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RelA Protein Is Involved in Induction of Genetic Competence in Certain *Bacillus subtilis* Strains by Moderating the Level of Intracellular GTP

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We found that the ability to develop genetic competence of a certain relaxed (*relA*) aspartate-auxotrophic strain of *Bacillus subtilis* is significantly lower than that of the isogenic stringent (*relA*$^+$) strain. Transcriptional fusion analysis utilizing a *lacZ* reporter gene indicated that the amount of the ComK protein, known as the key protein for competence development, is greatly reduced in the relaxed strain than in the stringent strain. We also found that the addition of decoyinine, a GMP synthetase inhibitor, induces expression of a competence gene (*comG*) in the relaxed strain, accompanied by a pronounced decrease in the level of intracellular GTP as measured by high-performance liquid chromatography. The transformation efficiency of the relaxed strain increased 100-fold when decoyinine was added at *t0* (the transition point between exponential to stationary growth phase). Conversely, supplementation of guanosine together with decoyinine completely abolished the observed effect of adding decoyinine on competence development. Furthermore, the impaired ability of the relaxed strain for competence development was completely restored by disrupting the *codY* gene, which is known to negatively control *comK* expression. Our results indicate that the RelA protein plays an essential role in the induction of competence development at least under certain physiological conditions by reducing the level of intracellular GTP and overcoming CodY-mediated regulation.

When *Bacillus subtilis* cells encounter adverse nutrient conditions, cells start to approach the stationary growth phase by initiating several development processes that contribute to its survival. Of these processes, genetic competence and sporulation have been studied in great detail. Genetic competence is a physiological state that enables cells to incorporate exogenous DNA, which is then incorporated into the endogenous DNA by crossing over. Competence development is tightly controlled by a complex regulatory network involving kinases, phosphatases, and transcriptional regulators (4). The most critical regulator is a transcriptional factor named ComK, which has been shown to be necessary for inducing the late competence genes such as *comG* (27, 28). It has been demonstrated that the cellular level of ComK is precisely controlled by several regulatory proteins at the transcriptional and posttranscriptional levels (2, 5, 6, 25, 26). During the exponential growth phase, ComK activity is inhibited by a complex of two proteins, MecA and ClpC (10, 26). The ComS protein, which is encoded by the *srfA* operon and is synthesized in response to quorum-sensing oligopeptide pheromones (13, 23), releases cells from ComK inhibition by dissociating the ternary complex with MecA and ClpC (25). In addition, Serror and Sonenshein (21) demonstrated that CodY, which is a global regulator for stationary-phase genes, directly binds to the *srfA* and *comK* promoter region. Thus, it is highly likely that CodY acts as a negative regulator for competence genes.

One of the most important adaptation systems for bacteria is the stringent response, which leads to the repression of stable RNA synthesis (reviewed by Cashel et al. [1]). The stringent response depends on the transient increase of hyperphosphorylated guanosine nucleotides [(p)ppGpp], which are synthesized from GDP or GTP by the *relA* gene product (called stringent factor [ppGpp synthetase]) in response to binding of uncharged tRNA to the ribosomal A site (reviewed by Cashel et al. [1]). Amino acid depletion in *B. subtilis* gives rise to the transient increase of (p)ppGpp, accompanied by the reduction of intracellular GTP, eventually leading to the induction of spore formation in *relA*$^+$ (stringent) cells but not in *relA* (relaxed) mutant cells (11, 12, 16–18). In the course of studying the ribosomal function of *B. subtilis*, we have recently found that genetic competence in a certain aspartate-auxotrophic *B. subtilis* strain is affected significantly by the *relA* mutation. In this study, attempts were made to demonstrate that the RelA protein plays an essential role in induction of genetic competence by reducing the intracellular GTP level in certain genetic backgrounds. This paper is the first demonstration showing that GTP functions as a nutritional signal for competence development, as predicted by the regulatory role of CodY, a GTP binding protein, in competence, and that the reduction in GTP levels during the onset of stationary phase is partially dependent upon RelA in nutritionally rich culture conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. Strain 168 is the standard strain of *B. subtilis* used for studying sporulation of this organism. The aspartate-auxotrophic *B. subtilis* strains 61884 and 61883 are isogenic with respect to *relA1* (17). This *relA1* mutation is a leaky *rel* mutation (11, 17) and has an amino acid alteration at position 240 (Gly to Glu) (as determined by DNA sequencing in this study). *B. subtilis relA* null mutants are known to demand valine for growth in synthetic medium and to be partially auxotrophic for isoleucine, leucine, and methionine (29). Unlike *relA* null mutants, the *relA1* strain does not demand any amino acids for growth on synthetic medium, possibly because of leaky *rel* character.

