Full Paper

Bacillus subtilis GntR regulation modified to devise artificial transient induction systems

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We modified GntR regulation in *Bacillus subtilis* to devise transient induction systems. GntR is the repressor antagonized by gluconate to induce transcription of the *gntRKPZ* operon for gluconate catabolism. On the other hand, the *gnt* operon is repressed by glucose via carbon catabolite repression involving CcpA/P-ser-HPr, which binds to two *cre* sites: one located in the *gnt* promoter region and the other within the *gntR* coding region. We initiated *gntKPZ* encoding of enzymes for gluconate catabolism expressed independently from the operon; this allowed constitutive degradation of gluconate. Both *cre* sites were mutated to abolish catabolite repression. The mutated *gnt* promoter was set up to drive the expression of the *lacZ* reporter under the control of GntR. Even in the presence of glucose, *lacZ* was induced upon the addition of gluconate and shut down again as gluconate was consumed. Thus, modified GntR regulation enables artificial transient induction. This will allow us to design a flexible metabolic engineering system with genes expressed only temporarily as desired.

Key Words: *Bacillus subtilis*; catabolite repression; gluconate; repressor

Introduction

Bacteria are exposed to an ever-changing environment

in which available nutrients may often change in quantity and composition. To adapt, bacteria can express different enzymes to optimize different physiological pathways depending on the quantity and types of nutrients available (Balleza et al., 2009). Bacterial transcription regulation is one of the main mechanisms that bacteria use to quickly switch their metabolism in changing environments. This regulation is achieved by various regulator proteins that bind to their respective and specific sites on DNA to switch target genes on or off. The inducible system is one of the most common mechanisms in which genes under regulation are shut off—unless specific agents functioning as inducers (that modify respective regulator proteins, repressors, or the activators) modulate their DNA-binding capacity, thus allowing genes to be expressed. Another important mechanism is the repressive system, in which genes under regulation are expressed, unless a specific repressor appears (sometimes together with an agent functioning as a corepressor) or an activator loses its functionality.

Recent developments in the field of metabolic engineering have led to an increased interest in the development of artificial transcriptional regulation systems to control endogenous and sometimes heterogeneous genes to eliminate or enhance specific metabolic pathways for the production and accumulation of desired metabolites (Lee et al., 2012; Na et al., 2013). Several inducible and repressive systems are available for artificial transcriptional control. However, to date, such systems were irreversible because once an inducible system is turned on, it cannot be shut off again because of the remaining inducer. Similarly, a repressive system is not rebootable once it is turned

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Table 1. Bacterial strains used in this study.

Strain	Relevant genotype	Reference
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
KU1001	<i>trpC2, ΔgntR::(erm PrpsO)</i>	This study
KU1011	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat PgntWT-lacZ)</i>	This study
KU1012	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat Pgntm1-lacZ)</i>	This study
KU1013	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat Pgntm2-lacZ)</i>	This study
KU1021	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat PgntWT-lacZ-gntRm spc)</i>	This study
KU1022	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat Pgntm1-lacZ-gntRm spc)</i>	This study
KU1023	<i>trpC2, ΔgntR::(erm PrpsO), amyE::P(cat Pgntm2-lacZ-gntRm spc)</i>	This study
KU1031	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat PgntWT-lacZ), pBE-S-PyhaM-gntRm</i>	This study
KU1032	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat Pgntm1-lacZ), pBE-S-PyhaM-gntRm</i>	This study
KU1033	<i>trpC2, ΔgntR::(erm PrpsO), amyE::(cat Pgntm2-lacZ), pBE-S-PyhaM-gntRm</i>	This study

off either. Therefore, the issue of irreversibility in artificial regulation systems has received considerable critical attention in the metabolic engineering field. To design and optimize a metabolic circuit aiming at maximal production of the desired products, in some cases the elimination of essential genes and overexpression of toxic ones have to be considered, and such manipulations can provoke disastrous problems in the maintenance of cellular integrity. Therefore, reversible induction and repression of target genes at any desired time can help us to control the pleiotropic effects and to circumvent the problems.

With respect to switchable gene expression, no reversible system involving chemical inducers has previously been reported. However, a light- and temperature-switchable system allows the rapid and reversible induction or repression of the target gene at any desired time (Drepper et al., 2011; Lee and Keasling, 2006; Lee et al., 2013; Rinas, 1996). Although such a system can function at smaller scales, it may be difficult to operate at larger industrial scales.

