Biochemical and Molecular Biological Analyses of the Effects of Host Factors on the Replication of Baculoviruses

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Contents

Introduction

A virus is an obligate parasite that requires the cellular machinery of a host cell to produce progeny (Lobo *et al.*, 2009; Wimmer *et al.*, 2009). Because of this dependence on the host cellular machinery, the interactions between the virus and host are continuously coevolving (Lobo *et al.*, 2009). The physiological condition of the host is an important factor for viral replication. The nutrient composition of food plays an important role in the growth rate of insects and affects the lethal time of virus-infected insects (Hoover *et al.*, 1997).

Baculoviruses are classified in the family Baculoviridae. About 600 insect species, primarily found in Lepidoptera, are hosts of baculoviruses (Herniou *et al.*, 2004). Based in part on the morphology of the baculovirus occlusion body, baculoviruses are divided into two major groups, nucleopolyhedrovirus (NPV) and granulovirus (GV). The GV virion contains only one nucleocapsid per envelope, the NPV virion on the other hand contains single or multiple nucleocapsids per envelope. After baculovirus genomic DNA sequence information has become more widely available, the family Baculoviridae has recently been divided into four genera: alphabaculovirus (lepidopteran NPVs), betabaculovirus (lepidopteran GVs), gammabaculovirus (hymenopteran NPVs), and deltabaculovirus (dipteran NPVs) (Rohmann, 2011). The genome of NPVs is double-stranded, circular, supercoiled DNA of 80-180 kbp. Baculovirus nucleocapsids are rod-shaped and composed of 11-25 kinds of structural proteins. The baculovirus virion is 20-50 nm in diameter and 200-500 nm in length. Baculoviruses have a unique biphasic replication cycle that involves the formation of two types of progeny virions: budded virus (BV) and occlusion-derived virus (ODV). BV is produced during an early stage of the

replication cycle and obtains its envelope as it buds through the cell plasma membrane. ODV on the other hand is formed during a late stage of the replication cycle and obtains its envelope "de novo" while in the cell nucleus. The ODV is subsequently occluded in a protein matrix resulting in the formation of a granule or a polyhedron. These occlusion bodies play important roles for protection and maintaining the activity of virions embedded within them (Smith *et al.*, 1983; Vlak and Rohrmann, 1985). ODVs are primarily involved in insect-to-insect transmission, whereas BVs are involved in cell-to-cell transmission within a single host. Each polyhedron is typically 0.5-1.5 μm in diameter and can be visualized by light microscopy (Summers and Arnott, 1969; Kelly, 1985). The polyhedrin gene (*polh*) is expressed by a very late and very strong promoter that produces a ca. 30 kDa protein that can constitute 30% of the mass of a cell during a late stage of infection. During the budding process, the BV envelope acquires the virus-encoded glycoprotein, gp64, which is a homotrimeric membrane glycoprotein and a low pH activated envelope fusion protein (Roberts and Faulkner, 1989; Volkman, 1986). Gp64 is produced during both the early and late phases of the infection cycle and essential for budding of the virion out of the cells as well as binding to cell surface.

Wild type NPVs and GVs are used as effective and selective biopesticides to protect crops and forest from harmful insects (Huber, 1986). Despite interest and attractiveness, the use of baculoviruses for pest insect control has been limited, because the wildtype viruses have slow speed of kill and limited host range in comparison to most synthetic chemical insecticides. The inherent insecticidal activity of the baculovirus can be increased by genetic engineering (Inecoglu *et al.*, 2006; Kamita *et al.*, 2005). In addition, NPVs are used as protein expression vectors (Summers, 2006).

Baculovirus-expressed proteins are biologically active and show correct post-translational modifications (Maeda, 1994).

Insects are the most diverse group of animals in the world and represent more than half of all living organisms. Most insects are phytophagous and others are carnivorous, omnivorous, or parasitic. The structure and physiology of the digestive system of insects are highly varied because insect feeding habits are diverse (Gillott, 1980). In insects, metamorphosis is the biological process of development, that is divided into two types: incomplete metamorphosis and complete metamorphosis (Gullan and Cranston, 2005). Insects that undergo complete metamorphosis display four distinct life stages: egg, larva, pupa, and adult. In contrast insects that undergo incomplete metamorphosis lack a pupal stage, and they develop through a series of nymphal stages. In general, ecdysone is a molting hormone, which is synthesized and secreted from the prothoracic gland by stimulation of prothoracicotropic hormone (PTTH) produced from the insect brain. Juvenile hormone (JH) is another key insect hormone that is secreted from the *corpora allata.* When JH is present in the hemolymph at low nanomolar levels the *status quo* is maintained so that the juvenile stage is maintained, i.e. a larva to larva or nymph to nymph molt occurs. In contrast, when JH titer is dramatically reduced to undetectable levels, and when there are two concurrent spikes of ecdysteroid the insect undergoes a developmentally more advanced molt, i.e., larva to pupa or nymph to adult (Gilbert, 1973). Insects can damage a crop plant by feeding on sap, leaves, or fruits. Insects also transmit disease to humans and livestock by biting and feeding on blood. In contrast beneficial insects provide ecological benefits or direct economic benefits. *Bombyx mori*, the silkworm, is a beneficial and domesticated insect which is classified in Hexapoda, Lepidoptera,

Bombycidae. The silkworm is a specialist that feeds exclusively on mulberry leaves (Konno *et al.*, 2006). Sericulture is the process of breeding silkworms for producing raw silk. The origins of sericulture are found more than 5,000 years ago in China and spread to Korea and Japan and later to India and the West (Barber *et al.*, 1992). In addition, silkworms are very useful as an experimental animal because genetically identical (or nearly genetically identical) strains are available and easily prepared as developmentally staged individuals (Banno *et al.*, 2010).

Lepidopteran larvae that are infected with NPV generally do not molt. The reason for this is that the NPV encodes a specific gene, *ecdysteroid UDP-glucosyl transferase* (*egt*) whose product can inactivate ecdysteroid in insects (O'Reilly, 1995). The *egt* gene encodes ecdysteroid UDP-glucosyltransferase (EGT) an enzyme that catalyzes the transfer of UDP-glucose to carbon-22 of ecdysteroids resulting in the loss of activity (O'Reilly and Miller, 1989; Burand and Park, 1992). The result is that an increase in the production of progeny viruses because molting and pupation is inhibited in the virus-infected insect (O'Reilly, 1995). Recently, Hoover *et al.* (2011) found that *egt* gene is responsible for controlling climbing behavior of the virus-infected host. Thus, the *egt* gene appears to be an evolutionarily advantageous gene.

O'Reilly and Miller (1991) have made a mutant *Autographa californica* multiple NPV (AcMNPV) which lacks its *egt* gene. Larvae infected with the mutant AcMNPV shows a decrease in food consumption and die faster in comparison with larvae infected with wild type virus. In addition, larval *B. mori* infected with a mutant *Bombyx mori* NPV (BmNPV) in which its *egt* gene is inactivated die 12 hours faster than those infected with wild type BmNPV (Kang *et al.*, 1998). Inactivation of the *egt* gene is

now a common method to reduce the time that a virus takes to kill its host (Georgievska *et al.*, 2010; Harison *et al.*, 2008; Inecoglu *et al.*, 2006).

Glycosidases such as sucrase, maltase, and trehalase break down complex carbohydrates that are found in insects, microorganisms, plants, and mammals (Nishimoto *et al.*, 2001). α-Glucosidase (EC 3.2.1.20) is a glycosidase that catalyzes the hydrolysis of carbohydrates having a 1,4-α-glucosidic linkage. In insects, α-glucosidases are generally found in the alimentary canal, salivary secretions and hemolymph. In insects most α-glucosidases such as maltase and sucrase are involved in the digestion process. Trehalase is responsible for the hydrolysis of trehalose, a major sugar in the hemolymph of insects and an important energy source for insect tissues (Sato *et al.*, 1997). α-Glucosidases have been purified and biochemically characterized, and their DNA sequences identified, from honeybee, sandfly, and whitefly (Gontijo *et al.*, 1998; Ohashi *et al.*, 1996; Salvucci, 2000).

1-Deoxynojirimycin (DNJ) is a polyhydroxylated alkaloid in which the ring oxygen is replaced by nitrogen (Asano *et al.*, 2001). DNJ has a function to inhibit α-glucosidases. DNJ is found at high levels in plants such as *Morus alba* L., mulberry tree, and *Omphalea diandra* (Asano *et al*, 2001; Kim *et al.*, 2003; Kite *et al.*, 1997) and in silkworm (Asano *et al.*, 2001). DNJ is produced by microorganism such as *Bacillus* and *Streptomyces* (Ezure *et al.*, 1985; Hardick and Hutchinson, 1993; Stein *et al.*, 1984; Watson and Nash, 2000). DNJ has been studied in terms of its ability to inhibit the increase in the blood sugar levels that occur after a meal. Because DNJ has only moderate α-glucosidases inhibitory activity, DNJ has been modified to various derivatives such as miglitol and sold as a drug for controlling diabetes (Asano *et al.*,

2001). DNJ is also known to inhibit virus replication by the inhibition of α-glucosidase I and II, two enzymes involved in the biosynthesis of glycoproteins found in viral envelope proteins. Thus, DNJ and its derivatives have been studied as antiviral agents for the control of bovine viral diarrhea virus, dengue virus, Japanese encephalitis virus, hepatitis B virus, human immunodeficiency virus, and human parainfluenza virus (Chang *et al.*, 2009; Jacob *et al*, 2007; Metha *et al.*, 2002; Tanaka *et al.*, 2006; Whitby *et al.*, 2005 Wu *et al.*, 2002).

Studies about the mass-production of DNJ in *Bacillus* and *Streptomyces* are actively reported. However, the DNJ biosynthetic pathway is still not completely known. A few research teams have studied the DNJ biosynthetic pathway by isotope labeling analysis. In *Bacilli* and *Streptomyces*, it has been proposed that DNJ is synthesized from a glucose molecule by C2/C6 cyclization (Hardick *et al.*, 1991, 1992; Hardick and Hutchinson, 1993). In the higher plant *Commelina communis*, it has been suggested that DNJ is biosynthesized through a different route involving the C1/C5 cyclization of the original glucose molecule in comparison with that found in *Bacilli* and *Streptomyces* (Shibano *et al.*, 2004). Currently, information regarding the genetics and biosynthetic enzymes that are involved at each step of the biosynthesis process of DNJ are unavailable.

In this thesis, I hypothesized that the hormonal and nutritional conditions of the host would affect the replication of baculoviruses. Thus, I investigated the effects of ecdysteroid application and starvation of the host on the replication of the baculovirus. In addition, I investigated how BmNPV is able to efficiently generate progeny in silkworms even in the presence of high DNJ concentrations. I performed comparative analyses of the effect of DNJ on the replication of BmNPV and AcMNPV, a virus that

is pathogenic to *Spodoptera frugiperda* a generalist in terms of the food plants that it will eat. I also investigated whether the high levels of DNJ that are found in silkworms results from high levels of biosynthesis or originates in the diet of the silkworm.

Chapter 1. Effect of starvation upon baculovirus replication in larval *Bombyx mori* and *Heliothis virescens*.

The availability of food in insects plays a critical role in larval growth and subsequent metamorphosis. When food resources are depleted during the juvenile stage, some insects possess evolutionary mechanisms to retard development, whereas others show accelerated metamorphosis (see Cymborowski *et al.* 1982; Shafiei *et al.* 2001; Munyiri and Ishikawa, 2005). In *B. mori*, DNA synthesis activity in prothoracic glands is nutrient-dependent during the last larval instar (Chen and Gu, 2006). A shortage of food during this period results in an increase in ecdysteroid production, which is hypothesized to help enhance survival. Hoover *et al.* (1997) have also shown that the nutrient composition of the diet not only affects the relative growth rate of the insect but also affects the median lethal time (LT_{50}) of a baculovirus that infects the insect.

In this chapter, larvae of the silkworm *Bombyx mori* (5^{th} instar) and tobacco budworm *Heliothis virescens* (1^{st} or 3^{rd} instars) were inoculated (orally with polyhedra or by injection of BV) with wild type or recombinant baculovirus. Following inoculation the larvae were starved for 12 or 24 h before being placed on diet. Alternatively, the inoculated larvae were placed on diet, starved for a 24 h-long period at 1 or 2 days post inoculation (p.i.), and then placed back on fresh diet. The effect of host starvation on baculovirus pathogenicity, larval mortality, and larval mass were quantified.

Materials and methods

Insects and viruses

Eggs of the silkworm *B. mori* and tobacco budworm *H. virescens* were obtained from the National Institute of Agricultural Science and Technology (Republic of Korea) and United States Department of Agriculture, Agricultural Research Service (Stoneville, MS), respectively. Larvae of *B. mori* and *H. virescens* were reared on artificial diet at 26°C as described previously (Choudary *et al.* 1995; Hoover *et al.* 1997). Wild type *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and AcAaIT, a recombinant AcMNPV carrying the insect-selective scorpion toxin gene AaIT (Choudary *et al.* 1995; Hoover *et al.* 1997), were propagated on Sf9 cells. Wild type *B. mori* NPV (BmNPV) (Maeda *et al.* 1985) and BmCysPD, a recombinant BmNPV in which the endogenous cysteine protease gene was replaced with a *lac*Z gene cassette (Ohkawa *et al.* 1994), were propagated on Bm5 cells. The Sf9 and BmN cells were maintained on TC-100 supplemented with 5% and 10% fetal bovine serum, respectively, as described previously (OReilly *et al.* 1992; Choudary *et al.* 1995). Viral titers were determined by end-point dilution on Bm5 or Sf9 cells as appropriate (OReilly *et al.* 1992).

Bioassay, starvation regimes, and hemolymph collection

Neonates of *H. virescens* were inoculated with AcMNPV by a droplet feeding method (Hughes *et al.* 1986). Each droplet (1 µl) contained 2000 PIBs and 5% (v:v) blue food coloring in distilled H_2O. Only larvae that completely ingested the droplet during a 1 h-long exposure were used in the bioassays. Following inoculation, the neonates were starved for 0, 12 or 24 h, and then transferred to fresh diet. Each treatment cohort consisted of 30 larvae. Mortality was checked at 4 to 6 h intervals until all larvae died. Third instar larvae of *H. virescens*, 30 or 48 larvae per cohort,

were inoculated by allowing the larvae to completely ingest a plug of artificial diet (during a 12 h-long exposure period) contaminated with AcMNPV (4000 PIBs) or AcAaIT (4000 PIBs). Following inoculation, the larvae were starved for 0 or 24 h, and then transferred onto fresh diet. Mortality was checked at 4 to 6 h intervals. The viral doses (2000 PIBs per neonate and 4000 PIBs per 3rd instar) were sufficient to induce 100% mortality by 6 days p.i.

Female larvae of *B. mori*, one day after the 4th larval ecdysis, were inoculated by hemocoelic injection of 20 µl of tissue-culture medium containing 2.9 x 10^4 $TCID_{50}$ of BmNPV or BmCysPD as described previously (Choudary *et al.* 1995). Following inoculation, the larvae were starved for 0, 12 or 24 h and then placed on fresh diet. Other larvae were placed on diet immediately after inoculation, starved for a 24 h-long period at 1 or 2 days p.i., and then returned to fresh diet. Larval mortality was scored at 4 to 6 h intervals. Larval mass was determined at 24 h intervals. At 3 or 4 days p.i., the hemolymph was collected into a chilled microfuge tube containing a few crystals of phenylthiourea by piercing a proleg with a sterilized pin as described previously (Choudary *et al.* 1995). The hemolymph was appropriately diluted and the titer of virus in the hemolymph was determined as described below. Median lethal time to death (LT_{50}) was determined using the POLO prohibit analysis program (LeOra).

SDS-PAGE and western blot analysis

Hemolymph proteins were separated by SDS-(12%)PAGE as described by Laemmli (Laemmli 1970). The hemolymph sample was pooled from 10 individuals (100 µl from each larva) for each treatment group. Prior to SDS-PAGE, the hemolymph sample was mixed with sample buffer (final concentration of 2% SDS, 6.25

mM Tris-HCl pH 6.7, 15% glycerol and 100 mM dithiothreitol) and boiled for 5 min. Following electrophoretic separation of the proteins, the gel was stained with Coomassie brilliant blue R-250. For western blot analysis, pooled hemolymph proteins from 10 individuals were separated by SDS-(12%)PAGE as described above and then transferred to a nitrocellulose membrane. Following protein transfer, the membrane was blocked with 3% (w:v) gelatin in Tris-buffered saline (TBS) for 1 h, and then incubated for 2 h with mouse anti-polyhedrin antibody (a gift from Prof. Yeon Ho Je, Seoul National University). The mouse anti-polyhedrin antibody was diluted 1:10,000 in TBS containing 0.02% Tween 20 (TBS-T) containing 1% (w:v) gelatin. Following incubation with the first antibody the membrane was washed with TBS-T, and then incubated for 1 h with rabbit anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad). The rabbit anti-mouse alkaline phosphatase-conjugated antibody was diluted 1:3,000 in TBS-T containing 1% (w:v) gelatin. Following incubation with the second antibody the membrane was washed with TBS-T, and then stained using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad) following the manufacturer's protocol.

Histochemical analysis of the transmission of BmCysPD in 5th instar B. mori

The expression of β-galactosidase, an indicator of BmCysPD transmission in tissues of *B. mori*, was visualized by staining with 5-bromo-4-chloro-3-indolyl-β-D-glactopyranoside (X-gal). At the appropriate time p.i., each larva was secured onto a block of Styrofoam with dissecting pins and a longitudinal incision was made through the dorsal cuticle. The dissected larva or tissue was immersed with PBS (0.5 M sodium phosphate, 0.5 M NaCl, pH 6.0) for 3 min. The PBS was then replaced with

fixative solution (4% paraformaldehyde in PBS). After 20 min, the larvae were washed twice with PBS and infused with staining solution [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in distilled H_2O] containing 2 mg/ml of X-gal and placed in total darkness for 6-12 h as described previously (Engelhard *et al.* 1994).

Determination of viral titer

The hemolymph of virus-infected larvae (following starvation for a 24 h-long period immediately following inoculation or at 1 or 2 days p.i.) was collected at 3 or 4 days p.i. into chilled microfuge tubes and centrifuged at 1000x*g* for 5 min to remove hemocytes and other large particles and stored at -80°C. The viral titers were measured by end-point dilution with serially 10-fold-diluted samples by modifications of the method described previously (OReilly *et al.* 1992).

Results and discussion

Effect of starvation on the time-mortality of virus-infected H. virescens *and* B. mori

The effect of starvation on the mortality of virus-infected hosts was investigated in neonate and 3rd instar *H. virescens* that were fed AcMNPV polyhedra and immediately starved for 12 or 24 h before placing them on a normal feeding regime. The LT_{50}s of neonate larvae that were starved for 12 and 24 h were 92.7 and 101.6 h, respectively (Table 1). In comparison to non-starved control larvae this corresponded to delays of roughly 13% (10.3 h) and 23% (19.2 h), respectively, in the LT_{50}. The LT_{50} of 3rd instar larvae that were starved for 24 h was 126.6 h (Table 1). This corresponded to a delay of roughly 12% (13.2 h) in the LT_{50} in comparison to non-

starved control larvae. Third instar larvae of *H. virescens* that were inoculated with AcAaIT, a recombinant AcMNPV carrying an insect-selective scorpion toxin gene, and starved for 24 h also showed a similar (13%) delay in LT_{50} (Table 1). Interestingly, this delay in LT_{50} that resulted from the 24 h-long starvation period was consistent (i.e., 12% and 13%) even though AcAaIT-infected *H. virescens* died 30% more quickly (e.g., 79.7 h versus 113.4 h, Table 1) than AcMNPV-infected *H. virescens*. Although the effect of a starvation period prior to virus inoculation was not investigated in this study, these findings suggest that the widely used methodology of starving insects to encourage consumption of a full inoculum may result in artificially longer lethal times.

Kawarabata *et al.* (1980) showed that prolonged starvation of *B. mori* reduces the ability of larval gut juices to dissolve polyhedra. This suggested that the delay in lethal time that I found following starvation might partially result from a reduction in the release of occlusion-derived virions. Thus, the effect of starvation on baculovirus-induced mortality was also investigated in 5^{th} instar *B. mori* that were inoculated by injection with BmNPV or BmCysPD. The LT_{50} of larvae that were injected with BmNPV or BmCysPD and then immediately starved for 24 h was 10% (9.5 h) and 14% (14.5 h) longer, respectively, than that of non-starved control larvae (Table 2). This delay in median lethal time was similar to that found in 3rd instar *H. virescens* that were inoculated *per os*. This suggested that a starvation-induced alteration in the midgut or midgut fluids was not a primary reason for the delay in median lethal time. In addition, these findings indicated that the delay in median lethal time is proportional to the duration of the starvation period (i.e., the delay in the median lethal time (19.2 h) with a 24 h-long starvation period was nearly twice that (10.3 h) following a 12 h-long starvation period).

The effect of the timing or onset of a 24 h-long starvation period was investigated in 5th instar *B. mori* that were inoculated with BmNPV. The larvae were injected with BmNPV and immediately placed on diet, then at 1 or 2 days p.i. the infected larvae were subjected to a 24 h-long starvation period. Control larvae were starved for 24 h immediately following injection, and then placed on fresh diet. A statistically significant difference was not found in the LT_{50} (98.9 h) of larvae that were starved for a 24 h-long period at 1 day p.i. and larvae that were not starved (Table 2). The LT_{50} (104.1 h) of larvae that were starved for a 24 h-long period at 2 days p.i. was longer by 6% (6.1 h) in comparison to larvae that were not starved (Table 2). These findings suggested that the early stages of infection are more sensitive to the effects of starvation. I hypothesize that once a critical viral load is surpassed that starvation has relatively little impact on larval mortality.

Effect of starvation on viral replication in the hemolymph of B. mori

The effect of starvation on hemolymph viral titer was investigated in 5th instar *B. mori* that were inoculated with BmCysPD. In these experiments, the larvae were starved for 24 h immediately after inoculation, and then placed on diet. At 3 days p.i., the hemolymph titer of BmCysPD was roughly 10-fold lower (i.e., 7.0 x 10^6 versus 6.9 x 10^7 $TCID_{50}$/ml) in larvae that were starved for 24 h in comparison to control larvae that were not starved (Fig. 1). By 4 days p.i., the hemolymph titer of BmCysPD increased in both starved larvae and non-starved control larvae by roughly 10- and 20-fold, respectively. These results suggested that the delay in median lethal times following starvation was the results of a reduction in hemolymph viral titer.

