *yktc1*, and *gutB1*, coding for putative aminotransferase, phosphatase, and zinc-dependent dehydrogenase enzymes. The *gabT1* gene is a member of the acetyl ornithine aminotransferase family[15] and a translated nucleotide BLAST of *gabT1* against the nr protein database revealed it shares 50% identity with ValM, the putative aminotransferase involved in validamycin biosynthesis in *Streptomyces hygroscopicus*.[16] The *yktc1* gene is a member of the FIG superfamily[17] which are metal-dependant phosphatases whose substrates include fructose and inositol phosphates. The final gene in the cluster, *gutB1*, is a member of the medium-chain reductase/dehydrogenase family,[18] which includes iditol and sorbitol dehydrogenases. Taking the putative functions of these genes into consideration along with the results from the labeling studies, we hypothesize that the three enzymes are involved in DNJ biosynthesis as shown in Scheme 1. The GabT1 enzyme could add the amino group to C2 of fructose-6-phosphate, after which Yktc1 removes the phosphate group, yielding 2-aminomannitol (3). GutB1 would then oxidize the unmasked hydroxyl group on C6 leading to formation of mannojirimycin (4). It is noteworthy to point out that the same gene cluster is also present in *B. atrophaeus* and *B. pseudomycoides*,[14,19] the former species is a known DNJ producer, and we consider the latter to be a candidate azasugar producer on the basis of the presence of the cluster.

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Inhibited Avian Viruses by 1-Deoxynojirimycin

Virus Family	Types of Virus
Coronaviridae	Avian Infectious Bronchitis Virus(IBV)
Herpesviridae	Avian Infectious Laryngotracheitis Virus(ILTV) Marek's Disease Virus(MDV)
Orthomyxoviridae	Avian Influenza A Virus(AIV)
Paramyxoviridae	Newcastle Disease Virus(NDV) Avain Paramyxoviruses(PMV) Avian metapneumovirus, AMPV)
Poxviridae (Avipoxviruses)	Fowl Pox Virus(FPV)
Retroviridae	Avian Leukosis-Sarcoma Virus(LSV)

Commercialized Supplement(MORI-MAX)

Synbiotic's Concept Supplement

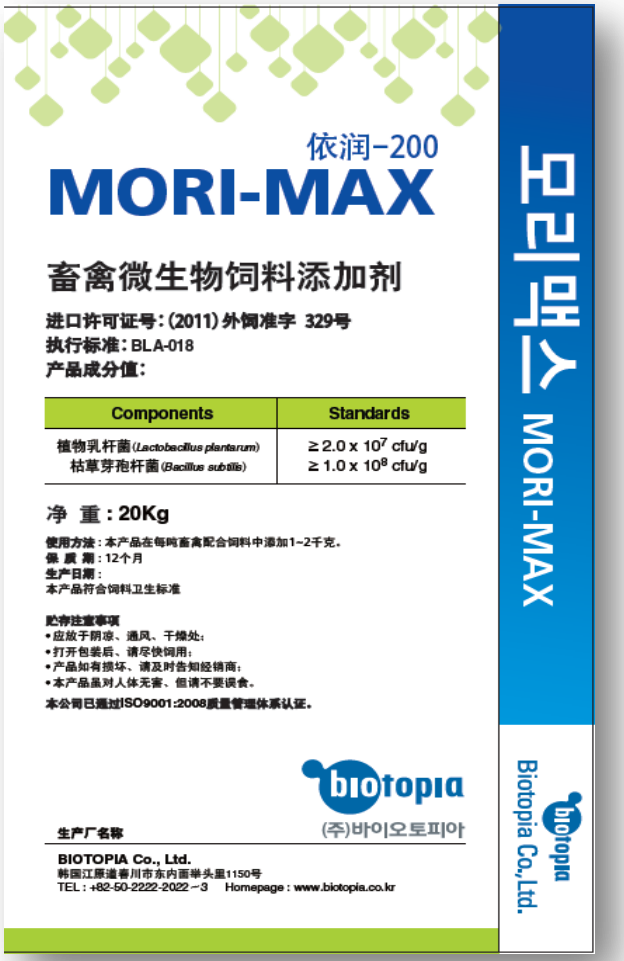
Anti-viral Activities by DNJ

Anti-bacterial Activities by PLA

Non-specific Immune Enhancing Activities

Probiotics Activities

Improve to Productivity



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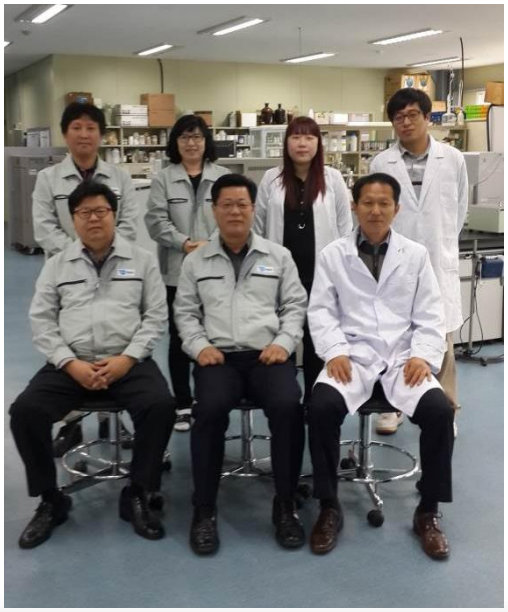

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