To study synthetic inducible and repressive operons, *Bacillus subtilis* represents an excellent model for the study of gene regulation and the metabolism of bacteria (Freyre-González et al., 2013). Its *gnt* operon is one of the well-characterized metabolic pathways responsible for gluconate catabolism in this bacterium and requires gluconate as an inducer that is intrinsically degradable. From the economical point of view, gluconate is beneficial to be used in chemical production at larger scales, because it is less expensive than the chemicals used conventionally as an inducer, such as isopropyl β -D-1-thiogalactopyranoside for LacI regulation.

Fujita et al. (1986) reported the nucleotide sequence of the gluconate operon in *B. subtilis*. The *gnt* operon, comprising *gntR*, *gntK*, *gntP*, and *gntZ*, encodes the transcriptional repressor of the operon (GntR), gluconate kinase (GntK), gluconate permease (GntP), and gluconate 6-phosphate dehydrogenase, respectively (Fujita et al., 1986; Miwa and Fujita, 1988). Moreover, Fujita et al. demonstrated that the *gnt* operon is negatively auto-regulated through the interaction between the GntR repressor and the operator within the *gnt* promoter (*Pgnt*) region preceding *gntR* (Fujita and Fujita, 1987; Fujita and Miwa, 1989). The operator is located between positions –10 and +15 (+1 is the transcription initiation site) and has a

palindromic structure with the half-symmetry sequence ATACTTGTA.

In another study, Fujita et al. (1987) recognized that the GntR protein became inactivated after interaction with gluconate. GntP is involved in substrate uptake, and GntK phosphorylates gluconate to give gluconate 6-phosphate, which is catabolized further through the pentose phosphate shunt involving GntZ (Reizer et al., 1991). The *gnt* operon is not expressed in the presence of glucose because it is subject to carbon catabolite repression mediated by a glycolytic intermediate (fructose-1,6-bisphosphate) that stimulates the production of P-ser-HPr (Nihashi and Fujita, 1984). P-ser-HPr and CcpA form a DNA-binding protein-protein complex CcpA/P-ser-HPr that binds to *cre* sites on DNA for negative regulation. The catabolite repression in the *gnt* operon involves two distinct modes: CcpA/P-ser-HPr binds to a *cre_{up}* site in *Pgnt* to repress the transcriptional initiation and CcpA/P-ser-HPr binds to another *cre_{down}* site within the *gntR* coding region to roadblock the transcriptional progression (Marciniak et al., 2012; Miwa et al., 1997).

Based on the intrinsic degradability of gluconate, the GntR regulation is reversible; after being induced in the presence of gluconate it shuts down again when the gluconate is consumed. However, it is under the strict control of catabolite repression and does not function in the presence of glucose that is supplied from cellulytic biomass and generally regarded as the practical starting material for microbial chemical production. In this study, we modified GntR regulation so that artificial transient induction systems circumventing catabolite repression could be devised.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains of *B. subtilis* were grown in LB (Sambrook et al., 1989) or S6 media (Fujita and Freese, 1981) containing 0.5% casamino acids as carbon sources, supplemented with 10 mM glucose and/or gluconate (1 mM or 10 mM). When needed, appropriate antibiotics were used: 5 μ g/ml chloramphenicol, 0.5 μ g/ml erythromycin, 5 μ g/ml kanamycin, and 100 μ g/ml spectinomycin.

Fig. 1. Construction of the strains in this study.

(I) The entire *gntR*, including its cognate promoter, was replaced with the constitutively active and strong promoter of *rpsO* (*PrpsO*) to allow the constitutive expression of *gntKZP* for the constant degradation of gluconate. For the construction of the strains, PCR fragments covering regions A–D were ligated by recombinant PCR and integrated into the *gntR* locus by a double-crossover event (Fabret et al., 2002). (II) *lacZ* reporter, under the control of the native promoter of *gntR* (*PgntWT*) or its mutated variations (*Pgntm1* and *Pgntm2*), was constructed and introduced into the *amyE* locus using pCRE-test plasmid. (III) *gntR* with a silent mutation (*gntRm*) was introduced into the strains with the *lacZ* reporter. PCR fragments covering regions E–I were ligated by recombinant PCR and integrated downstream of *lacZ*, which was integrated into the *amyE* locus as described above. (IV) pBE-S-*PyhaM*-*gntR* carrying *gntRm* under the control of the *yhaM* promoter was constructed by ligation of PCR fragments covering regions J–L. (V) Nucleotide substitutions introduced in the *Pgntm1*, *Pgntm2* and *gntRm* mutations. Part of the nucleotide sequences are shown. The mutated nucleotides are shown in lower case.