The effect of the timing or onset of a 24 h-long starvation period on hemolymph viral titers was investigated in 5th instar *B. mori* that were inoculated with BmNPV. The larvae were injected with BmNPV and immediately placed on diet. Then at 1 or 2 days p.i., the infected larvae were subjected to a 24 h-long starvation period before returning the larvae to fresh diet. At 3 days p.i., the hemolymph titer of BmNPV was 4.2-fold (3.8×10^7 $TCID_{50}$/ml versus 1.6×10^8 $TCID_{50}$/ml) lower in larvae that were starved immediately after injection, respectively (Fig. 2). However, when the BmNPV-infected larvae were starved for a 24 h-long period at 1 or 2 days p.i., the hemolymph of titer of BmNPV was similar or higher in comparison to control larvae. At 4 days p.i., a statistically significant difference (8.0×10^8 $TCID_{50}$/ml versus 2.9×10^9 $TCID_{50}$/ml) in the hemolymph titer was found in larvae that were starved immediately after inoculation (Fig. 2). These findings were consistent with those of BmCysPD-infected 5th instar *B. mori* and also suggested that the early stages of larval infection are more sensitive to the effects of starvation.

Effect of starvation on viral transmission within B. mori

The effect of starvation on virus pathogenesis was investigated by SDS-PAGE and western blot analyses of hemolymph proteins and by X-gal staining of whole larval mounts. In order to analyze the composition of hemolymph proteins, 5th instar *B. mori* were injected with BmNPV, then starved for 24 h immediately after injection, and then placed on diet. The hemolymph was collected at 3 and 4 days p.i. In general, the onset and abundance of virus-specific proteins in the hemolymph appeared to be delayed in larvae that were starved for 24 h in comparison to non-starved larvae (Fig. 3A). By western blot analysis, a band of approximately 31 kDa was detected by the

mouse anti-polyhedrin antibody. The polyhedrin band was clearly detected at 3 days p.i. in the hemolymph of non-starved control larvae. However, in larvae that were starved for 24 h polyhedrin was not detected until 4 days p.i. (Fig. 3B). The SDS-PAGE and western blot analyses indicated that starvation delays the expression of virus-specific proteins in hemolymph.

The effect of starvation on the progression of viral transmission in whole insects was observed by X-gal staining of 5th instar *B. mori* that were injected with BmCysPD. The BmCysPD-injected larvae were starved for 24 h immediately after injection, and then placed on diet. Whole larval mounts were made and stained with X-gal at 3 or 4 days p.i. At 3 days p.i., the X-gal staining of the fat body tissues, silk glands, and Malpighian tubules (Figs. 4A, E, and G, respectively) from the non-starved larvae was more pronounced in comparison to larvae that were starved for 24 h (Figs. 4B, F, and H, respectively). However, by 4 days p.i. (Fig. 4C and D), the intensity of X-gal staining of tissues from larvae that were starved for 24 h was similar to that observed in the non-starved larvae at 3 days p.i. Mock-infected control larvae displayed a distinctive ring of X-gal staining at the anterior end of the midgut. Weak X-gal staining was also observed in the foregut and midgut of some mock-infected larvae. The X-gal staining experiments suggested that starvation results in a delay in the spread of the baculovirus within the host.

Effect of starvation on the rate of growth and mass of virus-infected B. mori

The effect of starvation on the rate of growth and mass of virus-infected larvae was investigated in 5th instar *B. mori* that were injected with BmNPV and then starved for 0, 12 or 24 h before placing them on diet (Fig. 5A). BmNPV-infected larvae that

were not starved showed a uniform growth rate of 0.61 g/day until 3 days p.i. BmNPV-infected larvae that were starved for 12 h immediately following injection showed a reduced growth rate (0.28 g/day) until 1 day p.i., then showed a similar rate of growth (0.57 g/day) as larvae that were not starved until 3 days p.i. BmNPV-infected larvae that were starved for 24 h immediately following injection showed a similar pattern (Fig. 5A). These larvae showed no gain in mass during the 24 h-long starvation period, then showed a consistent gain in mass of 0.52 g/day between 1 and 3 days p.i. The average mass of 5^{th} instar *B. mori* that were starved for 0, 12 or 24 h was 2.4 ± 0.2, 2.0 ± 0.1, and 1.6 ± 0.1 g, respectively, at 3 days p.i. (Fig. 5A). No statistically significant differences in the mass of BmNPV-infected larvae were found in the observations made at 3 and 4 days p.i. under any of the treatment regimes.

The effect of the timing or onset of a 24 h-long starvation period on the mass of virus-infected larvae was determined using 5^{th} instar *B. mori* that were injected with BmNPV and then starved at 0, 1 or 2 days p.i. An increase in mass was not found during the 24 h-long starvation period regardless of when the starvation period was initiated (Fig. 5B). The average mass of BmNPV-infected larvae that were starved for 24 h at 0, 1 or 2 days p.i. was 1.6 ± 0.1, 1.8 ± 0.1, and 1.6 ± 0.1 g, respectively, when measured at 3 days p.i. In comparison the mass of non-starved, BmNPV-infected larva was 2.4 ± 0.2 g at 3 days p.i. These findings suggested that although starvation clearly interrupts larval growth, the timing of a 24 h-long starvation period has little effect on the final mass of the starved larvae.

Conclusions

These results indicated that starvation reduces the rate of insect growth regardless of whether the insect is virus infected or not virus infected. The starvation-induced reduction in insect growth was coincident with a reduction in virus replication and pathogenicity (i.e., an increase in median lethal time). This reduction in virus replication and pathogenicity was more pronounced when the starvation occurred during the early stages of virus infection suggesting that there is a critical viral load beyond which starvation has little impact on larval mortality.

Table 1. Effect of starvation immediately following inoculation on the time-mortality of virus-infected *H. virescens*

Instar	Virus	Duration of starvation (h)	LT_{50} (h)	95% Confidence limits (h)	
				Lower	Upper
1	AcMNPV	0	82.4	79.8	84.6
1	AcMNPV	12	92.7	89.3	96.0
1	AcMNPV	24	101.6	99.0	104.2
3	AcMNPV	0	113.4	107.5	119.2
3	AcMNPV	24	126.6	120.0	134.3
3	AcAaIT	0	79.7	77.4	82.3
3	AcAaIT	24	89.9	85.9	96.0

Table 2. Effect of starvation on the time-mortality of virus-infected 5th instar *B. mori*

Virus	Start of starvation period (days p.i.)	Duration of starvation (h)	LT_{50} (h)	95% Confidence limits (h)	
				Lower	upper
BmNPV	0	0	98.0	95.6	99.9
BmNPV	0	24	107.5	105.9	109.3
BmCysPD	0	0	100.6	99.1	102.0
BmCysPD	0	24	115.1	111.6	118.6
BmNPV	1	24	98.9	97.2	100.3
BmNPV	2	24	104.1	102.3	105.6

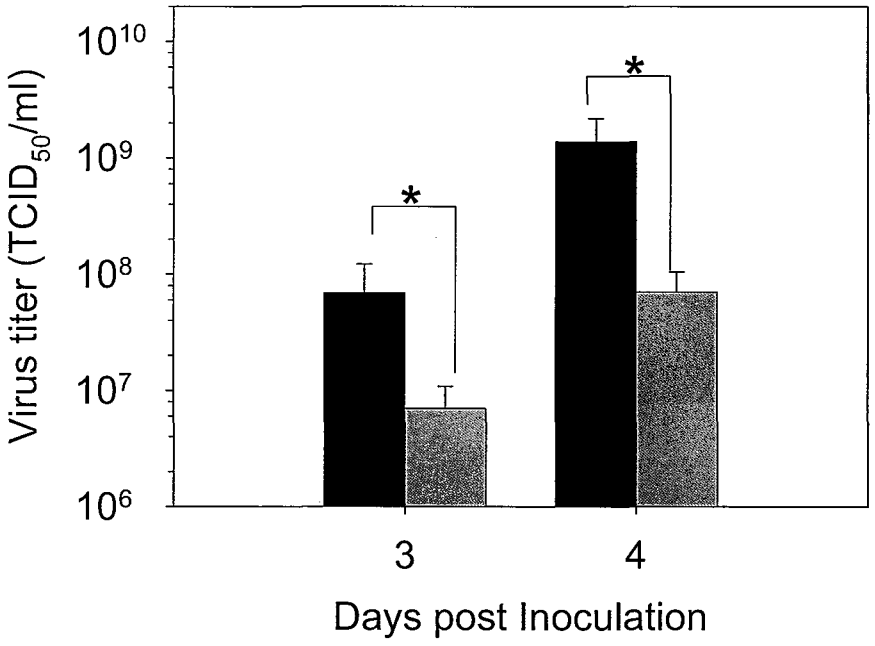

Fig. 1. The effect of starvation on the replication of BmCysPD in 5th instar *B. mori*. The larvae were starved for 0 h (black bars) or 24 h (grey bars) immediately following inoculation by injection with BV, then placed on fresh diet. At 3 or 4 days p.i., hemolymph was collected from 10 individuals and the titer of BmCysPD was determined. The error bars indicate the standard deviation of the mean viral titer. The asterisk (*) indicates a significant difference (p<0.01) between the mean viral titer in non-starved and starved larvae.

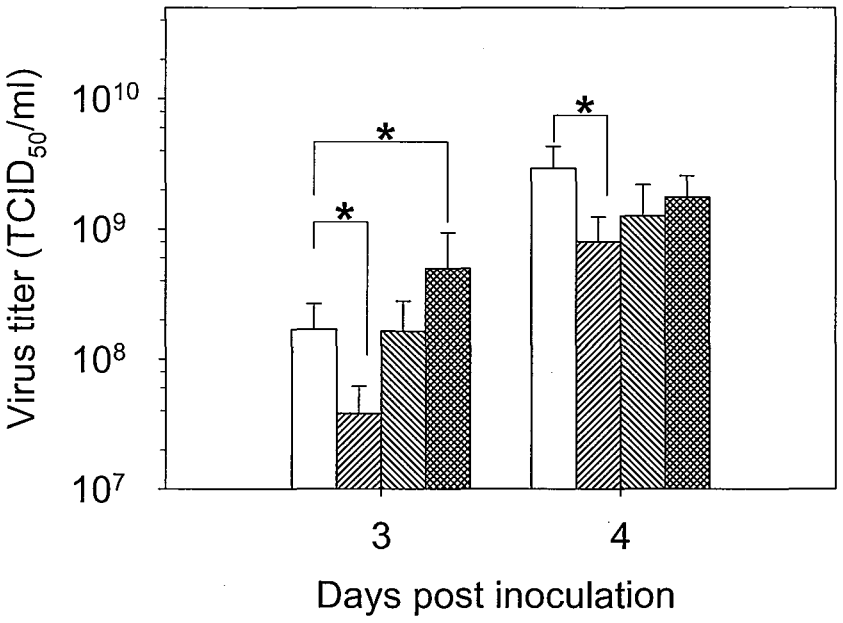

Fig. 2. The effect of the timing of the starvation period on the replication of BmNPV in 5th instar *B. mori*. The larvae were inoculated by infection with BmNPV BVs and then placed immediately on fresh diet (□) or were starved for a 24 h-long period at 0 (▨), 1 (▧) or 2 (▩) days p.i., and then placed on fresh diet. At 3 or 4 days p.i., hemolymph was collected from 10 individuals and the titer of BmNPV was determined. The error bars indicate the standard deviation of the mean viral titer. The asterisk (*) indicates a significant difference ($p < 0.01$) between the mean viral titer in non-starved and starved larvae.

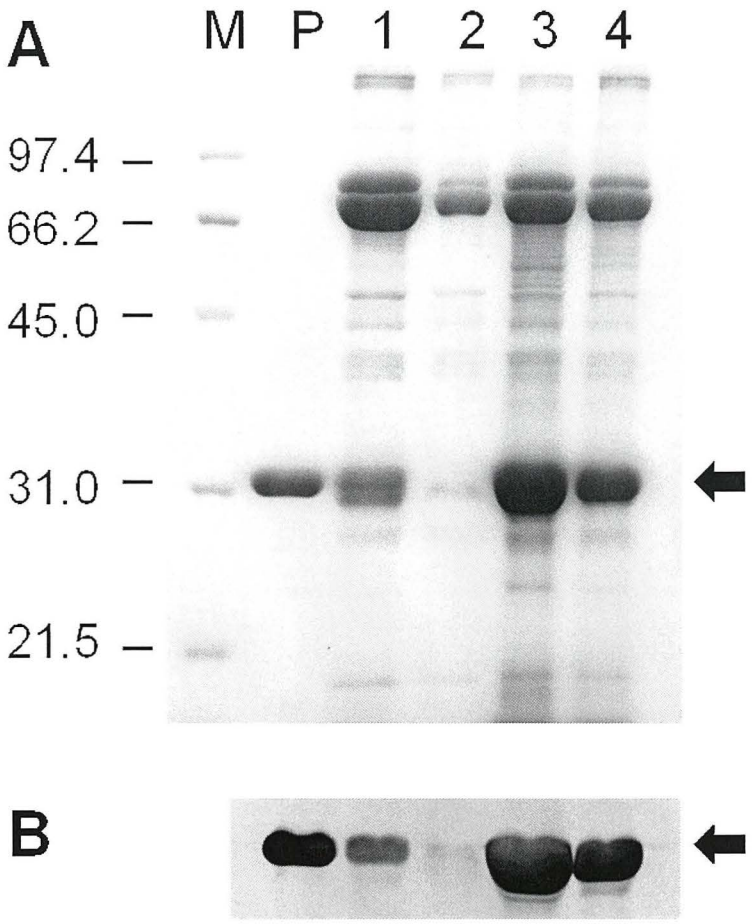

Fig. 3. SDS-(12%) PAGE (A) and western blot (B) analyses of hemolymph proteins from BmNPV-infected 5th instar *B. mori*. The larvae were starved for a 0 h- (lanes 1 and 3) or 24 h- (lanes 2 and 4) long period immediately following inoculation, and then placed on fresh diet. The hemolymph was collected at 3 (lanes 1 and 2) or 4 (lanes 3 and 4) days p.i. from 10 individuals for each treatment group and pooled. The arrow indicates the migration of BmNPV-expressed polyhedrin. The sizes (in kDa) of molecular weight standards (lane M) are indicated to the left. Purified polyhedrin protein (lane P) was also used as a size marker and positive control for the western blot analysis.

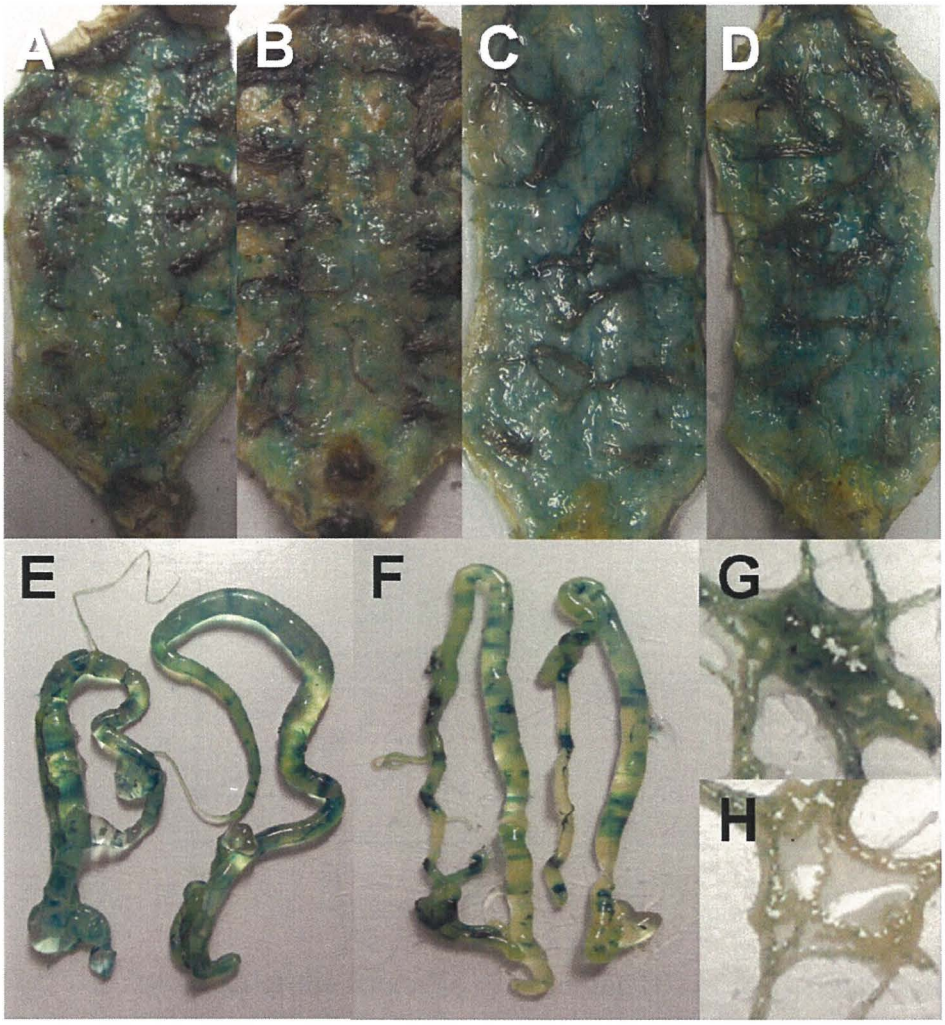

Fig. 4. Histochemical analysis of the progression of baculovirus infection in 5th instar *B. mori*. The larvae were inoculated with BmCysPD and then starved for a 0 h- (A, C, E, and G) or 24 h- (B, D, F, and H) long period immediately following inoculation, and then placed on fresh diet. The progression of BmCysPD infection at 3 (A and B) or 4 (C and D) days p.i. in fat body tissues, and at 3 days p.i. in silk glands (E and F), and Malpighian tubules (G and H) was visualized by X-gal staining. The images are representative of tissues from 20 individuals (i.e., 10 individuals per treatment) that were stained with X-gal.

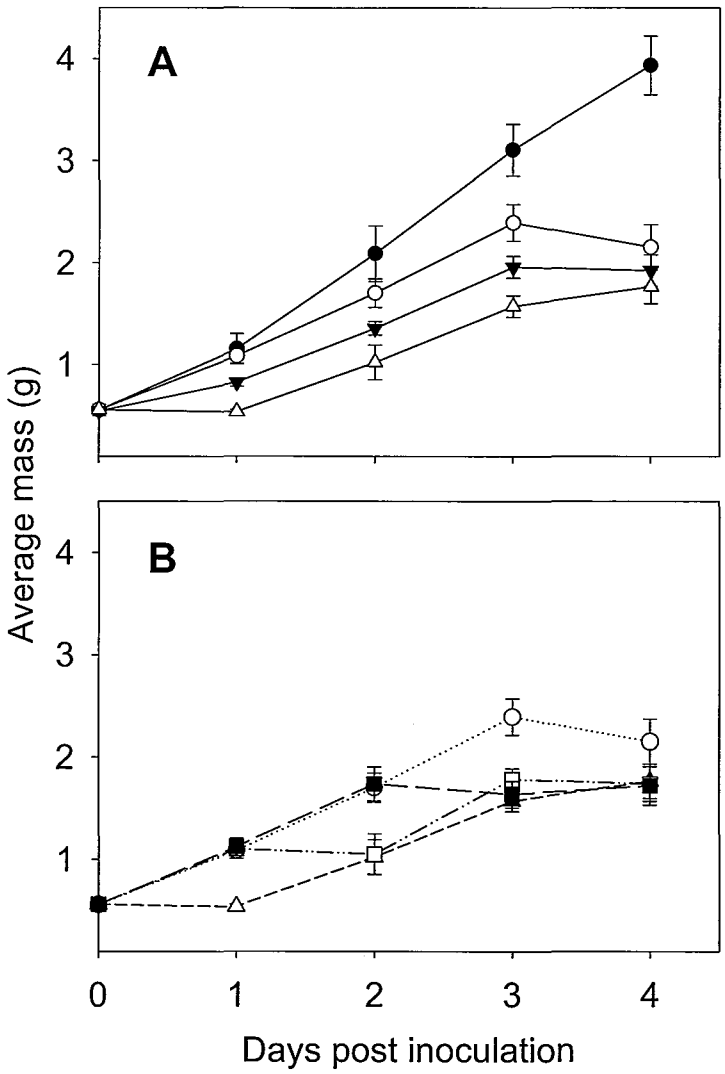

Fig. 5. The effect of the length (A) and timing (B) of the starvation period on the mass of 5th instar *B. mori* that are infected with BmNPV. (A) The larvae were starved for 12 h (▼) or 24 h (△) immediately following inoculation with BmNPV, and then placed on fresh diet. Control larvae were immediately placed on fresh diet following inoculation with BmNPV (○) or mock infection (●). (B) The larvae were inoculated with BmNPV, placed on fresh diet, and then starved for a 24 h-long period at 0 (△), 1 (□) or 2 (■) days p.i. prior to placing the larvae back onto fresh diet. Non-starved control larvae (○) were inoculated with BmNPV and then placed immediately on fresh diet. The error bars indicate the standard deviation of the mass of 20 larvae.

Summary

The progression of baculovirus (BmNPV, BmCysPD, AcMNPV or AcAaIT) infection in larval *Bombyx mori* and *Heliothis virescens* (1^{st}, 3^{rd} or 5^{th} instar) was investigated following various starvation regimes. When the larvae were starved for 12 or 24 h immediately following inoculation, the median lethal time to death (LT_{50}) was delayed by 9.5 to 19.2 h in comparison to non-starved controls. This corresponded to a delay of 10 to 23% depending upon the larval stage and virus that was used for inoculation. When a 24 h-long starvation period was initiated at 1 or 2 days post inoculation (p.i.), no statistically significant difference in LT_{50} was found indicating that the early stages of infection are more sensitive to the effects of starvation. Viral titers in the hemolymph of 5^{th} instar *B. mori* that were starved for 24 h immediately following inoculation were 10-fold lower ($p<0.01$) than that found in non-starved control larvae. Histochemical analyses indicated that virus transmission was reduced in 5^{th} instar *B. mori* that were starved for 24 h immediately following inoculation in comparison to non-starved control larvae. In general, the mass of larvae that were starved immediately after inoculation was 30% lower than that of non-starved control insects. These findings indicate that starvation of the larval host at the time of baculovirus exposure has a negative effect on the rate baculovirus transmission and pathogenesis.

Chapter 2. Ecdysteroid stimulates virus transmission in larvae infected with *Bombyx mori* nucelopolyhedrovirus

The virus-encoded enzyme (ecdysteroid UDP-glucosyltransferase (EGT)), which catalyzes the conjugation of a sugar molecule to the insect molting hormone ecdysteroid, is thought to be involved in this block since sugar-conjugated ecdysteroid is functionally inactive. Following the initial identification of the *egt* gene in the baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) (O'Reilly and Miller, 1989), an *egt* gene homologue was identified in most of the other baculoviruses (Clarke *et al.*, 1996).