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Construction or source
61884	*trpC2 aspB66*	Laboratory stock (17)
61883[a]	*trpC2 aspB66 relA1*	Laboratory stock (17)
168	*trpC2*	Laboratory stock
YO-005	*hisH101*	Laboratory stock (8)
K2-007	*trpC2 relA1*	61883→YO-005[b]
RIK720	*trpC2 lys-1 aprEΔ3 nprR2 nprE18 clpCΔS amyE*::(*comG-lacZ cat*)	F. Kawamura (14)
RIK51	*trpC2 lys-1 aprEΔ3 nprR2 nprE18 amyE*::(*comK-lacZ cat*)	F. Kawamura (5)
61884G	*trpC2 aspB66 amyE*::(*comG-lacZ cat*)	RIK720→61884
61883G	*trpC2 aspB66 relA1 amyE*::(*comG-lacZ cat*)	RIK720→61883
61884K	*trpC2 aspB66 amyE*::(*comK-lacZ cat*)	RIK51→61884
61883K	*trpC2 aspB66 relA1 amyE*::(*comK-lacZ cat*)	RIK51→61883
TI70	*trpC2 aspB66 codY*::*neo*	pCR2.1-*codY*::*neo*→61884
TI71	*trpC2 aspB66 relA1 codY*::*neo*	pCR2.1-*codY*::*neo*→61883

[a] The *relA1* mutation was originally found by Swanton and Edlin (24) in 1972.
[b] The strain to the right of the arrow was transformed with the chromosomal DNA or plasmid to the left of the arrow.

All of the strains used were grown aerobically at 37°C using L medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl [all amounts given per liter]) or Spizizen's salts medium [14 g of K_2HPO_4, 6 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 1 g of sodium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 5 g of glucose (all amounts given per liter)]. For *B. subtilis* transformation, cells were grown in TF medium (Spizizen's salts medium supplemented with 0.05% yeast extract, 0.03% Casamino Acids, 2 μM $MnCl_2$, and excess amounts of amino acids as required [50 μg of tryptophan per ml and 20 mM sodium aspartate]). For *B. subtilis* sporulation, TF medium was further supplemented with 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 mM $MnCl_2 \cdot 4H_2O$, and 1 μM $FeSO_4 \cdot 7H_2O$ to promote sporulation. If necessary, neomycin (3 μg/ml) (for selection of *codY* mutants) or ampicillin (50 μg/ml) (for *E. coli* transformants) was added to the medium.

Disruption of the *codY* gene. The *codY* gene was disrupted by insertion of the *neo* gene within the coding region as follows. The complete coding region of the *codY* gene (780 bp) was cloned into plasmid pCR2.1 after amplification by PCR with primers codY-F (5′-ATGGCTTTATTACAAAAAACAAGAAT-3′) and codY-R (5′-TTAATGAGATTTTAGATTTTCTAATTCAA-3′), and the resulting plasmid was designated pCR2.1-*codY*. A 1.3-kb *Sma*I fragment containing the *neo* gene derived from pBEST501 (9) was inserted at the *Ssp*I site within the *codY* gene in pCR2.1-*codY*, creating pCR2.1-*codY*::*neo*. Integration of pCR2.1-*codY*::*neo* into the chromosome of strain 61884 (*relA*$^+$) or 61883 (*relA*) by double-crossover recombination gave rise to strain TI70 or TI71, respectively. The disruption of the *codY* gene was confirmed by PCR amplification using the codY-F and codY-R primers and subsequent sequencing of the flanking region.