Construction of strains. Strain KU1001 was constructed by replacing the entire *gntR* gene, including the *gnt* promoter with a constitutively active and strong promoter of *rpsO* (*PrpsO*), to direct the transcription of *gntKZP*. First, two DNA fragments approximately 700-bp long, corresponding to the upstream (region A as indicated in Fig. 1-I) and downstream (region D) stretches of *gntR*, were amplified by a polymerase chain reaction (PCR) from the DNA of strain 168 with the primers a424/a425 and a426/

a427, respectively (Table 2).

Two additional DNA fragments, the erythromycin-resistant gene cassette derived from pMutin2 (region B) as well as the *rpsO* promoter region of strain 168 (region C), were amplified using the primers a118/a119 and a120/a121, respectively (Table 2). The four fragments of the regions A, B, C, and D were ligated by recombinant PCR using the primers a424/a427, and the resulting fragment transformed strain 168 to erythromycin resistance by a

Table 2. Oligonucleotide primers used in this study.

Name	Sequence (5' → 3')
a014	CACGATATTCACGGTTTACCCGATCAGACCAGTTTAAATTGTG
a067	GATAAGCTGTCAAACATGAGAATTGAAAGTGTTCATAAAAAGAAATATTCACG
a068	GATAAGCTGTCAAACATGAGAATTGAATAAACTTGACAAAAAGAAATATTCACGTTATCATACTTG
a069	GATAAGCTGTCAAACATGAGAATTGAAAGTGTTCGACAAAAAGAAATATTCACG
a070	GTGTATCAACAAGCTGGGGATCCAACAGGTCTTTGGAGTCTAGC
a072	AGTACCGCTTTCAATCAGACCTG
a073	CAGGTCTGATTGAAAAGCGTACTATTTTATCAGAAAATACAATCGCCGCC
a118	CTACATTCCTTTAGTAACGTGTAAC
a119	GAGTGTGTTGATAGTGCAGTATC
a120	GATACTGCACTATCAACACACTCATGGCATCAAAGAATTAAGTACG
a121	CCAAATCATATTTAGCCCCAGTTACC
a424	GGAATCAAGCCCCGCTCACATC
a425	GTTACACGTTACTAAAGGGAATGTAGGTATACAAGTATGATAACGTGAATATTC
a426	GGTAACTGGGGCTAAATATGATTTGGGGCTCTCACAACAAATGCTGGC
a427	CCGGGCTGCAGTGTGAAAG
a431	CCGCTGGATAACGACATTGGC
a432	CACCTTCCTCACTCAAGGAGTTTATTTTACACACCAGACCAACTGG
a433	ACTCCTTGAGTGAGGAAGGTG
a434	CTGCGCATATCGATTCTTAAC
a435	GTTAGGAATCGATATCGGCACGCTGCCCGGGGATCGATC
a436	CAAAATTCTCCAGTCTTCACATCGG
a750	CGAAATAACTATTTTCATACAGAAAAGAACAC
a751	GCGGTAGTTTATCACAGTTAAATTGC
a758	GCAATTTAACTGTGATAAACTACCGCCGTGCCGATATCGATTCTTAAC
a759	GTGTTCTTTCTGTATGAAAATAGTTATTTTCGGTATGATTTATCTGAAGGCGGTAGG
a760	CACCTTCCTCACTCAAGGAGTCATAATACCAGTATATCACATACCGATTTC
a900	GGGTAAACCGTGAATATCGTG

double-crossover event within the regions A and D. One of the transformants with the correct construction was selected as strain KU1001.