When larvae are infected with a mutant AcMNPV, which lacks a functional *egt* gene, these larvae stop feeding and prepare to molt or pupate (O'Reilly and Miller, 1989). O'Reilly *et al.* (O'Reilly and Miller, 1991; O'Reilly *et al.*, 1998) speculated that the *egt* gene product of the wild-type AcMNPV prolongs feeding, resulting in an enlarged larva which yields greater numbers of progeny virions. It was also been shown that larvae infected with an *egt* deletion mutant are killed faster than larvae infected with wild-type virus (O'Reilly and Miller, 1991; Park *et al.*, 1996). I previously reported that *Bombyx mori* larvae injected with BmEGTZ, a *B. mori* NPV (BmNPV) deletion mutant of *egt*, also die faster (by about 12 h) than larvae infected with wild-type AcMNPV (Kang *et al*, 1998). Flipsen *et al.* (1995) showed histochemically that an AcMNPV *egt* deletion mutant induces the degeneration of Malpighian tubules in *Spodoptera exigua* larva. Furthermore, since this phenomenon is not observed in wild-type AcMNPV infected larvae, they suggested that the Malpighian tubule degeneration is involved in the rapid killing. However, the mechanistic basis for this reduced time to death is still unclear. The aim of this chapter

is: (1) Elucidate the physiological basis for the reduced time to death of BmEGTZ-infected larvae, compared to BmNPV-infected larvae. (2) Examine the role, if any, of ecdysteroid in this process. In order to accomplish this, the profile of hemolymph proteins, and progression of virus infection, were examined by SDS-PAGE and histochemical analyses of newly molted fifth instar *B. mori* larvae; which were virus-infected, or virus infected and subsequently ecdysteroid treated.

Materials and Methods

Cells, viruses, and silkworms

The *B. mori*-derived BmN cell line was maintained in Grace's medium (Gibco BRL, New York, USA) supplemented with 0.26% tryptose broth and 10% fetal bovine serum. The wild-type BmNPV T3 isolate (Maeda *et al.*, 1985), as well as the BmNPV deletion mutant BmCysPD (Ohkawa *et al.*, 1994) (lacking the endogenous cysteine protease gene and BmEGTZ in which the endogenous *egt* gene was disrupted by insertion of a lacZ gene cassette (Maeda *et al.*, unpublished)), were propagated and titrated on BmN cells, as described previously (Maeda, 1989; Koh *et al.*, 1998). Silkworm, *B. mori*, larvae were reared on an artificial diet at 27°C, as described previously (Choudary *et al.*, 1995).

Virus infection of larvae

Female larvae on the third day, after the third or fourth larval ecdysis, were hemocoelically injected with 1 x 10^5 plaque forming units (PFU) of each virus (BmEGTZ, BmNPV or BmCysPD) in 20 µl of tissue culture medium. Control insects were injected with 20 µl of tissue culture medium.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

At 12, 24, 36, 48, 60, 72, 84, 96, 108 or 120 h postinfection (p.i.), a proleg of treated larva was pierced with a sterilized pin and the hemolymph was collected into a chilled microfuge tube containing a few crystals of phenylthiourea in order to prevent melanization, as described previously (Choudary *et al.*, 1995). Hemolymph proteins were analyzed by vertical gel electrophoresis (12% acrylamide gels) in the presence of SDS, as described by Laemmli (1970). All of the samples were boiled for 5 min in the presence of 2% SDS, 6.25 mM Tris-HCl pH 6.7, 15% glycerol and 100 mM dithiothreitol before loading them onto the gel. Following electrophoretic separation, the proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, USA).

Preparation and examination of larvae for lacZ expression

Larvae were prepared for X-Gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) staining by securing them individually onto styrofoam blocks with insect pins through the head capsule and terminal abdominal segment. Using scissors, a longitudinal incision was made through the dorsal cuticle. The cuticle was spread out to expose the internal tissues using four additional insect pins and the dissected larvae were covered with dissecting buffer (PBS: 0.5 M phosphate, 0.5 M NaCl, pH 6.0) for 3 min. The dissecting buffer was then removed and the tissues immediately infused with fixative (4% paraformaldehyde in PBS) for 20 min. Following fixation, the larvae were washed twice with PBS and infused with staining solutions [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in distilled H_2O] containing 2 mg/ml X-

Gal for 6-12 hr in total darkness, as described previously (Engelhard *et al.*, 1994). The stained larvae were observed under a dissecting microscope at 10-40x.

Ecdysteroid treatment

Female larvae (newly emerged fifth instar) were first injected with 1 x 10^5 PFU of each virus (BmEGTZ, BmNPV T3 or BmCysPD), as described above. At 24 h post virus injection, the larvae were reinjected with 10 µl of a solution containing either 50 ng or 100 ng [200- or 100-fold dilution of 1 mg/ml ecdysteroid (Sigma, St. Louis, USA) in 5% methanol] of ecdysteroid. At 72, 96 or 120 h post ecdysteroid treatment, the larval tissues were subjected to X-Gal, staining as described above, or the hemolymph proteins were collected and subjected to SDS-PAGE, as described above.

Results and Discussion

Comparison of the hemolymph protein profiles between BmEGTZ- and BmNPV T3- infected larvae

In order to establish a biochemical basis for the accelerated death of BmEGTZ-infected *B. mori*, the hemolymph protein profiles (between BmEGTZ- and BmNPV-infected larvae) were compared by SDS-PAGE. At 48 h p.i. and earlier, the differences in the hemolymph protein profiles of larvae (injected with either BmEGTZ or BmNPV on the second day after the third larval ecdysis) were not observed (data not shown). Thereafter, temporal differences were found in the profiles; virus-expressed or virus-induced proteins were found at least 12h earlier in BmEGTZ-infected larvae (Fig. 6A). For example, virus-specific proteins of 16, 31, 40, and 50 kDa were detected at 60 h p.i. in BmEGTZ-infected larvae; whereas, these proteins were

undetected in BmNPV-infected larvae until 72 h p.i. or later. The expression of polyhedron (a major, very late, virus structural protein of 31 kDa) in BmEGTZ-infected larvae at 84 h p.i., however, was not as high compared to that in BmNPV-infected larvae at 96 h p.i. A similar pattern of the accelerated appearance (by at least a 12 h) of virus-expressed, or virus-induced proteins, was found in the hemolymph of larvae injected with BmEGTZ on the third day, after the fourth larval ecdysis when compared to BmNPV-infected larvae (Fig. 1B).

Histochemical analysis of virus transmission following BmEGTZ or BmCysPD infection

Virus transmission in BmEGTZ- or BmCysPD-infected larvae was compared at 2, 3, 4 or 5 days p.i. by histochemical detection (with X-Gal) of beta-galactosidase expression. In these experiments, a control virus (BmCysPD) was used, which carries as a *lacZ* gene cassette identical to that of BmEGTZ at the cysteine protease gene locus. BmCysPD has indistinguishable growth characteristics compared to BmNPV (Ohkawa *et al.*, 1994). Following the injection of *B. mori* larvae (5^{th} instar, day 3) with either BmEGTZ or BmCysPD, beta-glucosidase expression was clearly detected in the fat body and tracheal tissue of BmEGTZ-infected larvae at 2 days p.i. (Fig. 7B), but only barely detectable in BmCysPD-infected larvae (Fig. 7A). At 3 days p.i., the staining of fat body and tracheal tissues in BmEGTZ-infected larvae intensified; staining was also detected in other tissues including midgut, silkgland, Malphigian tubules, nervous system, and gonads (Fig. 7D). The staining intensity of BmCysPD-infected larvae (Fig. 7C) was similar to that found in BmEGTZ-infected larvae at 2 days p.i. At 4 days p.i., similar differences were found in the staining intensities between BmEGTZ-

and BmCysPD-infected larvae (Fig. 7F and E). At 5 days p.i. all of the BmEGTZ-infected larvae had died and were not subject to histochemical analysis. In contrast, all of the BmCysPD-infected larvae were still alive and showed a staining intensify that was similar to BmEGTZ-infected larvae at 4 days p.i. (Fig. 7G).

Effect of ecdysteroid treatment on hemolymph proteins

In order to examine how ecdysteroid affected the profile of proteins in the hemolymph of virus-infected *B. mori*, BmEGTZ- or BmNPV-infected 5th instar larva were individual at 24 h post virus injection with a solution containing 0, 50, or 100 ng of ecdysteroid. Fifth instar larvae were used because at this larval stage endogenous ecdysteroid exists only in trace amounts (Kiaguchi, 1983). The hemolymph proteins were collected at 96 h post virus infection and analyzed by SDS-PAGE (Fig. 8A). In mock-infected larvae, ecdysteroid treatment had no apparent effect in the profile of hemolymph proteins. This suggests that ecdysteroid itself does not affect the expression of hemolymph proteins. Ecdysteroid treatment also had no apparent effect on hemolymph protein expression in BmNPV-infected larvae. This suggests that the injected ecdysteroid was inactivated by the BmNPV-expressed ecdysteroid treatment appeared to accelerate virus-specific protein synthesis in BmEGTZ-infected larvae. For example, polyhedron was detected in ecdysteroid treated larvae, but not in the non-ecdysteroid treated BmEGTZ-infected larvae at 96 h p.i. This level of polyhedron expression was not detected in the non-ecdysteroid treated BmEGTZ-infected larvae until 120 h p.i. (Fig. 8B). Furthermore, this effect may be concentration dependent since the expression of polyhedron appeared to be greater following ecdysteroid treatment at 100 ng/larva, when compared to treatment 50 ng/larva.

Histochemical analysis of the effect of ecdysteroid on virus transmission

Ecdysteroid was injected into larvae at 24 h post BmEGTZ infection on the day of the fourth larval ecdysis. At 3 days p.i., tracheal and fat body tissues from ecdysteroid treated larvae (Fig. 9B and C), showed slightly stronger X-Gal-staining compared to non-ecdysteroid-treated larvae (Fig. 9A). Staining was also slightly more intense in larvae injected with a high dose (100 ng/larvae) of ecdysteroid, in comparison to those injected with a low dose (50 ng/larvae). At 4 days p.i., the staining intensity increased for all treatments, but was slightly more intense in the larvae treated with 100 ng of ecdysteroid (Fig. 9F), compared to the other treatment (Fig. 9D and E). These histochemical observations coincided with the SDS-PAGE analysis of the hemolymph proteins (Fig. 8). Furthermore, these findings indicated that virus transmission is affected by not only ecdysteroid, but by its concentration.

Larvae infected with a baculovirus carrying an inactivated *egt* gene succumb to viral infection quicker than larvae infected with a wild-type virus carrying an active *egt* gene (O'Reilly & Miller, 1991; Park *et al.*, 1996; Kang *et al.*, 1998). In this study, I found that in *B. mori* larvae the expression of virus-encoded, or virus-induced proteins, is accelerated following infection with a mutant BmNPV, in which the endogenous *egt* gene is inactivated. Furthermore, this accelerated protein expression apparently causes the accelerated transmission of the virus. This results in the early death of BmEGTZ-infected larvae, in comparison to BmNPV-infected larvae. Keeley and Vinson (1975) reported that the injection of 20-hydroxyecdysone into baculovirus-infected *Heliothis virescens* results in a delay in the onset of virus-induced pathology, as well as a decrease in the mortality of the infected insects. Similarly, by overexpressing

prothoracicotropic hormone (PTTH) in baculovirus-infected larvae, O'Reilly *et al.*, (1995) suggested that ecdysteroid has a direct inhibitory effect on viral replication. The data, however, showed that following ecdysteroid treatment there is acceleration in virus-specific protein synthesis, as well as virus transmission. This suggests that ecdysteroid plays a direct role in enhancing virus replication.

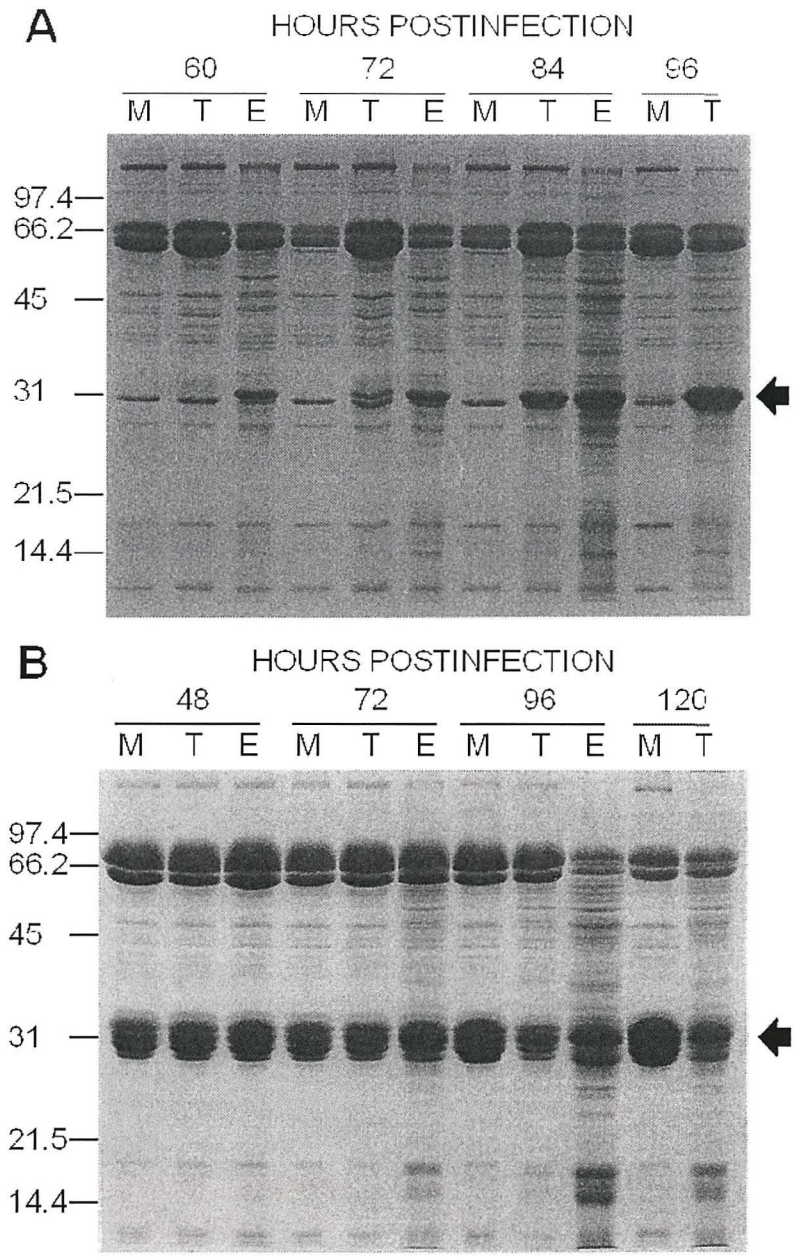

Fig. 6. SDS-PAGE analysis of hemolymph proteins from *B. mori* larvae that were mock- (M), wild-type BmNPV- (T) or BmEGTZ- infected (E) on the second day of the third ecdysis (A), or the third day of the fourth larval ecdysis (B). The hemolymph was collected at 48, 60, 72, 84, 96, or 120 h p.i. The arrowhead indicates the migration of polyhedrin. Protein molecular masses in kilo Daltons are shown at the left.

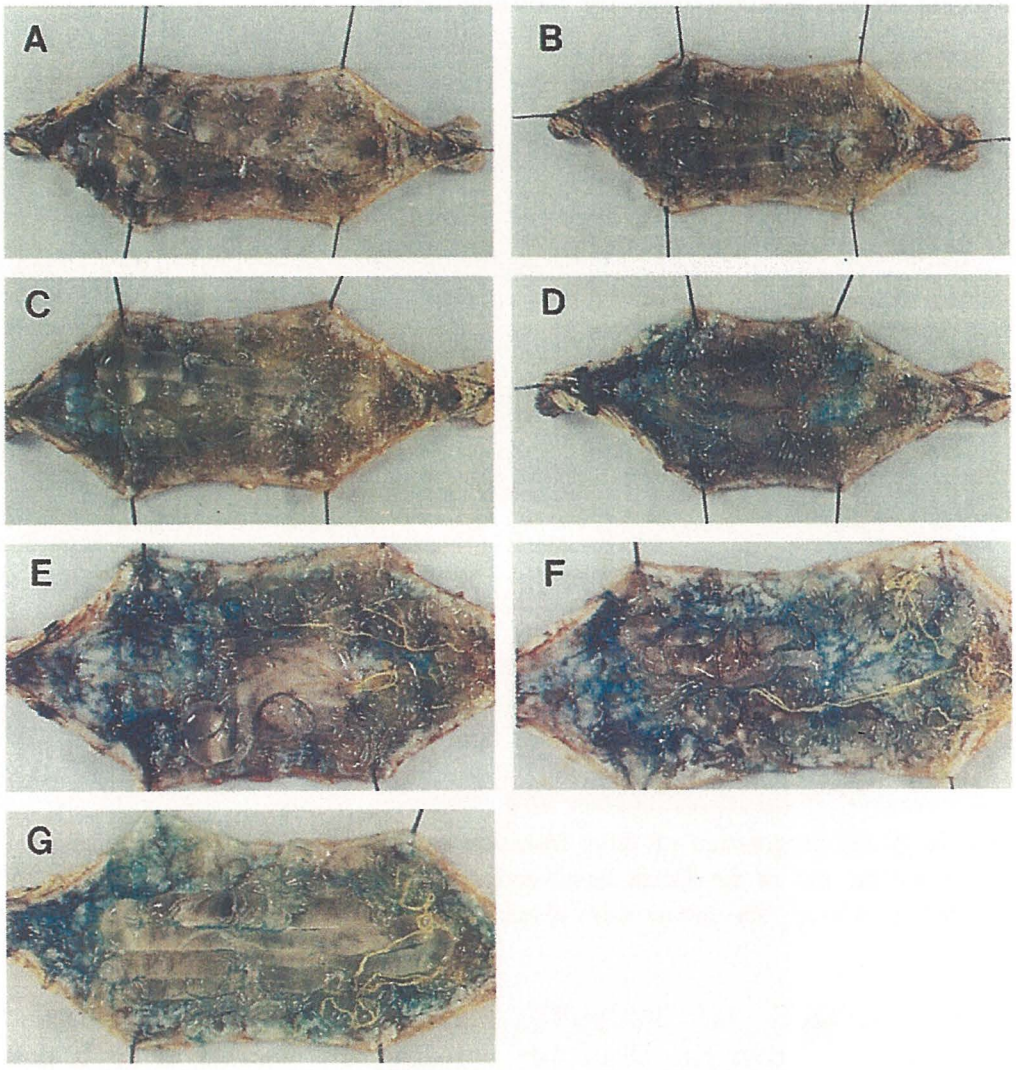

Fig. 7. Histochemical analysis of the progression of virus infection as determined by LacZ expression (blue staining) in *B. mori* larvae infected with control BmCysPD (A, C, E and G) or BmEGTZ (B, D and F) on the third day of the fourth larval ecdysis. Larvae were stained at 2 (A and B), 3 (C and D), 4 (E and F) or 5 (G) days p.i.

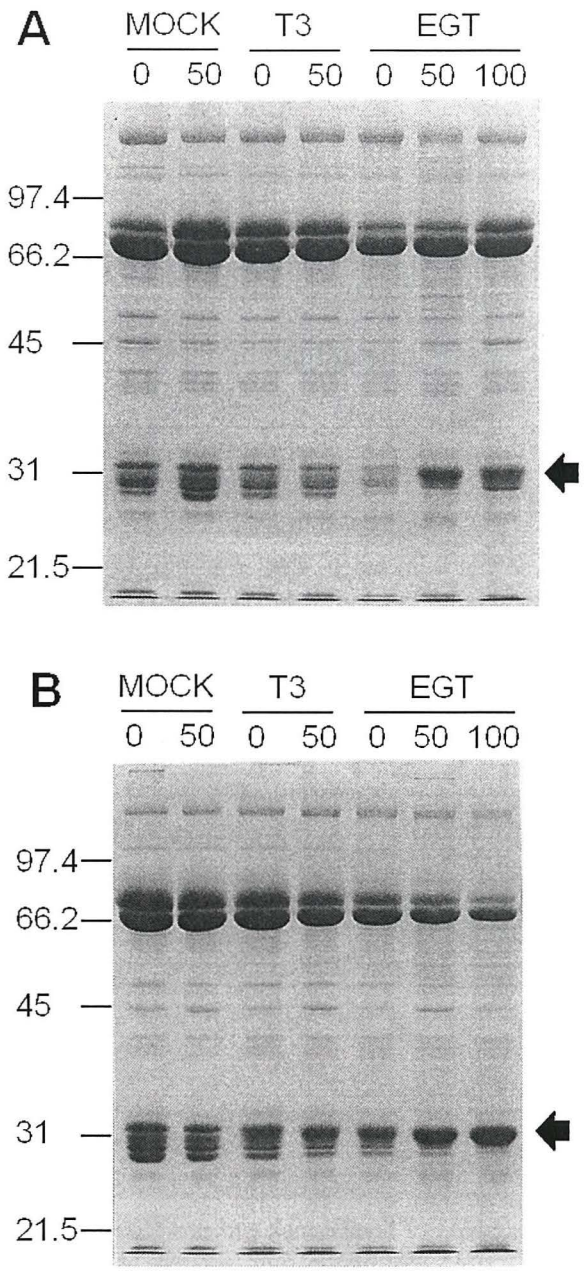

Fig. 8. SDS-PAGE analysis of hemolymph proteins at 96 h p.i. (A) or 120 h p.i. (B) from *B. mori* larvae that were mock- (M), wild-type BmNPV- (T3) or BmEGTZ-infected (EGT) on the day of the fourth larval ecdysis. At 24 h post virus or mock infection, individual larva was injected with zero, 50 or 100 ng of ecdysteroid as indicated. The arrowhead indicates the migration of polyhedrin. Protein molecular masses in kilo Daltons are shown at the left.

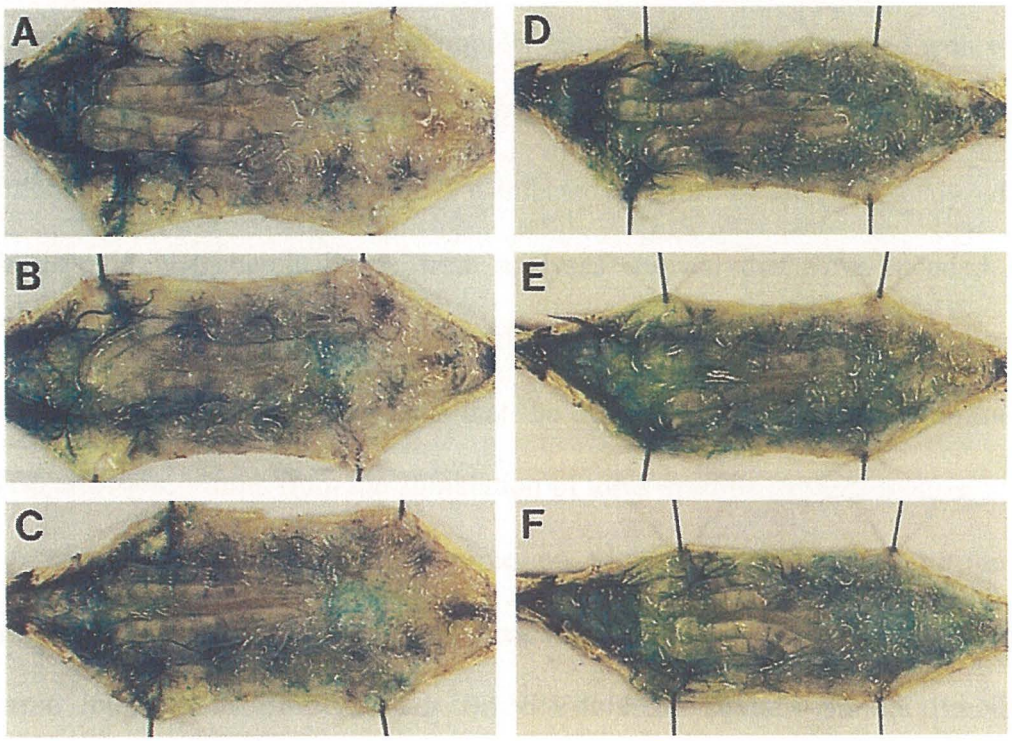

Fig. 9. Histochemical analysis of the progression of virus infection as determined by LacZ expression (blue staining) in *B. mori* larvae infected with BmEGTZ on the day of the fourth larval ecdysis and treated with zero (A and D), 50 (B and E) or 100 ng (C and F) of ecdysteroid per larva at 24 h p.i. The larvae were dissected at 3 (A, B, and C) or 4 (D, E, and F) days p.i.

Summary

Most baculoviruses have an ecdysteroid UDP-glucosyltransferase (*egt*) gene, whose product inactivates ecdysteroid within the infected host. *Bombyx mori* larvae infected with BmEGTZ, a mutant *B. mori* nucleopolyhedrovirus (BmNPV) in which the egt gene has been inactivated, die more rapidly compared to larvae infected with wild-type BmNPV. In this study, the profile of hemolymph proteins, and progression of virus infection in BmEGTZ- and BmNPV-infected *B. mori* larvae, was analyzed by SDS-PAGE and histochemically. These analyses showed that virus-encoded and virus-induced proteins were expressed quicker in BmEGTZ-infected larvae than in BmNPV-infected larvae. This suggests that the decrease in time to death, following BmEGTZ infection, results from the stimulation of virus-specific protein expression. In order to examine the effect of ecdysteroid on virus transmission, the profile of hemolymph proteins, and progression of virus infection, were analyzed following an ecdysteroid injection of BmEGTZ- or BmNPV-infected larvae. In the BmNPV-infected larvae, ecdysteroid treatment had no apparent effect on hemolymph protein expression. This suggests that the injected ecdysteroid was inactivated by the BmNPV-expressed ecdysteroid UDP-glucosyltransferase. An ecdysteroid injection into BmEGTZ-infected larvae increased the speed of virus-specific protein expression and virus transmission. These results suggest that ecdysteroid stimulates protein expression, which in turn results in the stimulation of virus transmission.

Chapter 3. Comparative analysis of α-glucosidase activity in *Bombyx mori* and *Antheraea yamamai.*

The mulberry silkworm *Bombyx mori* (Lepidoptera, Bombycidae) feeds exclusively on the leaves of the mulberry bush (*Morus alba*) or artificial diet containing mulberry leaf extract. *B. mori* has been domesticated for use in sericulture over a period of thousands of years. On the other hand, the Japanese oak silkmoth *Antheraea yamamai* (Lepidoptera, Saturniidae) feeds upon a wider range of diets including leaves of the deciduous oak tree and chestnut tree. *A. yamamai* is widespread in Korea and Japan. The composition and enzymatic activities of proteins in the hemolymph and midgut of *B. mori* are well characterized, whereas those of *A. yamamai* are significantly less well characterized.

In many insect species, iminosugars have been identified that inhibit various insect disaccharidases (Asano *et al.* 2001; Hirayama *et al.* 2007). Sucrase and trehalase of *B. mori* are, however, highly insensitive to the inhibitory effects of iminosugars in mulberry leaf (Hirayama *et al.* 2007).

In this chapter, α-glucosidase activity in the hemolymph and midgut tissues of *B. mori* and *A. yamamai* were characterized in terms of substrate preference, temperature and pH dependence, and sensitivity to the α-glucosidase inhibitors DNJ, acarbose, and voglibose.

Materials and Methods

Insect rearing and protein preparation

Eggs of the silkworm *B. mori* and fifth instars of *A. yamamai* were obtained from the National Academy of Agricultural Science (Korea). Larvae of *B. mori* were reared

on an artificial diet and larvae of *A. yamamai* were reared on oak leaves at 25°C as described previously (Kang *et al.* 2000). Hemolymph was collected by piercing a larval proleg with a sterile pin and allowing the hemolymph to drip into a chilled microfuge tube containing a few crystals of phenylthiourea as described previously (Choudary *et al.* 1995). Midgut tissues were collected from larvae that were chilled on ice for 15 min. A longitudinal incision was made and the midgut was gently removed from the larvae, rinsed in phosphate buffered saline (2 mM NaH_2PO_4, 2 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and homogenized using a hand-held glass homogenizer on ice. The midgut homogenates as well as hemolymph were centrifuged at 11,000 x *g* for 5 min at 4°C and the supernatant from these preparations was divided into small aliquots and stored at -80°C. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (Sigma, USA) as a standard.

α-Glucosidase activity

The ability of the hemolymph and midgut preparations to hydrolyze maltose, sucrose, and trehalose (Sigma, USA) were determined in a 200 μL reaction containing 0.285 to 185 μg of protein and 30 mM of each substrate in potassium phosphate buffer, pH 6.8. The enzyme solution was preincubated at 37°C for 5 min prior to the addition of substrate, then for 20 min at 37°C. The concentration of glucose following the hydrolysis of maltose, sucrose or trehalose was determined using a Glucose-E kit (Wako, Japan) following the manufacturer's protocol (incubation at 37°C for 18 min and measuring absorbance at 505 nm using a spectrophotometer (Molecular device, USA)). *p*-Nitrophenyl α-D-glucopyranoside (PNPG; Sigma, USA) was also used as a

general substrate for α-glucosidase activity. This assay was performed as above except that the reaction mixture (150 μL) contained 4 mM PNPG and was incubated at 37°C for 30 min. After incubation, the PNPG reaction was terminated by the addition of 50 μL of 234 mM Na_2CO_3 and absorbance was measured at 405 nm.

Dependence of α-glucosidase activity on pH and temperature

The effect of pH on α-glucosidase activity of midgut extracts was determined using 0.1 M citrate buffer (pH 3, 4, 5, and 6), 0.1 M sodium phosphate buffer (pH 7 and 8), and 0.1 M glycine-NaOH buffer (pH 9 and 10). The assays were performed as described above at 37°C.

The effect of temperature on α-glucosidase activity of midgut extracts was determined at pH 6.8 in the standard assay described above at various temperatures from 25 to 70°C at 5°C intervals.

Electrophoresis and staining

Native PAGE was routinely carried out using 7.5% polyacrylamide gels as described by Davis (1964). To visualize α-glucosidase activity, the gel was incubated in a solution containing 1.5 mM 4-methylumbellifyl α-D-glucopyranoside (MUG; Sigma, USA) in 0.1 M potassium phosphate buffer, pH 6.8.

Inhibition of α-glucosidase activity by DNJ, acarbose, or voglibose

1-Deoxynojirimycin (DNJ; Biotopia, Korea), acarbose (Bayer Korea, Korea), and voglibose (CJ, Korea) are inhibitors of α-glucosidase (Kuhlmann and Plus 1996). The ability of these compounds to inhibit α-glucosidase activity in midgut extracts from

B. mori or *A. yamamai* was determined using maltose, sucrose, and trehalose as substrates in a standard assay as described above. Each inhibitor (dissolved in water) was preincubated with the protein at 37°C for 5 min prior to the addition of the substrate. The concentration of compound that resulted in 50% inhibition (IC_{50}) was determined using a range of inhibitor concentrations (0.001 to 1000 μM) that bracketed the predicted IC_{50}. IC_{50} values were calculated using regression analysis (SigmaPlot, SPSS Scientific, USA).

Results and Discussion

Comparison of α-glucosidase activity in hemolymph and midgut preparations from B. mori *and* A. yamamai

α-Glucosidase activity in the hemolymph and midgut of *B. mori* and *A. yamamai* was determined using maltose, sucrose, trehalose, and PNPG as substrates. PNPG was a dramatically better substrate with both hemolymph and midgut preparations from both species (Tables 3 and 4). Dramatically higher α-glucosidase activity was found in the midgut preparations of both species for all of the substrates tested (Table 4). In general, the midgut preparation from *A. yamamai* showed higher α-glucosidase activity than that of *B. mori*. Maltose, sucrose, and trehalose are potential authentic α-glucosidase substrates that are found in the diets of both species. I hypothesize that the higher α-glucosidase activity in the midgut relative to that found in the hemolymph is necessary for the metabolism of carbohydrates found in the diet.

Dependence of α-glucosidase activity in midgut preparations from B. mori and A. yamamai on pH and temperature

The effect of pH on the α-glucosidase activity of midgut preparations from *B. mori* and *A. yamamai* was determined using maltose, sucrose, trehalose, and PNPG as substrates. In general the highest α-glucosidase activity was found at pH 6 and higher for all of the substrates tested (Fig. 10). A dramatic reduction in α-glucosidase activity was generally found with both midgut preparations at pH 6 and below. The midgut preparations from both *B. mori* and *A. yamamai* showed similar sensitivity to changes in pH with maltose (Fig. 10A), sucrose (Fig. 10B), and PNPG (Fig. 10D) as substrates. In contrast, the midgut preparation from *B. mori* showed relatively higher sensitivity to changes in pH in comparison to the midgut preparation from *A. yamamai* when trehalose was used as a substrate (Fig. 10C). α-Glucosidase activity from the midgut preparations of both *B. mori* and *A. yamamai* increased with increasing temperature up to 50°C when maltose (Fig. 11A) was used as a substrate and up to 70°C when sucrose (Fig. 11B) was used as a substrate.

Analysis of α-glucosidase in hemolymph and midgut preparations from B. mori and A. yamamai by native PAGE

Following native PAGE separation of proteins in the hemolymph and midgut of *B. mori* and *A. yamamai*, the gel was stained with MUG, a fluorescent α-glucosidase substrate. α-Glucosidase activity was not detected in preparations from the hemolymph of *B. mori* and *A. yamamai* using MUG (Fig. 12). This was consistent with the undetectable or very low α-glucosidase activity that was found in the hemolymph of *B. mori* and *A. yamamai* by the enzymatic assays using maltose, sucrose, and trehalose as substrates. In contrast, MUG staining was found in the midgut preparations from both species (Fig. 12). The staining was found at different locations

on the gel indicating that the midgut α-glucosidase activity originated from different proteins.

Inhibition of α-glucosidase in the midgut preparations from B. mori and A. yamamai

The ability of DNJ, acarbose, and voglibose to inhibit α-glucosidase activity in midgut preparations from *B. mori* and *A. yamamai* were determined using maltose, sucrose, and trehalose as substrates (Table 5). DNJ is an antiviral and antidiabetic compound that is found in mulberry leaf (Asano *et al.* 2001; Jacob *et al.* 2007). Acarbose and voglibose are pharmaceuticals for treating diabetes. These drugs function by reducing the metabolism of complex carbohydrates into glucose and other monosaccharides by α-glucosidase following a meal (Kuhlmann and Plus 1996). The reduced availability of glucose results in a corresponding reduction in the transfer of glucose into the blood stream. With midgut preparations from *B. mori*, DNJ showed IC_{50}s of 490 and 59 μM when maltose and trehalose, respectively, were used as substrates. In contrast, no DNJ inhibitory activity was detected when sucrose was used as a substrate. DNJ showed significantly higher potency against midgut preparations from *A. yamamai*, i.e., DNJ showed IC_{50}s of 20, 8.8, and 7.7 μM when maltose, trehalose, and sucrose, respectively, were used as substrates. DNJ has also been shown to be highly potent against α-glucosidases from other insects (Scofiled *et al.*, 1995). Acarbose showed low nanomolar potency against midgut preparations from both *B. mori* and *A. yamamai* when maltose was used as a substrate (Table 5). Acarbose also showed very high potency against the midgut preparation of *A. yamamai* when sucrose was used as a substrate. In contrast, acarbose showed dramatically

lower potency against the midgut preparation from *B. mori* when sucrose was used as a substrate. Acarbose was not able to inhibit trehalose hydrolysis with midgut preparations from either *B. mori* or *A. yamamai*. Voglibose showed an IC_{50} of 19 μM against the midgut preparation of *B. mori* when maltose was used as a substrate, but showed no inhibitory activity when sucrose and trehalose were used as substrates (Table 5). With the midgut preparation from *A. yamamai*, voglibose showed IC_{50}s of 8.7 and 8.6 μM when maltose and sucrose, respectively, were used as substrates. Voglibose showed no inhibition of trehalose hydrolysis with midgut preparations from *A. yamamai*. In general, these results indicated that the α-glucosidases in *B. mori* are less sensitive to inhibition by the general α-glucosidase inhibitors DNJ, acarbose, and voglibose.

B. mori is monophagous species that feeds exclusively on mulberry leaf or artificial diet containing mulberry leaf extract. Asano *et al.* (2001) have identified eighteen alkaloids from mulberry leaf, root bark, and fruit of which DNJ is present at the highest concentration. Interestingly, Asano *et al.* (2001), also showed that α-glucosidase activity of *B. mori* is much less sensitive to inhibition by DNJ in mulberry leaf in comparison to digestive enzymes from mammals. In contrast to *B. mori*, the Eri silkworm *Samia cynthia ricini* is a polyphagous species. Hirayama *et al.* (2007) have compared the inhibitory activity of DNJ against sucrase and trehalase of *S. cynthia* and *B. mori*, and found that sucrase and trehalase from the midgut of *B. mori* are less sensitive to DNJ in comparison to those of *S. cynthia*. Similarly, in the present study, I found that α-glucosidase activity in the midgut of *B. mori* (in comparison to that of *A. yamamai*) is relatively insensitive to inhibition by DNJ, acarbose, and voglibose. These results suggest that monophagy has resulted in α-glucosidases in *B. mori* that are poorly inhibited by DNJ, a highly abundant alkaloid in mulberry leaf.

Table 3. α-Glucosidase activity in the hemolymph of *B. mori* and *A. yamamai*

Substrate	Activity (nmole/min/mg protein)	
	B. mori	*A. yamamai*
Maltose	1.7	ND[a]
Sucrose	32	ND
Trehalose	1.4	1.4
PNPG	6,900	2,500

[a]ND: Not Detected

Table 4. α-Glucosidase activity in the midgut of *B. mori* and *A. yamamai*

Substrate	Activity (nmole/min/mg protein)	
	B. mori	*A. yamamai*
Maltose	160	760
Sucrose	1,000	1,500
Trehalose	82	110
PNPG	220,000	5,600,000

Table 5. Concentration of α-glucosidase inhibitors giving 50% inhibition of glucosidase activities in the midgut of *B. mori* and *A. yamamai*

Enzyme source	Substrate	IC_{50} (μM)		
		DNJ	acarbose	voglibose
B. mori	maltose	490	0.027	19
	sucrose	NI[a]	190	NI
	trehalose	59	NI	NI
A. yamamai	maltose	20	0.009	8.7
	sucrose	8.8	0.007	8.6
	trehalose	7.7	NI	NI

[a]NI: No Inhibition (less than 50% inhibition at 1000 μM)

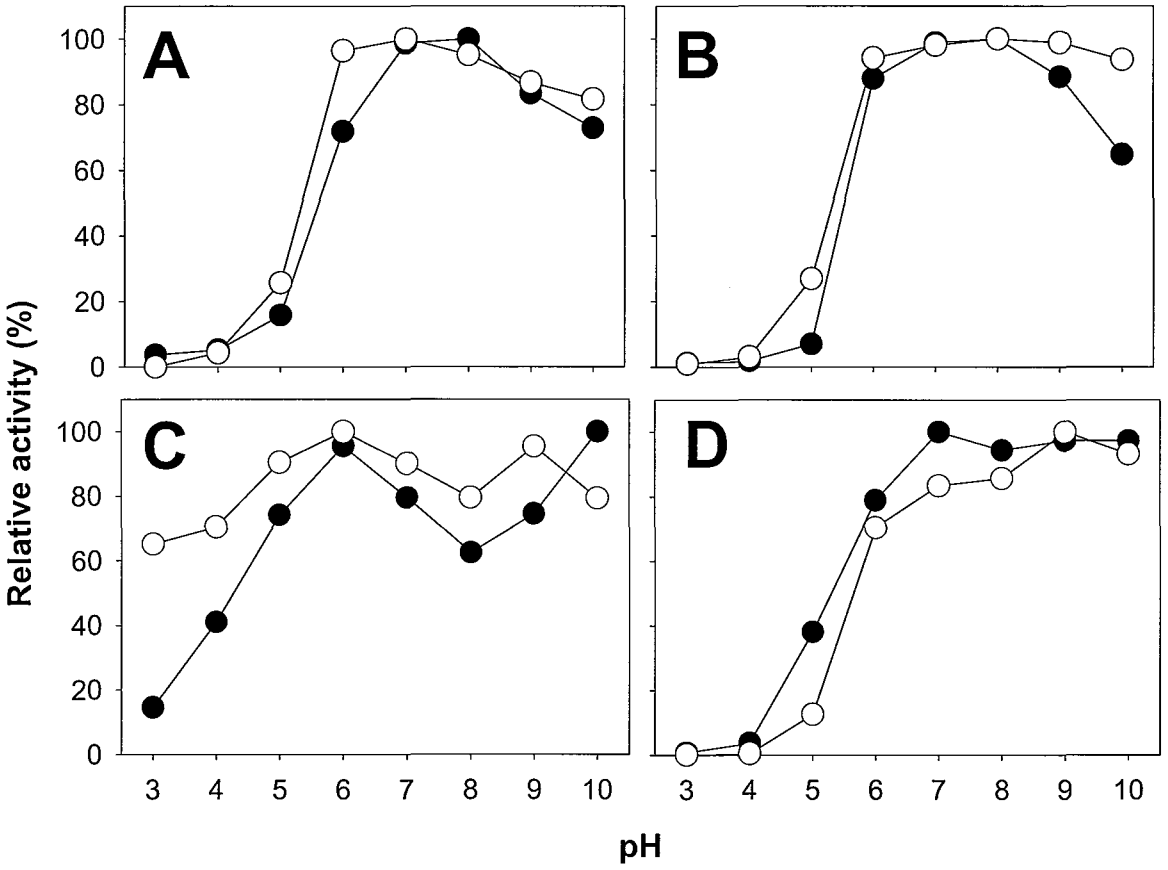

Fig. 10. The effect of pH upon the relative activity of α-glucosidase from the midgut of *B. mori* (•) and *A. yamamai* (○). Relative activity of 100% refers to the highest activity obtained for each enzyme using maltose (A), sucrose (B), trehalose (C), or *p*-nitrophenyl α-D-glucopyranoside (D) as a substrate.

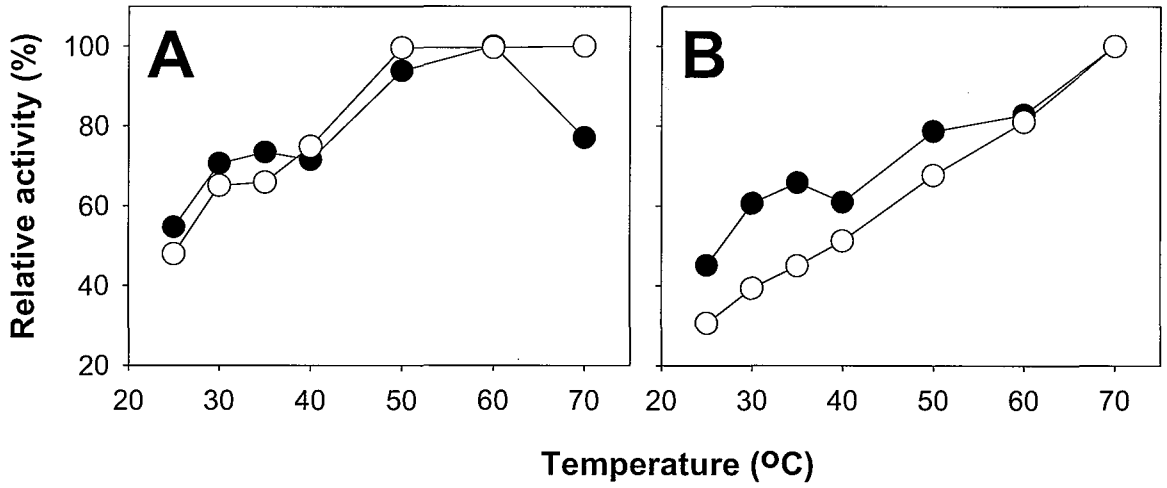

Fig. 11. The effect of temperature upon the relative activity of α-glucosidase from the midgut of *B. mori* (•) and *A. yamamai* (○). Relative activity of 100% refers to the highest activity obtained for each enzyme using maltose (A) or sucrose (B) as a substrate.