Strains KU1011, KU1012, and KU1013 were constructed as follows. A DNA fragment corresponding to the original *gnt* promoter region (PgntWT) was amplified using the primers a067/a070 (Table 2). Additionally, two kinds of DNA fragments containing the mutated versions of the *gnt* promoter (Pgntm1 and Pgntm2) were amplified with primers a068/a070 and a069/a070, respectively. The a068 and a069 primers were designed to confer alternations within the *cre_{up}* sequence (Table 2). Each of the three fragments were ligated using an In-Fusion HD Cloning Kit (Takara Bio) with the arms of pCRE-test (Miwa et al., 2000), previously digested by *Eco*RI and *Bam*HI and blunt-ended with T4 DNA polymerase, to obtain plasmids pCRE-PgntWT, pCRE-Pgntm1, and pCRE-Pgntm2, which contain a *lacZ* reporter under the control of the *gnt* promoter. The plasmid pCRE-PgntWT, pCRE-Pgntm1, and pCRE-Pgntm2 transformed KU1001 to chloramphenicol resistance with integration of the *lacZ* reporter into the *amyE* locus to yield KU1011, KU1012, and KU1013, respectively (Fig. 1-II).

The strains KU1021, KU1022, and KU1023 were constructed as follows. Five DNA fragments—the first corresponding to the C-terminal region of *lacZ* of pCRE-test (region E, Fig. 1-III); the second for the N-terminal region of *gntR* coding region (region F); the third for its C-terminal region (region G); the fourth for the spectinomycin-resistant cassette of *Staphylococcus aureus* SA2223 (region H); and the fifth for the N-terminal region of the *amyE* locus (region I)—were amplified by PCR with the primer pairs of a431/a432, a433/a072, a073/a434,

Fig. 2. Schematic organization of the *cis*- and *trans*-types of the modified GntR regulation systems.

a435/a900, and a014/a436, respectively (Table 2). Ligation of the regions F and G was designed to produce the entire *gntR* coding region with a silent mutation at the 40th codon from ACC to ACT to mutate the *cre_{down}* site for CcpA/P-ser-HPr binding (*gntRm*).

The five fragments were ligated in the order E-I by recombinant PCR using the primer pair of a431/a436. The resulting fragment transformed KU1011, KU1012, and KU1013 to spectinomycin resistance by a double-crossover event to integrate the mutated *gntR* co-transcribed with *lacZ* into the *amyE* locus to yield KU1021, KU1022, and KU1023, respectively (Fig. 1-III).

The recombinant plasmid pBE-S-PyhaM-*gntRm*, a derivative of pBE-S (Takara Bio) carrying *gntRm* under the control of *yhaM* promoter, was constructed as follows.

Fig. 3. Growth of *B. subtilis* strain KU1001.

Cells of KU1001 were inoculated into S6 medium containing 0.5% casamino acids (A) and the same medium supplemented with 10 mM glucose (B) and then allowed to grow to follow an increase in optical density for the cells at 600 nm. At the time points indicated with arrowheads, 1 mM (open squares) and 10 mM (closed triangles) gluconate was added or not added (closed diamonds). A representative data set is shown after three independent experiments with similar results.

Three DNA fragments—the first corresponding to the *yhaM* promoter of strain 168 (region J, Fig. 1-IV); the second for the *gntRm* gene from KU1021 (region K); and the third for the arms of pBE-S plasmid (region L)—were amplified by PCR with the primers a759/a760, a433/a758, and a750/a751, respectively. The three fragments were ligated using an In-Fusion HD Cloning Kit to obtain pBE-S-PyhaM-*gntRm*. KU1011, KU1012, and KU1013 were transformed with pBE-S-PyhaM-*gntRm* to kanamycin resistance to yield KU1031, KU1032, and KU1033, respectively.

Enzymatic assay. Cells were inoculated into the medium to an initial optical density at 600 nm of 0.05 and allowed to grow at 37°C with shaking at 150 rpm. β -Galactosidase activities in the cells were determined according to the methodology described by Griffith and Wolf (2002).

Results and Discussion

Two types of modified GntR regulation systems

We modified the GntR regulation to create artificial transient induction systems. From the beginning, the GntR regulation is reversible because of the intrinsic degradability of gluconate. However, since the GntR regulation is under strict catabolite repression, gluconate degradation is completely repressed in the presence of glucose that is the most abundant biomass resource in nature and generally assumed to be the promising starting material for microbial chemical production. To facilitate its constitutive degradation, *gntKPZ* in strain KU1001 was modified to be expressed as artificial operons under the control of a strong and constitutive promoter *PrpsO* (Nicolas et al., 2012), so that gluconate is taken into the cell and degraded through the pentose phosphate pathway.