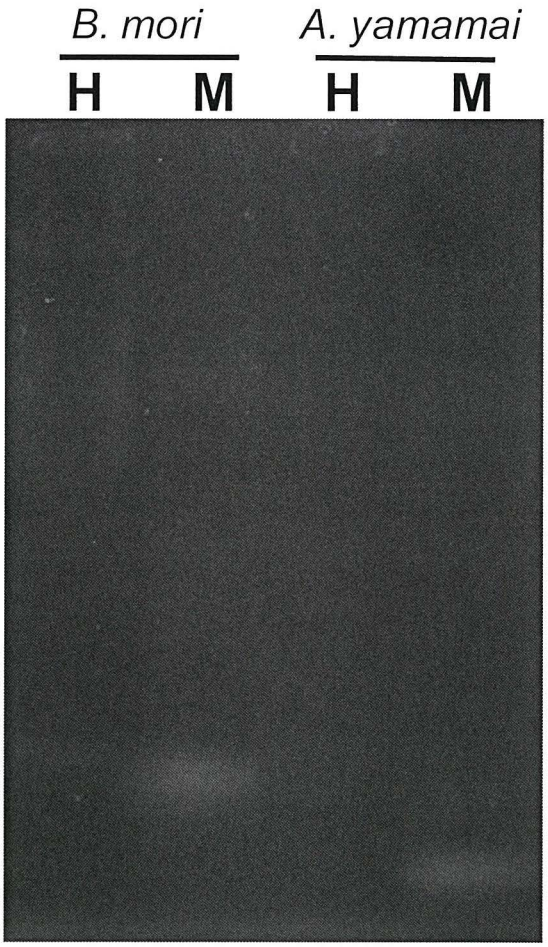

Fig. 12. In gel α-glucosidase activity staining. Hemolymph (lanes H) or midgut (lanes M) proteins from *B. mori* and *A. yamamai* were separated by 7.5% native PAGE and stained with 4-methylumbeliferyl α-D-glucoside.

Summary

α-Glucosidase (EC 3.2.1.20) is a glycosidase that hydrolyzes disaccharides, oligosaccharides, and polysaccharides resulting in the release of α-D-glucose. In this study, α-glucosidase activity in the hemolymph and midgut of the mulberry silkworm *Bombyx mori* and Japanese oak silkmoth *Antheraea yamamai* was measured using maltose, sucrose, trehalose, and *p*-nitrophenyl α-D-glucopyranoside as substrates. In general, hemolymph α-glucosidase activity was higher in *B. mori* than in *A. yamamai.* In contrast, midgut α-glucosidase activity was higher in *A. yamamai* than in *B. mori* for all of the substrates tested. α-Glucosidase activity in the midgut of both *B. mori* and *A. yamamai* showed similar responses to changes in pH and temperature for all of the substrates tested. Native (7.5%) PAGE of hemolymph and midgut proteins from *B. mori* and *A. yamamai* followed by staining with 4-methylumbelliferyl α-D-glucoside (MUG) indicated that the α-glucosidases of these related lepidopterans are functionally similar but structurally different. In comparison to α-glucosidase activity from *A. yamamai*, α-glucosidase activity from *B. mori* was generally less sensitive to the α-glucosidase inhibitors, 1-deoxynojirimycin (DNJ), acarbose, and voglibose when the activity was determined using maltose, sucrose, and trehalose.

Chapter 4. Effect of 1-deoxynojirimycin on the replication of baculoviruses, *Bombyx mori* nucleopolyhedrovirus and *Autographa californica* multiple nucleopolyhedrovirus

Glycosylation is an essential process in eukaryotic cells that attaches glycans to proteins and other biomolecules. In many proteins, appropriate glycosylation is essential for proper folding, authentic biological activity, and/or for normal protein stability. The most common types of glycoproteins are *N*-linked (glycans attached to a nitrogen of asparagine or arginine) and *O*-linked (glycans attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline). α-Glucosidase and mannosidase are the key enzymes involved in the *N*-linked glycosylation pathway (Francis *et al.* 2002). The first step of *N*-linked glycosylation generally involves the transfer of $Glc_3Man_9GlcNAc_2$ (a 14-sugar precursor formed by 3 glucose, 9 mannose, and 2 *N*-acetylglucosamine molecules) to a nascent protein in the endoplasmic reticulum (ER). Subsequently, the attached $Glc_3Man_9GlcNAc_2$ is processed by glycosidases such as α-glucosidase I, α-glucosidase II, α-mannosidase I, α-mannosidase II, and glycosyltransferases such as acetylglucosaminyltransferase I (Ren *et al.* 1997). The initial steps in the processing of complex oligosaccharides occur in the ER lumen and involve the removal of the terminal glucose of $Glc_2Man_9GlcNAc_2$ by α-glucosidase I. Subsequently, α-glucosidase II removes the next two glucose residues and α-mannosidase I removes one mannose residue. The resulting glycoprotein precursor ($Man_8GlcNAc_2$–protein) moves to the Golgi complex where three more mannose residues are removed by α-mannosidase I found within the Golgi. *N*-acetylglucosamine is then attached by *N*-acetylglucosaminyltransferase I and

subsequently the terminal mannose residues are removed by α-mannosidase II. The product, $GlcNAc_2Man_3GlcNAc_2$–protein can be further processed by various enzymes to generate more complex oligosaccharide structures.

1-Deoxynojirimycin (DNJ) is a naturally occurring alkaloid that inhibits glycosidases such as α-glucosidase I and α-glucosidase II that are involved in the early processing steps of $Glc_3Man_9GlcNAc_2$ (Moremen *et al.* 1994). Relatively high concentrations of DNJ are found in the leaves of mulberry trees and in silkworm, *Bombyx mori* (Asano *et al.* 2001), a caterpillar that feeds exclusively on mulberry. DNJ can also be purified from the culture medium of microorganisms including some species of *Bacillus* and *Streptomyces* (Kim *et al.* 2011). Since glycoproteins play important roles in the attachment of enveloped viruses to host cells DNJ may also be involved in altering viral surface glycoproteins so that recognition by receptor(s) on the host cell surface is altered.

A virus-encoded glycoprotein called GP64 is the major glycoprotein found in the envelope of BVs (Jarvis and Garcia, 1994). GP64 is required for binding of the BV to a host cell and subsequent fusion of the BV envelope and endosomal membrane during the penetration phase of the virus infection cycle (Volkman *et al.* 1984). During a later phase of baculovirus infection, the amount of GP64 that is found in the host cell membrane is related to the efficiency of BV production and rate of systemic infection in insects (Nagai *et al.* 2011).

In this chapter, the effect of DNJ on the replication of *B. mori* NPV (BmNPV) and *Autographa californica* multiple NPV (AcMNPV) was investigated. The host of BmNPV is the silkworm a specialist that feeds exclusively on mulberry, a plant with relatively high levels of DNJ. AcMNPV on the other hand replicates on generalist

insects that commonly feed on plants with relatively low DNJ levels (Hirayama *et al.* 2007). In addition, α-glucosidase activity in *B. mori*-derived Bm5 and *Spodoptera frugiperda*-derived Sf9 cell extracts were characterized. Bm5 and Sf9 are specific hosts of BmNPV and AcMNPV, respectively.

Materials and Methods

Chemicals

DNJ was purified from *Bacillus subtilis* MORI as described previously (Hirayama *et al.* 2007). The α-glucosidase inhibitors acarbose and voglibose were purchased from BayerKorea (Korea) and CJ (Korea), respectively. The α-glucosidase substrate *p*-nitrophenyl α-D-glucopyranoside (PNPG) was purchased from Sigma (USA).

Cells and viruses

Bm5 and Sf9 cells were maintained in TC-100 medium (Welgene, Korea) supplemented with 5% and 10% fetal bovine serum, respectively, at 27°C. *B. subtilis* MORI was propagated in TSB medium at 37°C as described previously (Kim *et al.* 2011). BmNPV and AcMNPV were propagated on Bm5 and Sf9 cells, respectively. Viral titer was determined by the end-point dilution method as described previously (O'Reilly *et al.* 1992).

Preparation of cell extract

Extracts of Bm5 and Sf9 cells were prepared from $1x10^7$ cells that were pelleted by centrifugation at 3,000 x g for 5 min at 4°C. Following the centrifugation the cells were resuspended in 50 mM sodium phosphate buffer (pH 7) and the cells were sonicated on ice for 1 min using a VCX130 ultrasonic processor (Sonics & Materials, USA). The extracts were centrifuged at 3,000 x g for 5 min at 4°C and then stored at -20°C prior to use. The protein concentration of the cell extract was measured by the method of Bradford (1976).

Virus replication assay

Bm5 or Sf9 cells were seeded into the wells of a 24 well plate (1×10^5 cells per well) and allowed to attach for 2 h. Subsequently, the cells were inoculated with virus at a multiplicity of infection (MOI) of 1. Following a 1 h-long viral attachment period, the inoculum was removed by aspiration and the cells were cultured in 0.5 mL of fresh medium containing 0, 1, 2.5, 5 or 10 mM DNJ. After a 4 day-long incubation period at 27°C, the virus titer in the cell culture supernatant was determined by the end-point dilution method.

Cell viability assay

Cell viability was determined using a colorimetric XTT assay (Cell Proliferation Kit; Biological industries, Israel) following the manufacturer's protocol. Bm5 or Sf9 cells were seeded into the wells of a 96 well plate (1×10^4 cells per well) and allowed to attach for 2 h. Following cell attachment, the old cell culture supernatant was replaced with 100 μL of fresh medium containing 0, 1, 2.5, 5 or 10 mM DNJ and incubated at

27°C for 48 h. After the 48 h-long incubation, 50 µL of activated XTT reagent was added to each well of the 96-well plate and incubated at 27°C for 4 h. Subsequently, the formation of reduced XTT was measured at 450 nm using a microplate reader (Molecular devices, USA) as described previously (Wu *et al.* 2002).

α-Glucosidase activity assay

α-Glucosidase activity in the cell extract was measured using PNPG as a substrate in the wells of a 96 well plate. The ability of the cell extract to hydrolyze PNPG was determined in a 150 µL reaction containing 80 µg of cell extract and 4 mM PNPG in 100 mM sodium phosphate buffer, pH 7. The reaction mixture was incubated for 30 min at 37°C and then 50 µL of 234 mM Na_2CO_3 was added to terminate the reaction. Absorbance was measured at 405 nm using a microplate reader as described previously (Kang *et al.* 2010).

The effect of pH on α-glucosidase activity was determined in 100 mM citrate buffer (pH 3, 4, 5, and 6), 100 mM sodium phosphate buffer (pH 7 and 8) or 100 mM glycine-NaOH buffer (pH 9, 10, 11). The effect of temperature on α-glucosidase activity was determined in 100 mM sodium phosphate buffer, pH 7, at 5°C intervals from 25°C to 70°C.

Inhibition of α-glucosidase activity by DNJ, acarbose, and voglibose

The ability of DNJ (0.03 – 1000 µM), acarbose (200 – 1,000 µM), and voglibose (3 – 200 µM) to inhibit α-glucosidase in Bm5 and Sf9 cell extracts was determined using PNPG as a substrate in 100 mM sodium phosphate buffer, pH 7, as described

above. Each inhibitor, dissolved in water, was preincubated with the Bm5 or Sf9 cell extract for 5 min at 37°C prior to the addition of PNPG. Following the addition of the PNPG, the reaction was incubated for 30 min at 37°C and then read at 405 nm as described above. The median inhibitory concentration (IC_{50}) of each compound was determined by regression analysis (SigmaPlot; SPSS Scientific, USA).

Results

Effect of DNJ on the replication of BmNPV and AcMNPV

DNJ is a known inhibitor of α-glucosidase activity as well as the replication of various viruses. The ability of DNJ to inhibit the replication of BmNPV and AcMNPV was determined in Bm5 and Sf9 cells that were grown in medium containing 1 to 10 mM DNJ. After 4 days, DNJ showed no significant effect on BmNPV replication in Bm5 cells at a concentration as high as 10 mM (Fig. 13). In contrast, AcMNPV replication was significantly reduced in the presence of 2.5 mM or higher concentrations of DNJ (in comparison to control Sf9 cells that were not treated with DNJ). AcMNPV showed a dose-specific inhibitory response up to 5 mM DNJ (i.e., no apparent inhibition at 1 mM DNJ, 40% inhibition at 2.5 mM DNJ, and 60% inhibition at 5 mM DNJ). The replication of AcMNPV, at 4 days post inoculation, was inhibited by approximately 67% when the host Sf9 cells were grown in medium containing 10 mM DNJ.

Effect of DNJ on the viability of Bm5 and Sf9 cells

The viability of Bm5 and Sf9 cells was measured by the colorimetric XTT assay. DNJ did not show any statistically significant effects on the viability of Bm5 and Sf9

cells that were grown for 48 h in medium containing up to 10 mM DNJ (Fig. 14). The results indicated that the viability of Bm5 and Sf9 cells is not affected by DNJ.

Effect of pH and temperature on α-glucosidase activity from Bm5 and Sf9 cell extracts

α-Glucosidase activity in Bm5 and Sf9 cell extracts was measured using PNPG as a substrate in 100 mM sodium phosphate buffer, pH 7. Under this condition α-glucosidase specific activity in the Sf9 cell extract (3.39 μmol/min/mg) was roughly 2-fold higher than that in the Bm5 cell extract (1.73 μmol/min/mg) (Table 6). When the effect of pH on α-glucosidase activity was analyzed, the highest activity in both Bm5 and Sf9 cell extracts was found at pH 10 (Fig. 15A). In comparison to the Bm5 cell extract, α-glucosidase activity in the Sf9 cell extract showed greater sensitivity to increases in pH. α-Glucosidase activity in the Bm5 cell extract showed relatively little sensitivity to changes in pH. Surprisingly, α-glucosidase activity in the Bm5 and Sf9 cell extracts showed strong thermo stability with increased activity up to 60°C (Fig. 15B).

Inhibition of α-glucosidase activity in Bm5 and Sf9 cell extracts by DNJ, acarbose, and voglibose

The ability of DNJ, acarbose, and voglibose to inhibit α-glucosidase activity in Bm5 and Sf9 cell extracts was measured using PNPG as a substrate in 100 mM sodium phosphate buffer, pH 7. α-Glucosidase activity in the Sf9 cell extract was 40-fold more sensitive to DNJ than α-glucosidase activity in the Bm5 cell extract (Table 7). Similarly, α-glucosidase activity in the Sf9 cell extract was more sensitive to inhibition

by voglibose but by only 2-fold in comparison to α-glucosidase activity in the Bm5 cell extract (Table 7). Acarbose did not show any detectable inhibitory activity with Bm5 and Sf9 cell extracts.

Table 6. α-Glucosidase activity in extracts from Bm5 and Sf9 cells

Extract source	Specific activity (μmol/min/mg protein)[a]
Bm5	1.73 ± 0.42[b]
Sf9	3.39 ± 0.58

[a]*p*-Nitrophenyl α-D-glucopyranoside was used as a substrate to measure α-glucosidase activity in 100 mM sodium phosphate buffer, pH 7.

[b]Values represent the mean ± standard deviation separate experiments.

Table 7. Inhibition of α-glucosidase activity in Bm5 and Sf9 cell extracts by DNJ, acarbose, and voglibose

Extract source	IC_{50} (μM)[a]		
	DNJ	acarbose	voglibose
Bm5	26.1 ± 3.0[b]	NI[c]	27.9 ± 2.1
Sf9	0.64 ± 0.06	NI	13.3 ± 4.0

[a]*p*-Nitrophenyl α-D- glucopyranoside was used as a substrate to measure α-glucosidase activity in 100 mM sodium phosphate buffer, pH 7.

[b]Values represent the mean ± standard deviation separate experiments.

[c]NI: No Inhibition (less than 50% inhibition at 1000 μM).

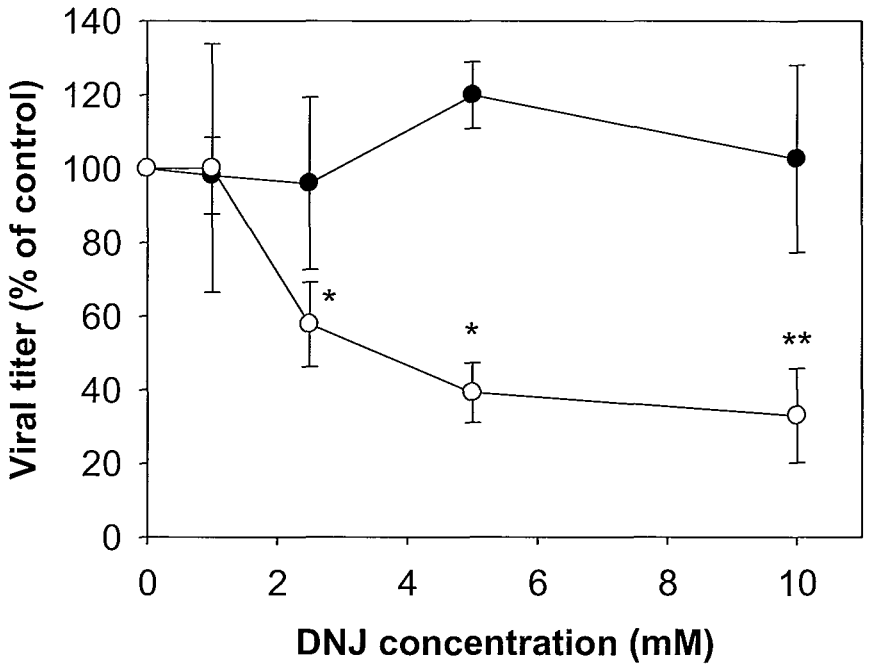

Fig. 13. The effect of DNJ on the replication of BmNPV (●) and AcMNPV (○). Bm5 or Sf9 cells were inoculated with BmNPV or AcMNPV, respectively, at an MOI of 1. Following inoculation, the cells were maintained in medium containing 0, 1, 2.5, 5 or 10 mM DNJ. At 4 days postinfection, the viral titer in each of the DNJ containing treatments was determined and compared to the viral titers in non-DNJ containing control infections. Each point represents the mean value of three independent experiments. The error bars indicate standard deviation of the mean. The asterisk indicates a significant difference between the mean viral titer in non-treated and DNJ-treated groups (*$P < 0.05$, **$P < 0.01$).

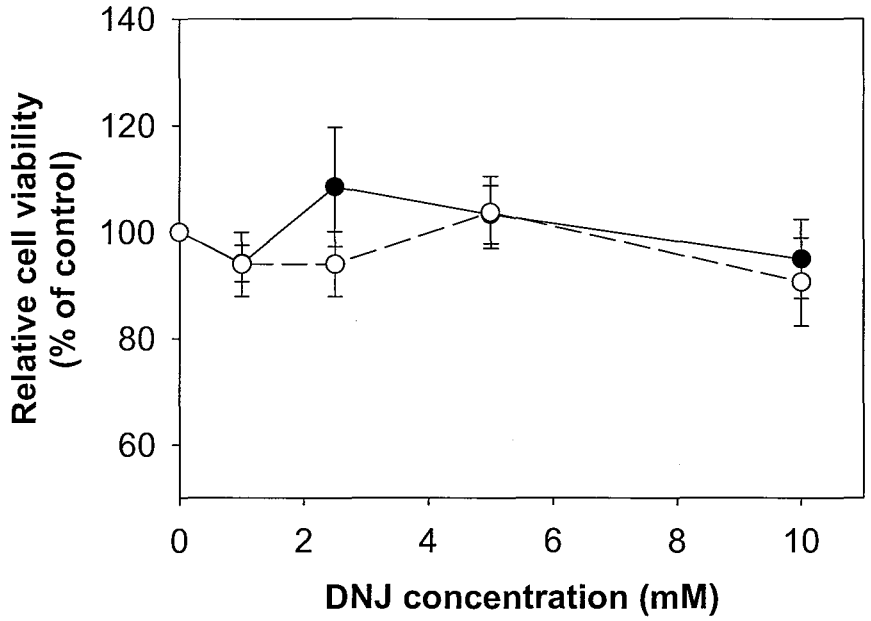

Fig. 14. Viability of Bm5 (•) and Sf9 (○) cells following treatment with DNJ. The viability of cells that were grown for 48 h in medium containing 0, 1, 2.5, 5 or 10 mM DNJ was compared to the viability of control cells that were grown in medium lacking DNJ. Cell viability was measured using XTT reagent. Each point represents the mean value of three independent experiments. The error bars indicate standard deviation of the mean. No statistically significant differences were found between cells grown in the absence or presence of DNJ up to 10 mM.

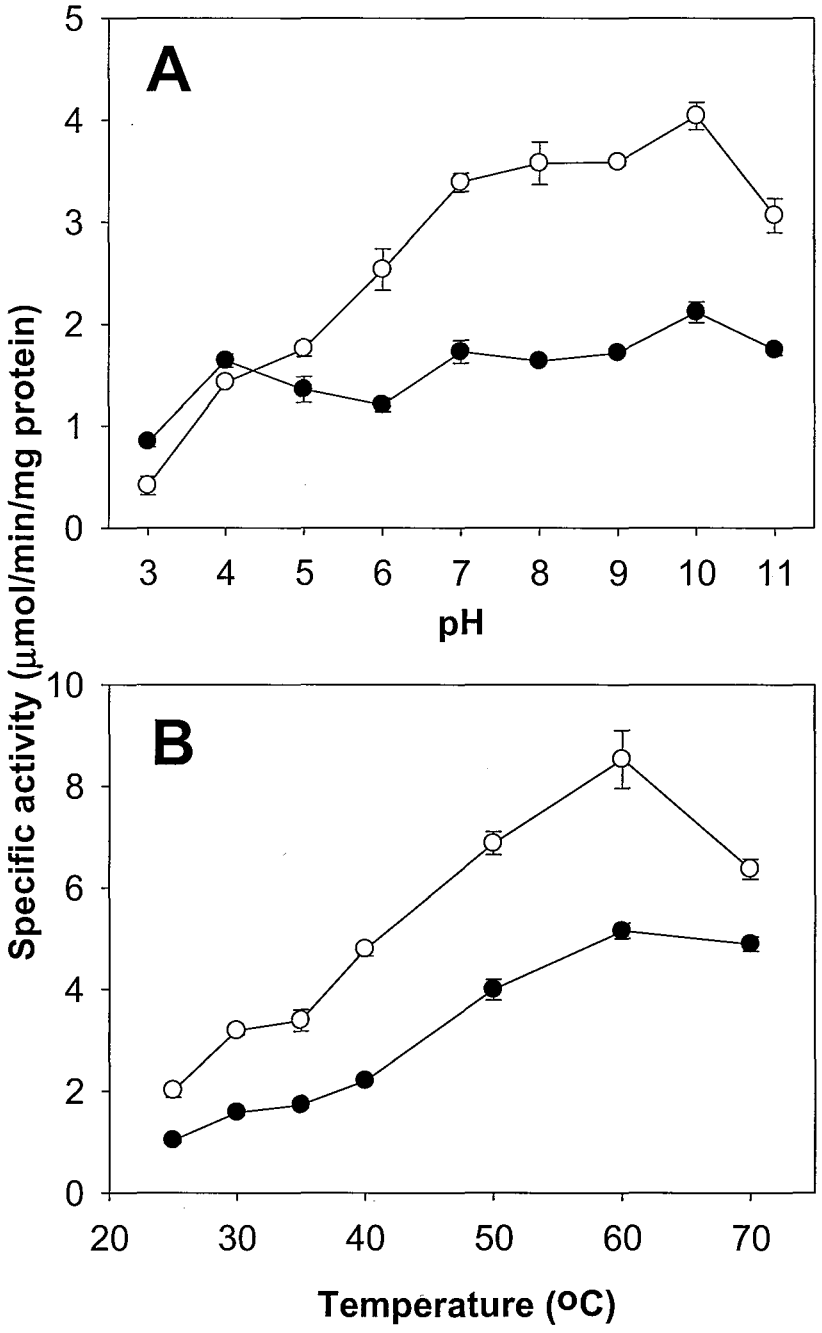

Fig. 15. The effect of pH (A) and temperature (B) on α-glucosidase activity in extracts of Bm5 (•) and Sf9 (○) cells. The α-glucosidase activity was measured with *p*-nitrophenyl α-D-glucopyranoside substrate. Each point represents the mean value of three experiments. The error bars indicate standard deviation of the mean.