The activity of the *gnt* promoter (regulated by the GntR repressor) was evaluated by the expression levels of *lacZ* reporter-encoding β -galactosidase. The *gnt* promoter re-

gion contains the *cre_{up}* site for CcpA/P-ser-HPr binding to exert carbon catabolite repression. The *cre_{up}* site was mutated in two ways to give the variation in *gnt* promoter regions: *PgntWT*, *Pgntm1*, and *Pgntm2* (Fig. 2). Within the *gnt* promoter, the GntR-binding site is located at the -10 region, whereas the *cre_{up}* is close to the -35 region. Because the -35 region is important for promoter activity, we needed to choose appropriate mutations to disrupt the *cre* sequence but not the -35 function. From the DBTBS database (Ishii et al., 2001), we found that the promoter region of *ysiAB-etfBA* is homologous to the *gnt* promoter (data not shown), with the characteristics of the -10 region similar to TATCA, a 17-bp spacer between the -10 and -35 regions, and the -35 region similar to TTGCAT.

To design *Pgntm1*, the original *gnt* -35 region was replaced with *ysiAB-etfBA*. On the other hand, *Pgntm2* was constructed by introducing a typical sequence of TTGACA for the -35 region. Therefore, the strains KU1011, KU1012, and KU1013 were constructed from KU1001 to place *PgntWT-lacZ*, *Pgntm1-lacZ*, and *Pgntm2-lacZ*, respectively, on the *amyE* locus.

In addition, *cre_{down}* within the *gntR* coding region was mutated. *cre_{down}* is located at the 40th codon ACC (Thr), which was mutagenized to ACT (Thr) without changing the amino acid sequence; thus, a silent allele *gntRm* was made to abolish the transcriptional roadblock of the catabolite repression mediated by CcpA/P-ser-HPr. *gntRm* was supplied into strains KU1011, KU1012, and KU1013 in two ways: the *cis*-type to place *gntRm* co-transcribed with the *lacZ* reporter as the expression levels of *gntRm* may be increased or decreased by the auto-regulation in the respective strains KU1021, KU1022, and KU1023 and the *trans*-type can express the plasmid-coded *gntRm* constitutively in the strains KU1031, KU1032, and KU1033, respectively.

Effects of forced and constitutive degradation of gluconate

In the case of the original *gntRKPZ* operon, *gntKPZ* for the enzymes of gluconate degradation (GntK and GntZ) and the specific transporter (GntP) is under the negative feedback regulation of GntR, which may moderate the induction to accommodate gluconate metabolism adequately. In the artificial systems explained above, however, it is possible that pools of intermediates appearing in the pentose phosphate pathway could increase suddenly and accumulate more than usual because of constitutive gluconate degradation. This may cause a disturbance in the global metabolic network and exert an adverse effect on cell growth. In particular, it is known that an excess accumulation of phosphorylated sugar intermediates is toxic to the cell (Sá-Nogueira and Mota, 1997).

The strain KU1001, expressing *gntKPZ* constitutively, grew normally in S6 minimal medium containing 0.5% casamino acids as the sole carbon source (Fig. 3). As anticipated, when 1 mM or 10 mM gluconate was added into the growth medium in the middle of the logarithmic growth phase, cell growth was arrested completely. On the other hand, when the medium was supplemented with 10 mM glucose, the growth arrest by gluconate became less obvious; the addition of 1 mM gluconate, especially, had almost no effect. Therefore, subsequent experiments were performed with the cells grown in S6 minimal medium containing both 0.5% casamino acids and 10 mM glucose, and 1 mM gluconate was added for the induction of the *gnt* promoters.

The observation above implies the possible underlying mechanisms. When the cells grow using casamino acids (a mixture of amino acids and some very small peptides obtained from acid hydrolysis of casein) as the sole carbon source, they are supposed to live depending on the degradation of the mixture of amino acids and mostly on gluconeogenesis. As described above, upon the addition of gluconate, the forced and constitutive expression of *gntKPZ* could cause a sudden and possibly excessive increase in intermediates appearing in the pentose phosphate pathway. Therefore, it is likely that the cells grown with casamino acids are intolerant to an increase in pentose phosphate pathway intermediates. On the other hand, in the presence of glucose, the cells grow mainly depending on glycolysis. The better growth observed with glucose suggests that the cells in glycolytic growth might be able to better tolerate the excess intermediates. It is known that not only the *gnt* operon but also a large number of genes are repressed or induced by glucose through various regulations including catabolite repression, which exerts pleiotropic effects and might be able to coordinate the global metabolic network to be more robust against the metabolic disturbance caused by the artificial gluconate degradation.

cis-Type GntR regulation

The *cis*-type GntR regulation was evaluated experimentally (Fig. 4). The strains KU1021, KU1022, and KU1023 were grown in S6 minimal medium containing 0.5% casamino acids and 10 mM glucose, and 1 mM gluconate was then added to induce the *lacZ* reporter. In KU1021

Fig. 4. *cis*-Type of the modified GntR regulation system.

Cells of KU1021 (A), KU1022 (B), and KU1023 (C) were inoculated into S6 medium containing 0.5% casamino acids and 10 mM glucose and allowed to grow. At the time points indicated with arrowheads (at the early log phase), *lacZ* was induced with (open squares) and without (closed diamonds) 1 mM gluconate. β -Galactosidase activities in the cell were measured hourly afterwards. A representative data set is shown after three independent experiments with similar results.

cells, *PgntWT* containing the intact *cre_{up}* sequence directed no expression of the *lacZ*, either with or without gluconate addition. The results indicate that transcription from *PgntWT* was shut off by catabolite repression involving the *cre_{up}* site in the presence of glucose. On the other hand, both *Pgntm1* and *Pgntm2*, in which the *cre_{up}* was disrupted by respective mutations, directed the induction of *lacZ* to elevate β -galactosidase activity in response to the addition of gluconate early in the logarithmic phase.

The results indicate that both *Pgntm1* and *Pgntm2* indeed functioned as promoters with the altered -35 region and were regulated by GntR properly, but neither was affected by catabolite repression because of the respective disruptions in *cre_{up}*. It should be noted that transcription

Fig. 5. *trans*-Type of the modified GntR regulation system.

Cells of KU1031 (A), KU1032 (B), and KU1033 (C) were inoculated into S6 medium containing 0.5% casamino acids and 10 mM glucose and allowed to grow. At the time points indicated with arrowheads (at the early log phase), *lacZ* was induced with (open squares) and without (closed diamonds) 1 mM gluconate. β -Galactosidase activities in the cell were measured hourly afterwards. A representative data set is shown after three independent experiments with similar results.

from *Pgntm1* and *Pgntm2* was shut off almost completely in the absence of gluconate (Fig. 4). Because *gntRm* is auto-regulated together with the *lacZ* reporter under the control of the *gnt* promoter and its derivatives in these *cis*-type regulation systems, the intracellular amount of GntR could be maintained at low levels in the absence of gluconate. Nevertheless, the results indicate that GntR was enough to exert its repressor function. In addition, the silent mutation in the *cre_{down}* sequence did not affect the translational efficiency of *gntRm* to produce sufficient GntR. On the other hand, both in the cases of *Pgntm1* and *Pgntm2*, the induction of β -galactosidase was maintained up to 2 h after the addition of gluconate and then the activity became repressed again.

Fig. 6. Effects of the growth phase on the modified GntR regulation systems.

Cells of KU1022 (A) and KU1032 (B) were inoculated into S6 medium containing 0.5% casamino acids and 10 mM glucose and allowed to grow; the growth courses are shown with closed diamonds. At the time points indicated with arrowheads, *lacZ* was induced with 1 mM gluconate in the middle log phase (open squares), at the entry to stationary phase (closed square), or without (open diamonds). β -Galactosidase activities in the cell were measured hourly afterwards. A representative data set is shown after three independent experiments with similar results.