Discussion

α-Glucosidase I removes the terminal glucose residue of $Glc_3Man_9GlcNAc_2$ that is attached to a nascent glycoproteins, a critical initial step of the *N*-linked glycosylation process. The inhibition of α-glucosidase interrupts the maturation of glycoproteins that are found on the envelope of enveloped viruses resulting in reduced viral particle assembly, suppression of virion secretion, and interference of the attachment of virions to host cells (Jacob *et al.*, 2007; Pelletier *et al.*, 2000). DNJ is an iminosugar which can competitively inhibit α-glucosidase I and II in the ER (Wojczyk *et al.* 1995). Inhibition of the formation of mature virions and reduced infectivity have been found in enveloped viruses such as HBV, human immunodeficiency virus (HIV), herpes simplex virus type 1, influenza virus, bovine viral diarrhea virus (BVDV), dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus, and HCV following exposure to DNJ and its derivatives (Durantel *et al.* 2001; Dwek *et al.* 2002; Mehta *et al.* 2002; Woodhouse *et al.* 2008). In this chapter, the results showed that the presence of DNJ reduces the budded virus titer of AcMNPV but not BmNPV. This suggested that α-glucosidase activity that processes the glycoproteins of AcMNPV is dramatically more sensitive to DNJ.

In chapter 3, I showed that α-glucosidase activity in the midgut of *B. mori*, a specialist insect that feeds exclusively on mulberry leaf, is less sensitive to DNJ in comparison to α-glucosidase activity in the midgut of *Antheraea yamamai*, a generalist insect. Hirayama *et al.* (2007) have also shown that sucrase and trehalase in the midgut of *B. mori* are less sensitive to DNJ in comparison to the corresponding enzymes of *Samia ricini*, a generalist herbivore. I found similar results in this study. The sensitivity of α-glucosidase activity in the Bm5 cell extract was significantly lower (i.e.,

40-fold higher IC_{50}) in comparison to that found in the Sf9 cell extract when PNPG, a known substrate of α-glucosidase II (Moremen *et al.* 1994), was used as a substrate. Since it appeared that DNJ did not induce any effects on cell viability, I hypothesized that the reduction in AcMNPV titer that was found in Sf9 cells grown in DNJ containing medium resulted from the inhibition of α-glucosidase I and/or α-glucosidase II that are important for the formation and/or release of mature BVs. In contrast, in Bm5 cells these enzymes appeared to be less sensitive to the effects of DNJ, so that the formation and/or release of BmNPV BVs from Bm5 cells that were grown in DNJ containing medium was unaffected. My findings are the first to show a differential response in terms of the inhibitory effects of DNJ on α-glycosidase activity in insects and on the ability of baculoviruses to efficiently replicate. Elucidation of the molecular mechanism behind this differential sensitivity to DNJ will provide a probe for the study of the comparative evolution of insects and nucleopolyhedroviruses.

Summary

1-Deoxynojirimycin (DNJ) is an alkaloid that is found at relatively high concentrations in mulberry leaf and tissues of the silkworm, *Bombyx mori*. DNJ is a well known inhibitor of α-glucosidase, an enzyme that is involved in the early stages of the *N*-linked glycoprotein synthesis pathway. α-Glucosidase activity in the cell extract from *B. mori*-derived Bm5 cells showed approximately 40-fold less sensitivity to DNJ than α-glucosidase activity in the cell extract from *Spodoptera frugiperda*-derived Sf9 cells. The replication of *B. mori* nucleopolyhedrovirus (BmNPV) was not inhibited when it was propagated in BmN cells that were grown in medium containing up to 10 mM DNJ. In contrast, the replication of *Autographa californica* multiple NPV

(AcMNPV) was reduced by 67% when it was propagated in Sf9 cells that were grown in medium containing 10 mM DNJ. The viability of Bm5 and Sf9 cells that were grown in medium containing up to 10 mM DNJ was not affected. These results suggested that the reduced replication of AcMNPV was the result of the higher sensitivity of α-glucosidase activity in Sf9 cells to DNJ.

Chapter 5. Identification of the Genes Involved in 1-Deoxynojirimycin Synthesis in *Bacillus subtilis* MORI 3K-85

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 can be synthesized by removing the anomeric hydroxyl group of nojirimycin (Asano *et al.*, 2000; Watson *et al.*, 2001). DNJ has been isolated from the roots of mulberry trees and called moranoline (Asano *et al.*, 2001; Watson and Nash, 2000). DNJ is also produced by several strains of *Bacilli* (Hardick and Hutchinson, 1993; Stein *et al.*, 1984; Watson and Nash, 2000) and *Streptomyces* (Asano *et al.*, 2000; Ezure *et al.*, 1985; Hardick *et al.*, 1991; Paek *et al.*, 1997; Schedel *et al.*, 2008; Watson *et al.*, 2001; Watson and Nash, 2000). However, the enzyme or gene responsible for DNJ biosynthesis has not been identified.

In this chapter, the DNJ biosynthetic genes of *Bacillus subtilis* MORI 3K-85, a DNJ-producing strain were identified. Furthermore, the recombinant *Escherichia coli* strain harboring DNJ biosynthetic genes can produce DNJ in the culture supernatant .

Materials and Methods

Bacterial strains, plasmids and culture conditions

The *B. subtilis* MORI 3K-85 strain was selected after the γ–irradiation of *B. subtilis* MORI (KCCM-10450) at dose of 3 KGy. *E. coli* EPI 300 [F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*dlacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL* (Str^R) *nupG trfA dhfr*] (Epicentre, USA) and DH5α (*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZ ΔM15*) (Clontech, USA) were used as host strains for genomic library construction and gene cloning, respectively. CopyControl pCC1BAC Cloning-Ready Vector (Epicentre) and pEXT20 (Dykxhoorn *et al.,* 1996) were used for genomic library construction and the induced expression of the operon, respectively. *E. coli* was cultured in LB broth at 37°C with shaking at 180 rpm. When necessary, chloramphenicol (12.5 μg/ml), ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) or isopropyl-β-D-thiogalactopyranoside (IPTG) (50 μg/ml) was added to the culture medium. The plasmids and chromosomal DNA were prepared using the GeneAll XPREP™ Plasmid SV Mini kit (GeneAll biotechnology, Korea) and the Wizard Genomic DNA Purification kit (Promega, USA), respectively. Restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation were all carried out as described previously (Sambrook *et al.* 1989).

Construction and screening of the genomic DNA library

For chromosomal DNA extraction, the *B. subtilis* MORI 3K-85 strain was cultured in Difco™ YM broth [Becton Dickinson (BD) and Company, USA] at 37°C with shaking at 180 rpm. The genomic DNA of *B. subtilis* MORI 3K-85 prepared using a Promega kit was partially digested with *Bam*HI and the DNA fragments were then ligated into the CopyControl pCC1BAC Cloning-Ready Vector. The ligation mixture was then transformed into TransforMax™ EPI300™ Electrocompetent *E. coli*

cells by electroporation (Dower *et al.*, 1988). The transformed cells were incubated for 1 h at 37°C with shaking at 220 rpm, and the aliquots were spread onto an LB agar plate containing chloramphenicol (12.5 μg/ml), X-Gal (40 μg/ml), and IPTG (50 μg/ml). After overnight incubation at 37°C, white colonies were selected and inoculated into LB broth containing chloramphenicol (12.5 μg/ml). The cells were incubated for 12 h at 37°C to determine the α-glucosidase inhibitory activity to screen clones harboring the DNJ biosynthetic genes of *B. subtilis* MORI 3K-85. Five positive clones showing α-glucosidase inhibitory activity were selected and their α-glucosidase inhibitory activities were compared. Among them, the clone C36-4, which exhibited the highest α-glucosidase inhibitory activity, was chosen for further analysis.

Sequence analysis

The recombinant plasmid isolated from the *B. subtilis* MORI 3K-85 genomic DNA library clone C36-4 was termed pCC1BAC-DNJbb, and the nucleotide sequence of the inserted DNA fragment was determined by Solgent Co. (Korea). The resulting sequence information was used in a search of the GenBank at the National Center for Biotechnology Information (NCBI) using the BLAST program. An additional sequence analysis and a comparison were also performed using web-based programs (http://www.uniprot.org and http://gib.gen-es.nig.ac.jp). The promoter sequence and putative transcription factor binding sites on the upstream region of the DNJ biosynthetic genes were predicted with the web-based DBTBS (a database of *B. subtilis* promoters and transcription factors) analysis program (http://dbtbs.hgc.jp/) (Sierro *et al.*, 2008). The rho-independent terminator was predicted by ARNold, a web-based

terminator finding program (http://rna.igmors.u-psud.fr/toolbox/arnold/) (Naville *et al.*, 2011).

Subcloning and expression of DNJ biosynthetic genes

To identify the minimum set of genes required to direct DNJ biosynthesis in the heterologous host *E. coli*, various sizes of genomic DNA fragments from pCC1BAC-DNJbb were subcloned. First, a 5.56 kb *Hind*III fragment containing four putative genes was ligated into pCC1BAC, resulting in pCC1BAC-DNJhh. A 4.2 kb *Hind*III-*Nru*I fragment containing three genes was cloned into the *Hind*III site of pCC1BAC, resulting in pCC1BAC-DNJhn. To subclone the putative DNJ biosynthetic genes into the multi-copy expression vector pEXT20 under the IPTG-inducible P_{tac} promoter, a 3.3 kb DNA fragment containing *gabT1-yktc1-gutB1* genes was PCR amplified using pCC1BAC-DNJbb as template and the primer pairs DNJt1-N (5'AAAGAGCTCATGTTGGTAGTGGGGACTA-3', *Sac*I) and DNJt-C (5'-AAAGGATCCTACGCAAGGTGAATGCTG-3', *BamH*I). The PCR product was digested with *Sac*I and *BamH*I and ligated with *Sac*I-*BamH*I-digested pEXT20, resulting in pEXT20-DNJ0. Likewise, a 3.2 kb DNA fragment containing *gabT1-yktc1-gutB1* genes but starting from an ATG codon at 69 bp downstream from the GTG codon was PCR-amplified with pCC1BAC-DNJbb as the template and primer pairs DNJt2-N (5'-AAAGAGCTCATGGAAAGAGG TGAAGGC -3', *Sac*I) and DNJt-C. The PCR product was digested with *Sac*I and *BamH*I and ligated with *Sac*I-*BamH*I-digested pEXT20, resulting in pEXT20-DNJ1.

Induction of the expression of the gabT1-yktc1-gutB1 operon

To investigate the effects of an increase in the gene dosage on the expression of the α-glucosidase inhibitory activity, the copy numbers of CopyControl pCC1BAC-based plasmids were induced using a copy number autoinduction solution according to the manufacturer's instructions. The relative amounts of the pCC1BAC control vector, pCC1BAC-DNJbb, and pCC1BAC-DNJhn isolated from a control culture and autoinduced cultures were compared by separation on 0.8% agarose gel by electrophoresis.

To determine the effects of the induced expression of the *gabT1-yktc1-gutB1* operon on DNJ production in *E. coli*, pEXT20-DNJ0 was transformed into the *E. coli* DH5α strain. The transformant was incubated in LB broth containing ampicilline (100 μg/ml) at 37°C with shaking at 180 rpm. To induce expression of the *gabT1-yktc1-gutB1* operon cloned under the P_{tac} promoter, 0.1 mM IPTG was added to the exponentially growing cultures. Samples were taken after 4 h of induction and subjected to α-glucosidase inhibition assay. To determine the expression of the *gabT1-yktc1-gutB1* operon, whole cell extracts of the induced and uninduced cultures were separated on SDS-polyacrylamide gel by electrophoresis.

Analytic procedures

To screen the clones harboring recombinant vectors containing DNJ biosynthetic genes, the α-glucosidase inhibitory activities were measured. To prepare the α-glucosidase solution, 0.8% rat intestinal acetone powder (Sigma, USA) was dissolved in 0.1 M potassium phosphate buffer (pH 6.8) and the spun supernatant was collected. The culture broths of the genomic DNA library clones were heated at 100°C for 10 min. The supernatants were harvested by centrifugation (6,000 × g, 10 min). The reaction

mixture for the α-glucosidase inhibition assay was prepared by the addition of 5 μl of the boiled culture supernatant, 75 μl of 0.1 M potassium phosphate buffer (pH 6.8), and 50 μl of 12 mM *p*-nitrophenyl-α-glucopyranoside. Finally, 20 μl of 0.8% α-glucosidase solution was added into the mixture. After incubation at 37°C for 35 min, the reaction was terminated by the addition of 50 μl of 200 mM sodium carbonate and the absorbance at 405 nm was measured using a microplate reader (Molecular Devices, USA). To calculate the α-glucosidase inhibitory activity, the formula shown below was used. Clones having more than 20% of α-glucosidase inhibitory activity were selected for further analysis (Cho *et al.*, 2008; Scofield *et al.*, 1986).

$$\text{Inhibition (\%)} = \left(1 - \frac{A_{405}\ \text{(inhibition)} - A_{405}\ \text{(control)}}{A_{405}\ \text{(enzyme)} - A_{405}\ \text{(blank)}}\right) \times 100$$

To test the possibility of DNJ production in the supernatant of the selected clones, a thin-layer chromatography (TLC) analysis was performed by spotting 10 μl of culture broths onto Silica gel 60 F_{254} TLC plates (Merck, Germany) and separating the samples in a TLC chamber containing a solvent mixture of propanol-acetic acid-water (4 : 1 : 1) at room temperature. As the standard control, 0.5 μl of 10 mM DNJ (Sigma, USA) was spotted under the same conditions. DNJ spots were detected by the spraying of a chlorine-*o*-tolidine reagent.

To identify the nature of the α-glucosidase activity inhibitor in the culture supernatant, the clone C36-4 was inoculated into 5 ml of LB medium in a 50 ml conical tube and incubated overnight at 37°C with shaking at 220 rpm. One milliliter of the overnight culture broth was inoculated into 500 ml of LB medium in a 1 L Erlenmeyer flask and incubated at 37°C with shaking at 220 rpm for 24 h. The culture broth was heated at 100°C for 10 min and centrifuged at 6,000 × g for 10 min. The supernatant

was concentrated to 50 ml using a vacuum evaporator (Büchi, Switzerland) to partially purify DNJ by two-step ion exchange chromatography using a previously described method (Asano *et al.*, 2001; Cho *et al.*, 2008).

Fifty milliliters of the concentrated supernatant was loaded into an ion-exchanger Amberlyst 15 (Sigma, USA) column (100 × 10 mm, H^+). To remove the unbound part of the concentrated supernatant, the column was washed with 10 ml of deionized water. The column was eluted with 80 ml of 0.5 N NH_4OH and 1 ml fractions were collected. The eluate in each fraction was subjected to a TLC analysis to detect DNJ as described above. Active fractions were combined and concentrated to 4 ml by a vacuum evaporator. The concentrated eluate was loaded into a Dowex 1 × 2-100 (Sigma, USA) column (100 × 10 mm, OH^-), and the column was eluted with 40 ml of deionized water. Again, active fractions were selected, combined, and concentrated to 4 ml using a vacuum evaporator.

For a qualitative determination of DNJ, the partially purified samples were labeled using 9-fluorenylmethyl chloroformate (FMOC-Cl) and were then analyzed via a HPLC analysis, as described previously (Cho *et al.*, 2008; Kim *et al.*, 2003). First, 10 μl of ion exchange fraction, 10 μl of 0.4 M potassium borate buffer (pH 8.5) and 20 μl of 10 mM FMOC-Cl in CH_3CN was mixed into Eppendorf tube and was allowed to react at 20°C for 20 min. The reaction was then terminated by the addition of 10 μl of 0.1 M glycine. To stabilize the DNJ-FMOC, 950 μl of 0.1% acetic acid was added into the reactant and then filtered through a 0.2 ㎛ syringe filter (Nalgene, nylon filter). A 10 μl aliquot of the filtrate was injected into a Capcell Pak C_{18} MG HPLC column (φ: 4.60 × 250 mm) (Shiseido, Japan) and separated in a mobile phase of acetonitrile : 0.1% acetic acid (1 : 1, v/v) at a flow rate of 1 ml min^{-1}. The DNJ-FMOC fluorescence

(excitation 254 nm, emission 322 nm) was monitored using a FL3000 fluorescence detector.

To confirm the isolation of DNJ via the molecular mass, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted using an Agilent 1200 series HPLC system and an Agilent 6410B system (Agilent Technologies, USA). Chromatographic separation was performed on an Agilent Epic HILIC-HC column (4.6 × 150 mm, 3 μm). The column temperature was maintained at 40°C and the sample injection volume was 2 μL. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) using gradient elution (0-0.1 min, 5% A and 95% B; 0.1-15 min, 80% A and 20% B; 15-20 min, 20% A and 80% B). It was delivered at a flow rate of 0.8 ml/min. Mass spectrometry was determined in positive mode by selected ion monitoring (SIM) and Scan mode. The mass spectrometer condition was set with a gas (N_2) flow rate of 35 L/min, a gas temperature of 320°C, a capillary voltage of 4 kV, and a fragmentor voltage of 150 V (Nakagawa *et al.*, 2010).

Results

Construction of genomic DNA library of B. subtilis MORI 3K-85

In the previous attempt to isolate a new DNJ-producing bacterial strain, several hundred bacterial strains isolated from soil and traditional Korean foods were screened. *B. subtilis* MORI, which was isolated from Chungkookjang, a Korean traditional fermented soybean food, was identified as a new DNJ-producing strain (Kim *et al.*, 2011). Subsequently, I carried out random mutagenesis by γ-irradiation to improve the DNJ production of the *B. subtilis* MORI strain. As a result, I obtained a *B. subtilis*

MORI 3K-85 strain whose DNJ production was increased by more than 10 fold compared to that of its parental strain *B. subtilis* MORI (Kim *et al.*, 2011). In this study, in an effort to isolate the genes involved in DNJ biosynthesis, a genomic DNA library was constructed by cloning the *Bam*HI digested genomic DNA of *B. subtilis* MORI 3K-85 into the CopyControl pCC1BAC Cloning-Ready vector followed by transformation into *E. coli* EPI300 competent cells. More than 20,000 transformants harboring the genomic DNA library vectors were selected and analyzed in terms of their inhibitory activities against α-glucosidase. Five clones, C5-26, C26-40, C36-4, C88-29 and C91-31, exhibited over 20% of α-glucosidase inhibitory activity (Table 8). Because clone C36-4 showed the highest α-glucosidase inhibitory activity of 48%, it was used for further characterization.

Identification of DNJ

First, to test whether this clone produced DNJ, the culture supernatant was analyzed by analytical TLC using a silica gel plate. It has been shown that the supernatant of clone C36-4 contains a substance which has an Rf value identical to that of the standard DNJ of 0.375 (data not shown). To investigate the identity of the α-glucosidase inhibitor further, the culture supernatant of clone C36-4 was subjected to two-step ion exchange chromatography for DNJ purification. When the fractions showing α-glucosidase inhibitory activity were pooled and analyzed by HPLC after being derivatized with FOMC-Cl, it was clearly shown that the fraction contained DNJ, which was detected at the same retention time (RT) of 3.8 min noted with the standard substance (Fig. 16).

Nucleotide sequence analysis of the genomic library clone conferring DNJ biosynthesis

To identify the genes that allowed the production of DNJ in *E. coli*, the size of the genomic DNA fragment inserted in the pCC1BAC-DNJbb isolated from the genomic DNA library clone C36-4 was determined by restriction enzyme digestion. It was found that the pCC1BAC-DNJbb contained a *B. subtilis* MORI 3K-85 genomic DNA fragment of about 10 kb (data not shown). The nucleotide sequences of the inserted DNA were determined, and the result showed that it contained a 10,086 bp DNA fragment flanked by *Bam*HI restriction sites at both ends. Based on the nucleotide sequence of this fragment, seven open reading frames encoding a polypeptide longer than 100 amino acid residues were predicted. When the entire nucleotide sequence was used to search the NCBI databases, two groups of genomic DNA stretches were identified to have strong homology with the insert of pCC1BAC-DNJbb. The sequence of the insert in pCC1BAC-DNJbb detected a homologous sequence with a similar length on the genome of *B. amyloliquefaciens* FZB42. According to the genome sequence information, in this region of *B. amyloliquefaciens* FZB42, seven ORFs were predicted to code for the following proteins, respectively: GlcP1 (404 aa, transmembrane transport), GabT1 (425 aa, transaminase), Yktc1 (316 aa, phosphatase), GutB1 (348 aa, oxidoreductase), YbaR (434 aa, transmembrane transporter), YbaS (327 aa, bile acid:sodium symporter), and YbaA (210 aa, unknown) (Table 9). In addition, it was noted that the sequence of the insert in pCC1BAC-DNJbb has the homology to genome sequence of *B. subtilis* 168. However, the homology pattern was different from that of *B. amyloliquefaciens* FZB42, and it could be divided into three regions depending on the homology to the *B. subtilis* 168 genome.