The results also suggest that the 1 mM gluconate added into the media was degraded within 2 h so that concentrations of intracellular gluconate decreased below the level necessary to inhibit DNA binding of GntR. However, β -galactosidase activities did not disappear completely when GntR was supposed to regain its function to repress the promoters. This may be due to the incomplete degradation of gluconate and/or the β -galactosidase enzyme under the experimental conditions. In any case, the patterns of induction and repression demonstrated the transient induction experimentally as intended. Comparing the performances of *Pgntm1* and *Pgntm2*, *Pgntm1* tended to exhibit a higher promoter activity, although the difference in *cis*-type regulation was not prominent.

trans-Type GntR regulation

Subsequently, the *trans*-type GntR regulation was also evaluated experimentally (Fig. 5). In this case, GntR is supplied from *gntRm* encoded by the plasmid pBE-S-PyhaM-*gntRm* so that a constant number of GntR could present intracellularly, and the *lacZ* reporter gene is lo-

cated at the *amyE* locus in the chromosome and transcribed from the *gnt* promoter or its derivatives under the regulation of GntR (Fig. 2). *PyhaM* was chosen because it is constitutively active and as strong as *Pgnt* according to the transcriptomic analysis (Nicolas et al., 2012).

No β -galactosidase activity was detected in the cells of strain KU1031 with *Pgnt*WT, regardless of the addition of gluconate because glucose caused catabolite repression via the intact *cre_{up}* to abolish the promoter function. This was similar to what was observed in the *cis*-type regulation. On the other hand, in the cases of KU1032 and KU1033 with *Pgntm1* and *Pgntm2*, respectively, the β -galactosidase activity was induced to reach a peak 1 h after gluconate was added and was repressed again thereafter. Interestingly, *Pgntm1* produced very high β -galactosidase activities—the peak was approximately 2.5 times higher than that given by *Pgntm2*—and it produced almost the same levels as those observed in the *cis*-type regulation.

These results suggest that *Pgntm1* is more efficient as a transcriptional promoter than *Pgntm2* (Fig. 5), although its *cis*-type regulation efficiency was less obvious (Fig. 4) where the promoter was under the negative feedback regulation of GntR (Fig. 2). The *gnt* promoters were not auto-regulated by GntR in the *trans*-type regulation constructs (Fig. 2), and both *Pgntm1* and *Pgntm2* may be able to exert their full activity once the repression is released. On the other hand, both *trans*-type systems with *Pgntm1* and *Pgntm2* exhibited a quicker induction and more gradual re-repression than the *cis*-type systems (Figs. 4 and 5). The re-repression by GntR did not recover immediately, as discussed with *cis*-type regulation, probably because the gluconate was not degraded completely and/or the large amount of β -galactosidase accumulating in the cell may not have decomposed readily.

Effects of growth phase

Finally, gluconate was added at different time points to examine whether artificial transient induction might be affected by growth phases (Fig. 6). Cells of the strains KU1022 (*cis*-type *Pgntm1* regulation) and KU1032 (*trans*-type *Pgntm1* regulation) were grown, and 1 mM gluconate was added into the medium at two different time points: in the middle of logarithmic growth, as well as at the start of the stationary phase. In the middle of the logarithmic growth phase, the transient induction of β -galactosidase activity was clearly observed both in *cis*-type and *trans*-type regulations. In the case of the stationary phase, however, the *cis*-type system became much less responsive in inducing higher activity, whereas in the *trans*-type system, although an obvious induction of the activity was observed, it was hardly repressed again later.

Therefore, both the *cis*-type and *trans*-type transient induction systems were less functional in the stationary phase under the tested conditions. To improve the induction levels, the addition of higher concentrations of gluconate would be worth testing because the growth arrest (Fig. 3) is no longer the problem during the stationary phase. With respect to the less efficient re-repression, the degradation of gluconate could be enhanced by expressing *gntKPZ* with another promoter activated during the

stationary phase.

From a different point of view, β -galactosidase may be less degraded during the stationary phase. It was demonstrated that protein degradation could be induced using heterologous peptide tags (Griffith and Grossman, 2008), which may be useful in clarifying the efficiency in re-repression not only during the stationary phase but also in the other growth stages.

Our future research will focus on fine-tuning the artificial transient induction systems as well as developing their practical application to metabolic engineering in *B. subtilis*. For example, transient induction systems could be used for shutting down one of the essential reactions to accumulate a metabolite to obtain a desired compound. In the presence of gluconate, the induction system allows the expression of the enzyme responsible for the particular reaction to support normal growth, but once the gluconate is consumed after obtaining a sufficient amount of cells, the reaction is turned off automatically to accumulate the metabolite. When needed, the reaction that was once shut down can be resumed transiently as required by the addition of some gluconate. Based on the mechanisms described above, a practical metabolic engineering circuit has been designed and will be tested, and the results will be reported elsewhere in the near future.

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