The first 2.3 kb sequence of the pCC1BAC-DNJbb insert has a homologous region in the genome of *B. subtilis* 168. However, the next 4.2 kb stretch of the pCC1BAC-DNJbb insert does not have any homologous sequence in the genome of *B. subtilis* 168. The last 3.6 kb stretch of pCC1BAC-DNJbb insert showed 93% identity to the region directly next to the first homologous region (Fig. 17). It is noteworthy that the *B. subtilis* 168 strain was not able to synthesize DNJ, whereas *B. amyloliquefaciens* FZB42 was able to synthesize DNJ. Thus, we closely examined the pCC1BAC-DNJbb sequences having a homologous region in the genome of *B. amyloliquefaciens* FZB42 but not in the genome of the *B. subtilis* 168 strain. This region was determined to have a putative operon consisting of the three open reading frames of *gabT1*, *yktc1* and *gutB1* (Fig. 18). The nucleotide sequence homologies between the *gabT1*, *yktc1* and *gutB1* ORFs of the *B. subtilis* MORI 3K-85 strain and those of *B. amyloliquefaciens* FZB42 were 86%, 78% and 75%, respectively (data not shown). Interestingly, the nucleotide sequence of the *gabT1*, *yktc1* and *gutB1* genes of *B. subtilis* MORI 3K-85 strain was identical to that of *B. subtilis* MORI strain (data not shown).

Identification of DNJ biosynthetic genes

To determine the gene sets required to confer the ability to produce DNJ to *E. coli*, the DNJ production of *E. coli* transformants harboring different sets of *B. subtilis* MORI 3K-85 genes from pCC1BAC-DNJbb were monitored. It was found that the *E. coli* transformant harboring subclone pCC1BAC-DNJhh containing *gabT1*, *yktc1*, *gutB1* and *ybaR* genes shows α-glucosidase inhibitory activity. Furthermore, when the subclone pCC1BAC-DNJhn containing *gabT1*, *yktc1*, and *gutB1* genes was transformed, the resulting recombinant *E. coli* strain was also able to produce α-glucosidase

inhibitory activity. As shown in Table 10, both transformants of pCC1BAC-DNJhh and pCC1BAC-DNJhn showed increased α-glucosidase inhibitory activity of about 70% compared to that of clone pCC1BAC-DNJbb. To confirm the production of DNJ by the recombinant *E. coli* strain harboring pCC1BAC-DNJhn, DNJ was purified by two-step ion exchange chromatography and analyzed by analytical LC MS/MS, as described in the Materials and Methods section. It was found that DNJ existed in the culture supernatant at the same RT of 6.16 noted with the standard DNJ (Fig. 19). Furthermore, about 0.2 mg/L of DNJ was detected in the purified sample (data not shown). Thus, when considering the possible loss of DNJ during purification, it can be predicted that the culture supernatant of this transformant contained about 6 mg/L of DNJ. Taken together, these results suggest that the *gabT1*, *yktc1*, and *gutB1* genes of *B. subtilis* MORI 3K-85 are sufficient to lead to DNJ synthesis in the heterologous host *E. coli*.

Growth-phase-dependent synthesis of DNJ in recombinant E. coli strain

To investigate whether it is possible to increase DNJ production by inducing the expression of DNJ biosynthetic genes, different approaches were employed. Based on the property of the pCC1BAC vector system, whose copy number could be induced by treatment of the inducer, the α-glucosidase inhibitory activities of transformants harboring pCC1BAC-DNJbb or pCC1BAC-DNJhn were compared with and without copy number induction. I was not able to observe the expected increase in the α-glucosidase inhibitory activities upon copy number induction. It was rather found that the α-glucosidase inhibitory activities increased at the stationary phase of the growth (Fig. 20). To increase the expression of all three genes involved in DNJ biosynthesis

further, the *gabT1-yktc1- gutB1* genes were cloned into the multi-copy expression vector pEXT20 under a strong IPTG-inducible P_{tac} promoter, resulting in pEXT20-DNJ0 (Fig. 21A). The *E. coli* DH5α strains harboring pEXT20-DNJ0 were induced by IPTG treatment, and whole cell lysates were separated on 10% PAGE (Fig. 21B). Some proteins have been clearly shown to be overexpressed by induction; however, the level of α-glucosidase inhibitory activity was not affected by IPTG induction but instead increased at the stationary phase of growth (Fig. 21C). To assess the possibility of utilizing the ATG codon, which is located 69 bp downstream from the originally predicted GTG codon, for translational initiation of the *gabT1* gene, the α-glucosidase inhibitory activity of the transformant harboring pEXT20-DNJ1 was determined, but no activity was detected, suggesting that the first 23 aa residues of the gabT1 encoded protein is important for its activity.

Table 8. Inhibitory activities against α-glucosidase by the culture broths of various selected clones

Clone	α-Glucosidase inhibitory activity (%)[b]
Control[a]	0.0
C5-26	20.0
C26-40	32.0
C36-4	48.0
C88-29	36.0
C91-31	38.0

[a]*E. coli* transformant harboring the empty CopyControl pCC1BAC Cloning-Ready Vector (Epicentre).

[b]The cells were incubated for 12 h at 37°C to determine the α-glucosidase inhibitory activity as described in the "Materials and Methods" section.

Table 9. Experimentally defined gene functions in the *B. amyloliquefaciens* FZB42 which is matched with DNA sequence of the inserted DNA in the clone C36-4

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
ybaR	434	YbaR	Integral to membarne and transpoter activity	Transmembrane transport
ybaS	327	YbaS	Bile acid:sodium symporter activity	Sodium ion transport
ybaA	210	YbaA	Unknown	Unknown

Table 10. Inhibitory activities against α-glucosidase by the culture broths of the selected clones harboring recombinant plasmids containing various sizes of DNA fragments

Type of DNA fragments (Kb)	**α-Glucosidase inhibitory activity (%)**
pCC1BAC-DNJbb (10.07)	51.0
pCC1BAC-DNJhh (5.56)	71.7
pCC1BAC-DNJhn (4.20)	74.5

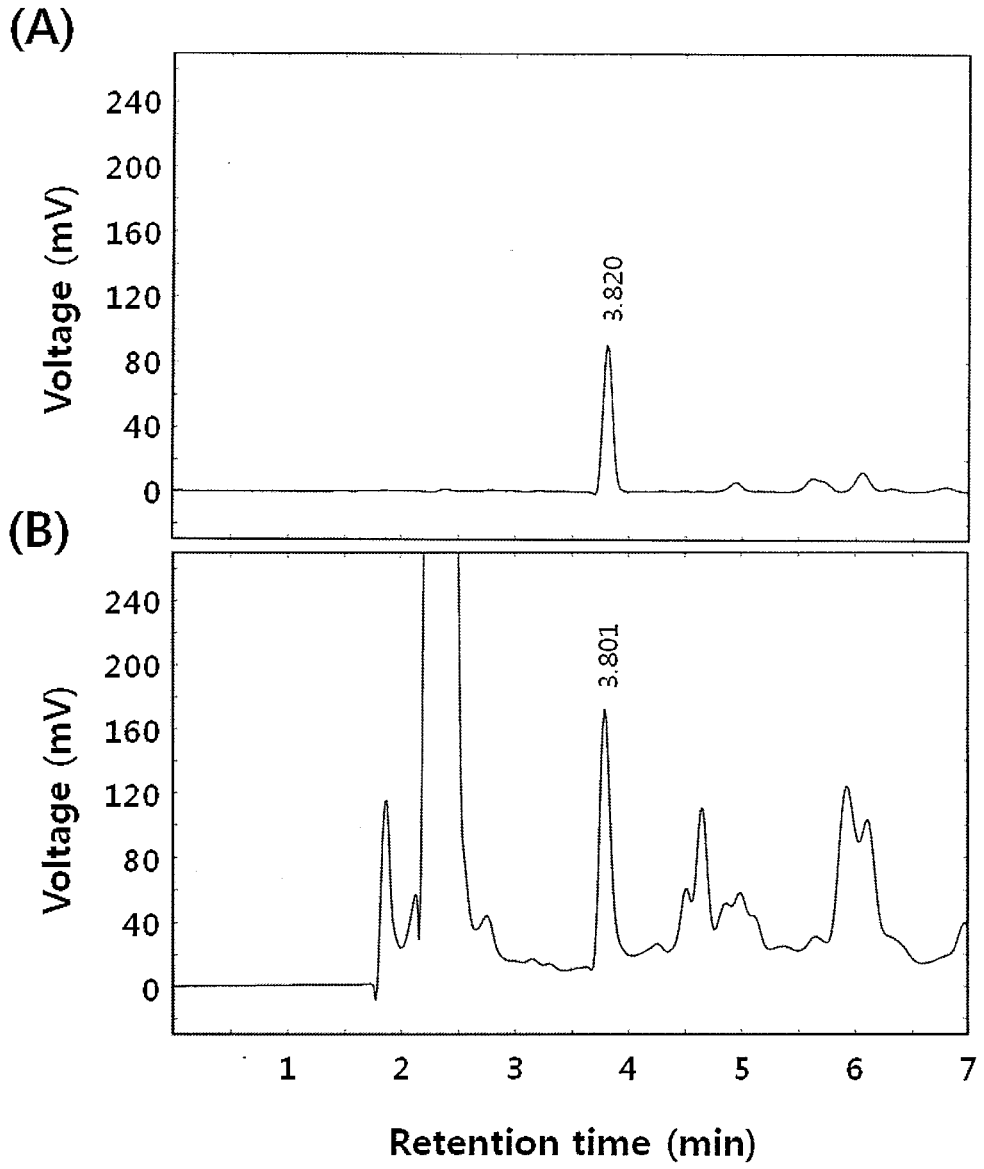

Fig. 16. Identification of DNJ in the culture broth of the recombinant *E. coli* strain harboring pCC1BAC-DNJbb. A. HPLC chromatogram of standard DNJ, B. HPLC chromatogram of the culture broth of *E. coli* strain harboring pCC1BAC-DNJbb (clone C36-4).

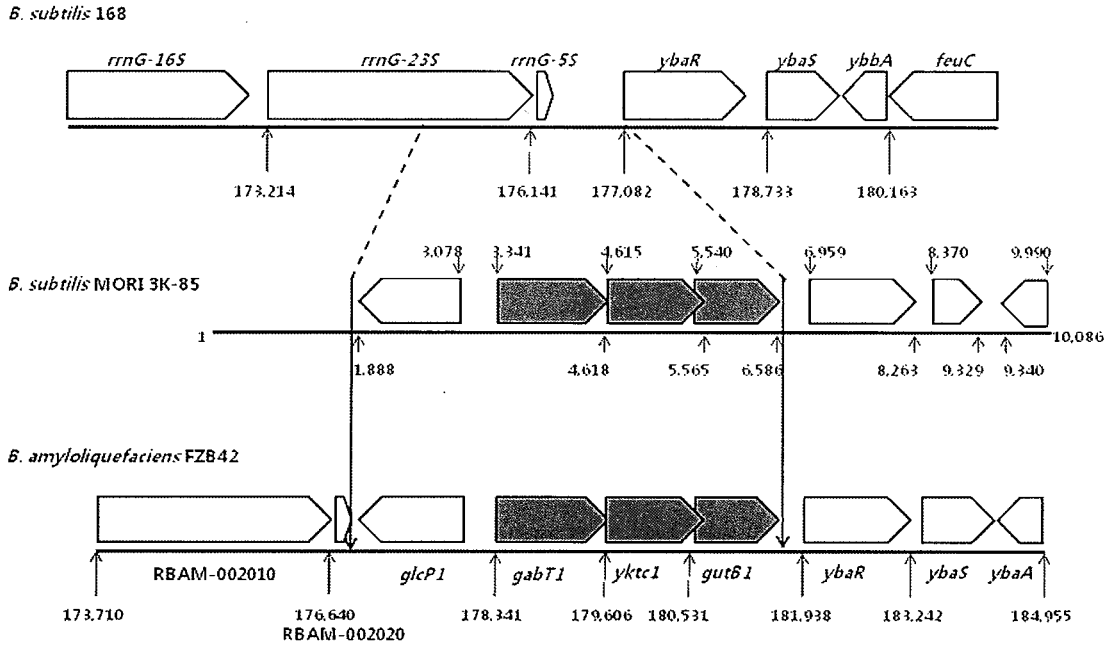

Fig. 17. Comparative organization of the DNJ biosynthetic genes of different bacilli strains. Homologous sequences of the putative DNJ biosynthetic genes of *B. subtilis* MORI 3K-85 were identified in the genome sequence of *B. amyloliquefaciens* FZB42, but they were absent in the genome sequence of *B. subtilis* 168. The shaded boxes represent *gabT1*, *yktc1* and *ybaR* genes which share 86%, 78% and 75% of their nucleotide sequence homologies between corresponding genes of *B. subtilis* MORI 3K-85 and *B. amyloliquefaciens* FZB42.

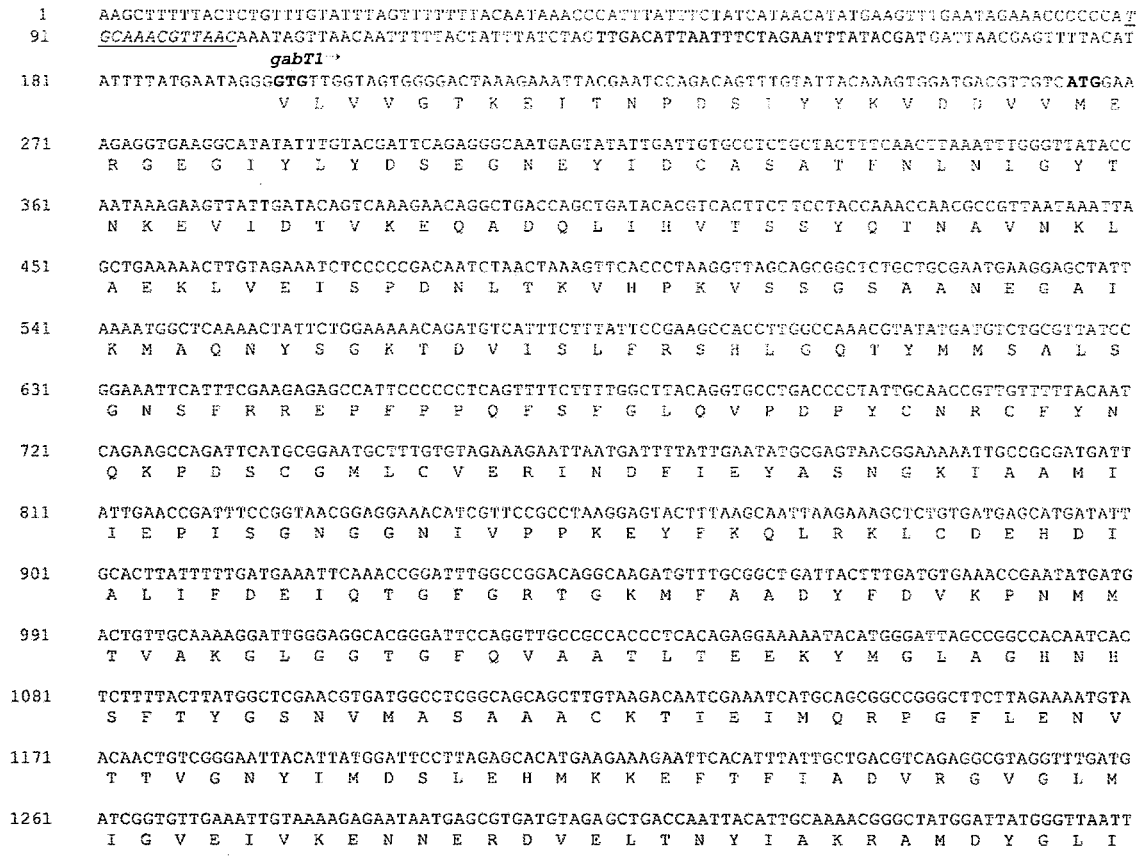

Fig. 18. The nucleotide sequence and deduced amino acid sequence of the DNJ biosynthetic genes from *B. subtilis* MORI 3K-85. The nucleotide sequence in the shaded box is the putative Sigma factor A (SigA) dependent promoter predicted by the web-based DBTBS (a database of *Bacillus subtilis* promoters and transcription factors) analysis program (http://dbtbs.hgc.jp/) (Sierro *et al.*, 2008). A putative CcpA binding sequence was identified upstream of the promoter (underlined italic letters). The putative ribosome binding sites and initiation codons of each gene are marked as boxed letters and bold letters in shaded boxes, respectively. The alternative ATG initiation codon for the *gabT1* gene is marked in bold letters. The rho-independent terminator (the underlined sequences where bold letters represent the stem region) was predicted by ARNOLD, a web-based terminator finding program (http://rna.igmors.u-psud.fr/toolbox/arnold/) (Naville *et al.*, 2011).

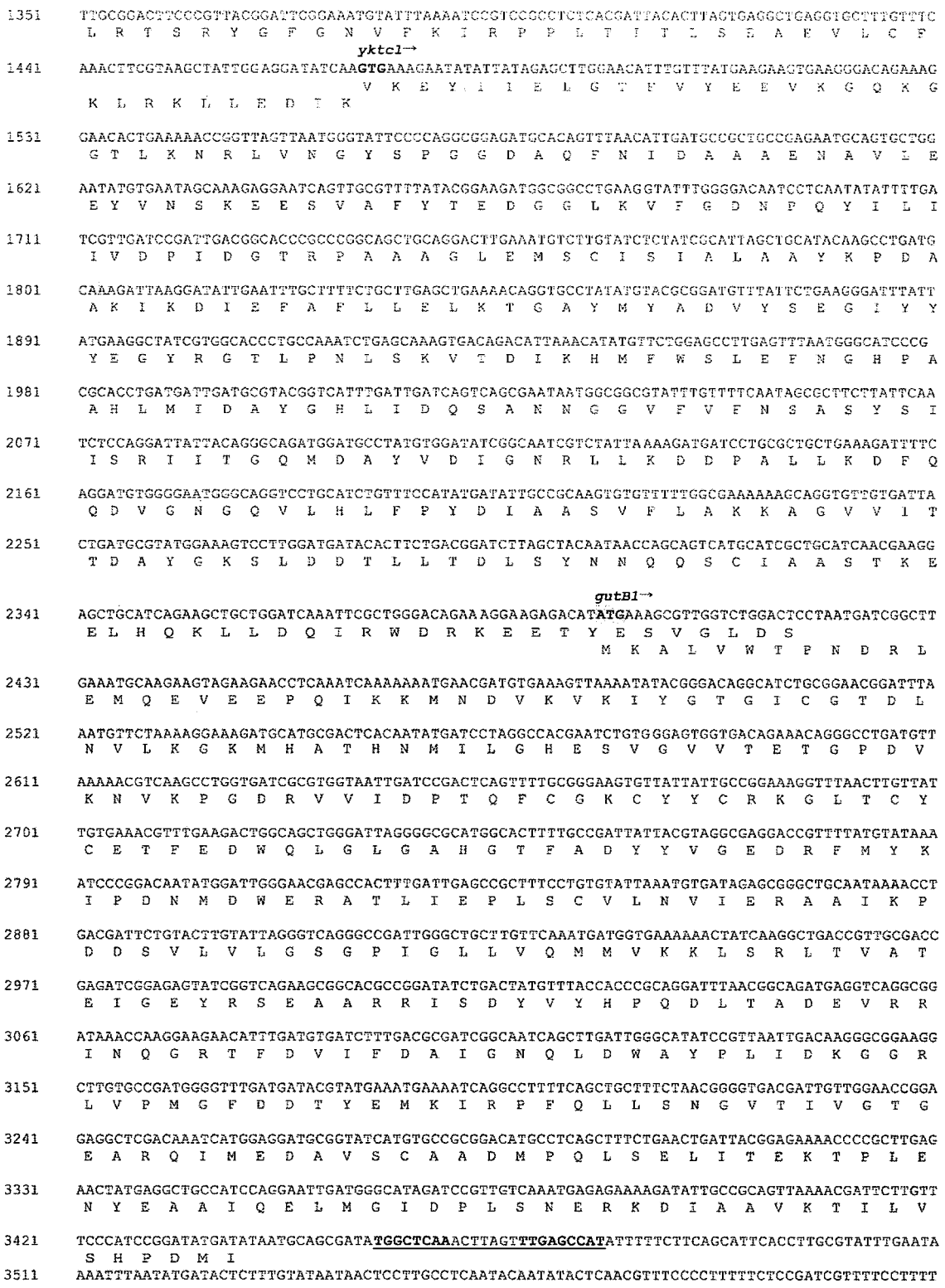

Fig. 18. Continued.

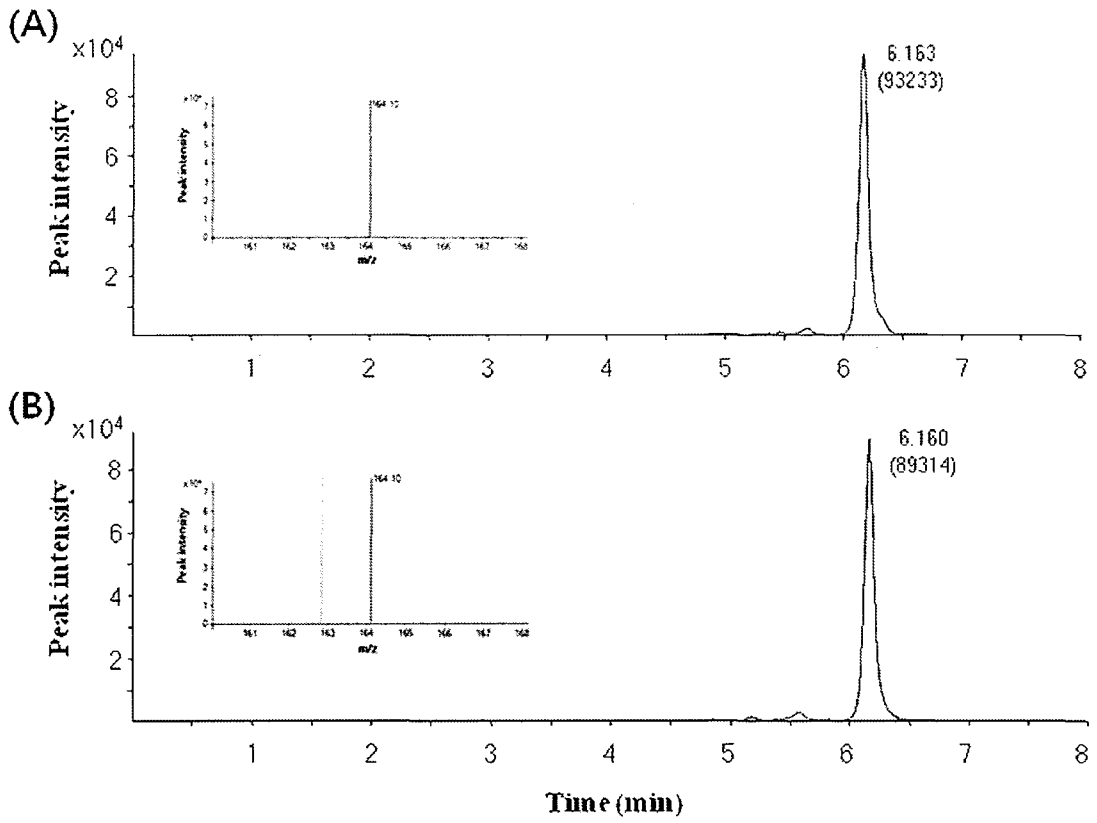

Fig. 19. Typical selected ion monitoring (SIM) chromatograms of DNJ. Standard DNJ (A) and the culture broth of *E. coli* transformant harboring pCC1BAC-DNJhn (B) were analyzed by LC-MS/MS. Mass spectrometry was determined in positive mode by SIM and Scan mode. Inserts represent the MS spectra of each sample.

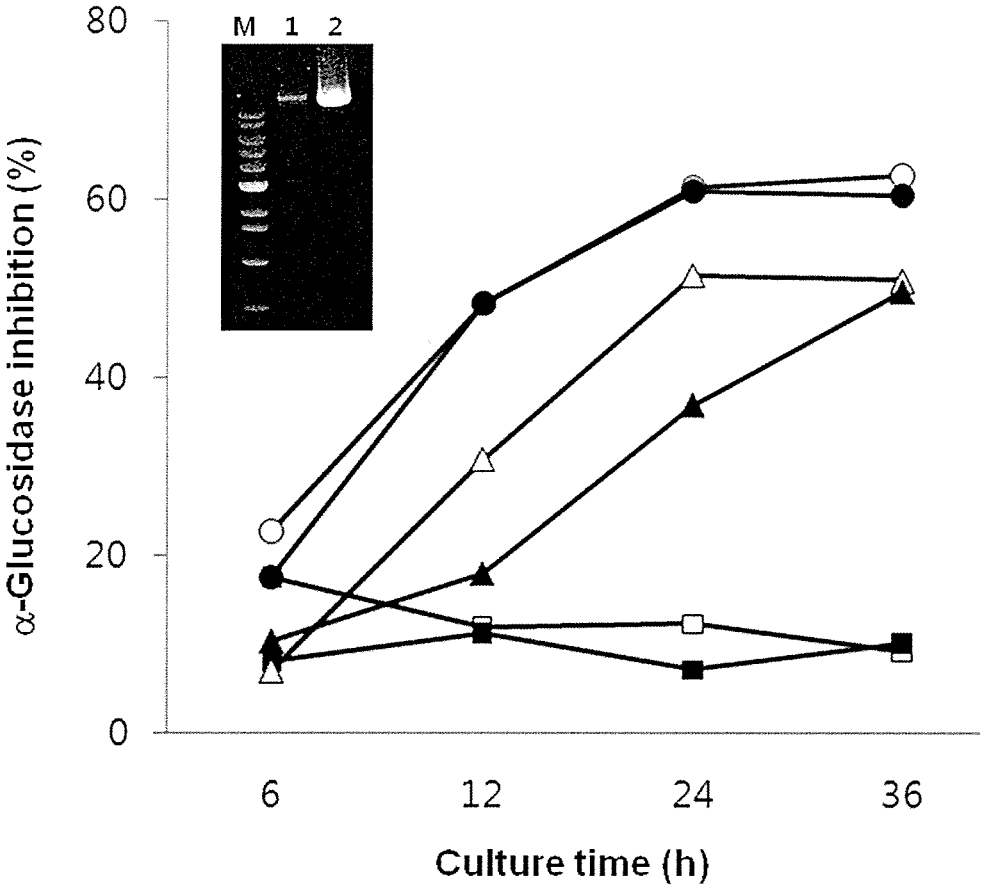

Fig. 20. Effects of the induced copy number of recombinant plasmids on the α-glucosidase inhibitory activities. *E coli* EPI300 strains harboring pCC1BAC (square), pCC1BAC-DNJbb (triangle), and pCC1BAC-DNJhn (circle) were cultured in the absence (open symbols) or presence of plasmid copy number induction (closed symbols). Samples were taken at the indicated time points and assayed for α-glucosidase inhibitory activity. In the insert, to confirm the plasmid copy number induction, pCC1BAC-DNJbb vectors prepared from the same volumes of uninduced (lane 1) and induced (lane 2) cultures were separated by electrophoresis on 0.8% agarose gel.

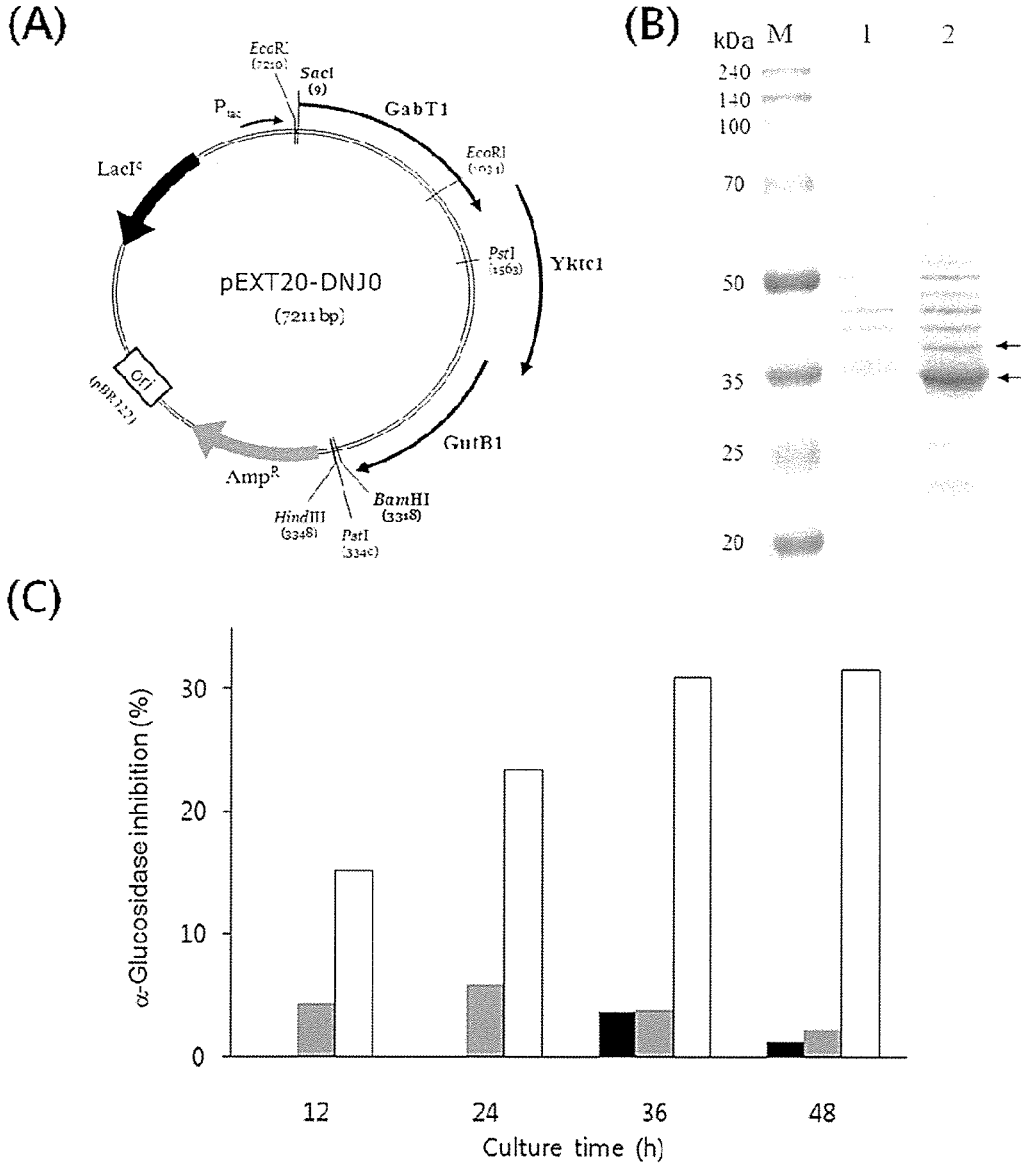

Fig. 21. Overexpression of DNJ biosynthetic genes. A. DNJ biosynthetic genes, *gabT1*, *yktc1*, and *gutB1*, were cloned under the IPTG-inducible P_{tac} promoter in the medium copy number expression vector pEXT20. B. *E. coli* DH5α strains harboring pEXT20 (lane 1) or pEXT20-DNJ0 (lane 2) were induced and whole cell lysates were separated on 10% PAGE. C. α-glucosidase inhibitory activities in a culture broth of *E. coli* DH5α strain harboring, pEXT20 (black bars), pEXT20-DNJ1 (grey bars), or pEXT20-DNJ0 (white bars) were determined at the indicated times of cultivation.

Discussion

Based on isotope labeling studies, putative DNJ biosynthetic pathways have been postulated in several different organisms. In *bacilli* and *streptomyces*, it has been suggested that the precursor of DNJ biosynthesis, glucose, undergoes C2/C6 cyclization in which amination, oxidation, and epimerization played major roles (Hardick *et al.*, 1992; Hardick and Hutchinson, 1993). In the higher plant dayflower *Commelina communis* (Commelinaceae), DNJ was proposed to be biosynthesized through a different route involving the C1/C5 cyclization of the original glucose molecule (Shibano *et al.*, 2004). Despite detailed proposals for DNJ biosynthetic pathways in both bacteria and plants, no report is available on the genetics or enzymes of DNJ biosynthetic pathways.

In this chapter, for the first time, three genes, *gabT1*, *yktc1* and *gutB1*, was isolated from the DNJ-producing *B. subtilis* MORI 3K-85 strain. When a recombinant plasmid harboring these genes was transformed into *E. coli*, the transformants produced DNJ into the culture medium. This suggests that the products of these three genes were enough to direct DNJ synthesis in the heterologous host *E. coli*. Further evidence of the involvement of these genes in DNJ biosynthesis was provided by the fact that these genes are missing in the genome of the DNJ non-producing *B. subtilis* 168, while homologous genes showing more than 75% of nucleotide sequence homologies were detected in the genome of the DNJ-producing *B. amyloliquefaciens* FZB42 strain. These genes were predicted to encode putative transaminase, phosphatase, and oxidoreductase, respectively. It is noteworthy that the putative DNJ biosynthetic pathway in *Bacillus subtilis var niger* was predicted to use a sugar-phosphate as an intermediate and involve amination and oxidation steps (Hardick and Hutchinson, 1993).

According to the nucleotide sequence analysis, upstream of the SigA dependent promoter of the *gabT1-yktc1-gutB1* operon, a catabolite-responsive element (*cre*) has been predicted. It is well known that the expression of genes containing *cre* in *B. subtilis* is regulated by the carbon catabolite protein A (CcpA) transcription factor in response to carbon metabolism (Fujita, 2009). Interestingly enough, it has been reported that the maximal level of DNJ production in the *B. subtilis* DSM704 strain was observed at the onset of sporulation at the late stage of growth (Stein, 1984).

When DNJ biosynthetic genes were introduced into *E. coli*, DNJ production was observed clearly but only in a limited amount. The maximal level of production was only observed at the stationary phase of growth. Moreover, it was not possible to increase DNJ production further by inducing the copy number of the recombinant plasmid or by expressing these genes under the control of a strong inducible promoter. This may indicate that there is a limiting step to increase the level of DNJ production in recombinant *E. coli*, such as shortage of a substrate or insufficient expression of one of the DNJ biosynthetic genes in active form. In fact, the expression level of the *gabT1* gene was extremely low compared to those of other two genes and it could be due to its five rare Arg codons (AGA).

There are increasing demands for DNJ due to its potential to be developed as a new therapeutic based on its α-glucosidase inhibition activity. Although many reports describing the chemical synthesis of DNJ have been published, there remain several obstacles to be overcome before it can be put into large-scale production. For example, chemical synthesis requires a complicated process and lacks stereochemical selectivity (Schedel, M. 2008). Thus, to produce DNJ economically, it is likely necessary to employ a biotechnological fermentation process. The DNJ biosynthetic genes isolated

in this study for the first time will allow an understanding of the physiological roles of DNJ as well as the fabrication of the bioprocesses for mass production of DNJ. Further studies regarding the biochemical properties of enzymes encoded by each gene and their substrates and cofactors will be required. In addition, to establish an economical production system using a heterologous host such as *E. coli*, it will be necessary to analyze and optimize the related metabolic pathways with molecular genetic and functional genomic tools.

Summary

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 the previous study, a DNJ-producing bacterium, *Bacillus subtilis* MORI, was isolated from the traditional Korean fermented food Chungkookjang. In the present study, I 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, I 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. These 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.

General Discussion

The overall theme of my dissertation research has been investigation of the role of host factors that influence baculovirus replication. In particular I have focused my studies on the effects of host starvation, as well as the levels of ecdysteroid, α-glucosidase, and DNJ on the replication of the baculoviruses BmNPV and AcMNPV. I have also investigated the biosynthetic pathway of DNJ in the bacterium *B. subtilis*.

Cory (2010) reported that one of the emerging areas in host-virus ecology is the role of the host condition in determining susceptibility. In insects, the host condition is primarily affected by plant secondary chemicals in the insect's diet. The nutritional state of an insect can have significant impacts in its development, physiology, behavior, fecundity, and other life parameters. When larvae of *Drosophila melanogaster* are transferred from a nutritious diet to a minimal diet, endoreplication cells stop their growth (Britton *et al.*, 2002). Chen and Gu (2006) also showed that starvation *in D. melanogater* reduces protein levels of certain glands. The nutritional content of an insect's diet is likely to affect its disease resistance through influences on its immune function (Siva-Jothy and Thompson, 2002; Yang *et al.*, 2007). Clearly, the "host condition" is important for viral replication. In my dissertation, I examined the effect of starvation on baculovirus pathogenicity, larval mortality, and larval mass. I found that starvation results in a decrease in the growth rate of infected larvae. Consequently, the decrease of the growth rate leads to a reduction in viral replication and delay in the lethal time to death. Hoover *et al* (1997) have also shown that slower relative growth rates of baculovirus-infected larvae which fed on old-stored diet were correlated with slower lethal time. These results suggested that nutritional condition of host affects the replication of baculovirus.

Ecdysteroids control molting and metamorphosis in insects. Pulses of ecdysteroid determine all development transitions (Colombani *et al.*, 2005). Most baculovirus produce ecdysteroid UDP-glucosyltransferase which inactivates ecdysteroids resulting in the inhibition of molting and pupation in infected larvae (O'Reilly and Miller, 1989). In my study, the growth rate of *B. mori* larvae infected with an *egt*-deleted mutant or wild-type BmNPV were compared. Mutant-infected larvae showed decreased food consumption and body weight, and died about 12 hours faster than wild-type virus infected larvae. In addition, mutant-infected larvae showed the initiation of normal molting and pupation processes unlike the wild-type virus-infected larvae. These results are similar to those found previously in caterpillars infected with other NPVs (O'Reilly and Miller, 1991; Eldridge *et al.*, 1992; Burand and Park, 1992).

In chapter 2, the effect of ecdysteroid on viral replication was investigated by treating the larvae with ecdysteroid on the day of the fourth larval ecdysis. Biochemical and histochemistrical methods were used to analyze how ecdysteroid treatment affects viral replication. Ecdysteroid treatment increased the speed of virus-specific protein expression and virus transmission suggesting that ecdysteroid stimulates virus transmission. From an evolutionary point of view, the ancestral NPV likely did not have an *egt* gene. The modern NPV likely obtained the gene from a host insect resulting in the inactivation of ecdysteroid function (Rohrmann, 2011). The inactivation of ecdysteroid inhibits molting and pupation and extends the feeding stage of host insects, resulting in an increase in the number of progeny viruses that are produced (O'Reilly and Miller, 1991). An ancestral NPV without an *egt* gene might be able to transmit faster than the modern NPV, but the amount of progeny produced by

the primitive NPV-infected larvae is likely to be lower. Therefore, the virus appears to have chosen a strategy to produce more progeny. Inhibition of molting and pupation, extension of feeding duration, and increase of body weight following ecdysteroid inactivation might be the best choice for a baculovirus to mass produce progeny.

As the sericulture industries in Korea and Japan become smaller, the focus of sericulture in Korea and Japan is evolving from one that is centered on the production of silk for the generation of fabric to one that is more diverse. For example, the application of silk and silkworm-derived technologies was used as an artificial eardrum, bone, and hypoglycemic agents (Banno *et al.*, 2010). DNJ, a compound that is abundantly found in silkworms and mulberry trees, is a recent focus in applied sericultural sciences. DNJ is an alkaloid which inhibits α-glucosidase in the small intestine of human resulting in a reduction in the rate of glucose uptake after a meal (Ahn *et al.*, 2005; Kong *et al.*, 2008). In addition, DNJ has been known as an antiviral agent against animal viruses. Various DNJ derivatives have been synthesized and tested against different viruses (Chang *et al.*, 2009; Hazama *et al.*, 2003; Jacob *et al.*, 2007; Mehta *et al.*, 2002; Tanaka *et al.*, 2006; Whitby *et al.*, 2005; Wu *et al.*, 2002). Normally silkworms feed on mulberry leaves which can contain 1 g or more of DNJ per kg (w/w) of leaf (Asano *et al.*, 2001; Kim *et al.*, 2003). A key question I had was how BmNPV can efficiently replicate under this high concentration of DNJ.

α-Glucosidase plays a role in removing the terminal glucose residue of the N-linked glycosylation process. The inhibition of α-glucosidase interrupts the maturation of glycoproteins on the envelope of enveloped viruses resulting in the reduction of viral particle assembly, suppression of virion secretion, and interference of the attachment of virions to host cells (Jacob *et al.*, 2007; Pelletier *et al.*, 2000). In chapter 3, α-

glucosidase activity in *A. yamamai*, an insect that commonly feeds on plants with relatively low DNJ levels, and *B. mori* were compared biochemically. α-Glucosidase inhibitory activities of *A. yamamai* and *B. mori* homogenates were measured in comparison to DNJ, acarbose and voglibose which are α-glucosidase inhibitors as a drug for treating diabetes. As a result, α-glucosidases in *B. mori* were shown to be less sensitive to inhibition by α-glucosidase inhibitors. However, the reasons why α-glucosidases of *B. mori* are less sensitive to DNJ are not clear. Hirayama *et al* (2007) also showed that sucrase and trehalase in the midgut of *B. mori* are less sensitive to DNJ in comparison to those in *Samia cynthia ricini.* In addition, other studies show that similar insensitivity of disacchridase to alkaloids was found in the *Uraniafulgens*, a specialist feeding on alkaloid-containing plant, *Omphalea diandra* (Kite el al., 1997). In both studies, they did not show the reasons of the insensitivity. Although the reasons are still unclear, I hypothesize that the α-glucosidase of *B. mori* might have a different structure which does not efficiently bind DNJ. Therefore, *B. mori* can feed mulberry leaves and grow normally although mulberry leaf has high concentrations of DNJ.

DNJ has been actively studied as an antiviral agent, because DNJ can inhibit α-glucosidase I and II related with synthesis of glycoproteins resulting in the interruption of the synthesis of viral envelope proteins (Chang *et al.*, 2009; Jacob *et al*, 2007; Tanaka *et al.*, 2006; Whitby *et al.*, 2005). For example, the IC_{50} of DNJ against BVDV and HBV is 150-300 μM and 100-500 μM, respectively (Mehta *et al.*, 2002). In this study, the inhibitory effect of DNJ on α-glucosidase activity was quantified in order to reveal how BmNPV efficiently produces progeny in silkworms in the presence of high concentrations of DNJ, a known antiviral. Bm5 and Sf9 cells have no

cytotoxic effects even when grown in medium containing 10 mM of DNJ. The titer of BmNPV, however, was not affected by up to 10 mM DNJ, while that of AcMNPV was reduced by 67% in medium containing 10 mM DNJ. In addition, α-glucosidase activity in Bm5 cells was less sensitive to DNJ in comparison Sf9 cell extracts. These results suggested that *B. mori* might have less sensitive α-glucosidase I and/or α-glucosidase II which are/is related with the formation and release of BV. Therefore, BmNPV can replicate under high concentration of DNJ.

During the late 1990s researchers started to study ways to control blood glucose levels using mulberry leaf extracts and silkworms. A key driver for this was a dramatic reduction in the scale of sericulture industry in Korea (Jang and Rhee, 2004). The key ingredient that was responsible for reducing blood glucose levels in mulberry and silkworm extracts is DNJ (Hwang *et al.*, 2008). In addition, DNJ is produced by *Bacilli* and *Streptomyces* (Ezure *et al.*, 1985; Hardick and Hutchinson., 1993; Stein *et al.*, 1984; Watson and Nash, 2000). No report has been published about how DNJ is biosynthesized in mulberry leaves until now. Recently, our laboratory isolated *Bacillus* producing DNJ from the traditional Korean fermented food Chungkookjang (Kim *et al.*, 2011). In prokaryotic genetic elements, genes coding for related enzymes are often clustered together in operons (Madigan *et al.*, 2000). So it is generally easier to find the genes related with biosynthesis of metabolite in prokaryotes. If the genes related with the biosynthesis of DNJ are found in *Bacillus*, these genes can be used to trace genes associated with the biosynthesis of DNJ in silkworms and mulberry. Thus, I focused on the identification of genes related DNJ biosynthesis in *Bacillus* and subsequently used this information to search for genes related to DNJ synthesis in silkworm by various molecular methods. As a result, clones having α-glucosidases

inhibitory activity were selected from a genomic DNA library which was constructed in *E. coli*. I identified several genes in this library including *gabT1*, *yktc1*, and *gutB1*, genes that are predicted to encode putative transaminase, phosphatase, and oxidoreductase, respectively. For the biosynthetic route of DNJ, amination and oxidation are described as essential reactions, but depholylation is not expected (Hardick and Hutchinson, 1993; Shibano *et al.*, 2004). My studies are the first identification of genes that are related with DNJ biosynthesis in *Bacillus*. 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. Currently the characteristics of the enzyme encoded by each gene are being investigated. The enzyme will be used for enzymatic synthetic and biomimetic synthesis of DNJ and its derivatives.

In conclusion, the present study examined the effects of the role of host factors that influence baculovirus replication. The results obtained in my study show that the hormonal and nutritional condition of hosts can affect the replication of baculovirus. In addition, I show that BmNPV can efficiently replicate in silkworms regardless of the presence of high DNJ concentration unlike AcMNPV and other animal viruses. This occurs because α-glucosidase, an enzyme associated with biosynthesis of virus envelope in silkworm is less sensitive to the inhibitory activity of DNJ in comparison to α-glucosidase activity in other lepidopterans and animals. I found three genes involved in DNJ biosynthetic pathway of *B. subtilis,* which will be useful for investigating the corresponding pathway in silkworm and/or mulberry. My findings show that virus survival is intricately dependent upon host and/or environmental condition.

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