Estimation of the transformation efficiency of *B. subtilis*. *B. subtilis* strains 61883 and 61884 were grown at 37°C in TF medium containing 20 mM aspartate. Samples were taken at appropriate times as indicated in the figures and exposed to the chromosomal DNA (1 μg/ml) from strain 168 for 30 min at 37°C with gentle shaking. The cells were then spread on Spizizen's salts agar plate containing 50 μg of tryptophan per ml. After incubation for 2 days at 37°C, Asp$^+$ transformants were scored.

β-Galactosidase assay. β-Galactosidase assay was performed as described previously (14). The specific activity was calculated as follows: (A_{420} per minute per milliliter of culture per A_{650}) × 1,000, where A_{420} is the absorbance of *o*-nitrophenol, the product of cleavage of the β-galactosidase substrate, *o*-nitrophenol-β-D-galactoside, and A_{650} is the absorbance of the culture at the time of sampling.

Measurement of the intracellular GTP and ppGpp pool sizes. The intracellular GTP and ppGpp pool sizes were determined as described previously (17). The content of GTP (and also of ppGpp) was determined by high-performance liquid chromatography analysis using a Partisil-10 SAX column (GL Science). Concentrations of these nucleotides are expressed as picomoles per AM_{650} (picomoles per milliliter of culture per A_{650}).

RESULTS

Effect of the *relA* mutation on competence development. The aspartate-auxotrophic *B. subtilis* strain 61884 demands a high concentration (20 mM) of aspartate for full growth. Introduction of the relaxed *relA* mutation has a deleterious effect on growth (and sporulation) because more aspartate drains into the purine and pyrimidine nucleotide biosynthetic pathway (17). To determine the effect of the *relA* mutation on competence development in *B. subtilis*, we first compared the abilities to develop competence of the stringent (61884) and relaxed (61883) strains, since these isogenic strains offer a feasible system for studying the secondary metabolism of this organism (19). The transformation efficiency in the stringent strain peaked at *t1* (1 h after the transition from exponential to stationary phase) (Fig. 1). In contrast, the transformation efficiency in the *relA* strain was shown to be significantly lower

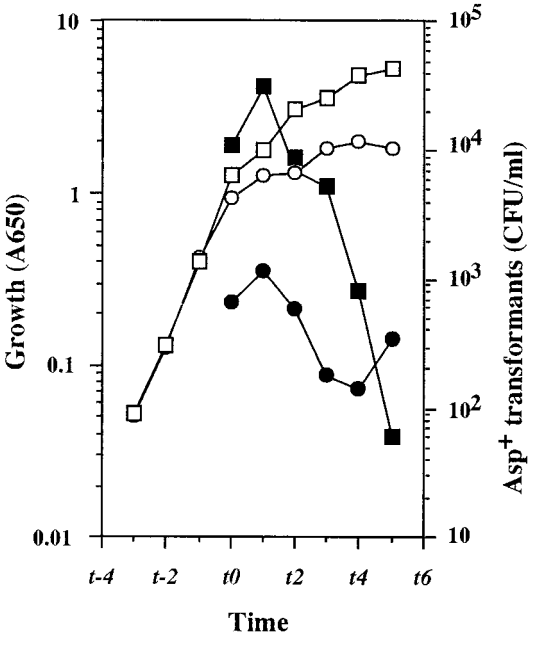

FIG. 1. Transformation of relaxed (*relA*) and stringent (*relA*$^+$) strains. *B. subtilis* strains 61884 (stringent) and 61883 (relaxed) were grown in TF medium containing 20 mM aspartate. Samples were taken at the indicated times to determine culture density (A_{650}) and transformation efficiency (as discussed in Materials and Methods). The transformation efficiency is expressed as the number of *asp*$^+$ transformants generated after exposure to chromosomal DNA from strain 168 (1 μg/ml) for 30 min. Growth (open symbols) and transformation efficiency (closed symbols) of strain 61883 (circles) and strain 61884 (squares) are shown.

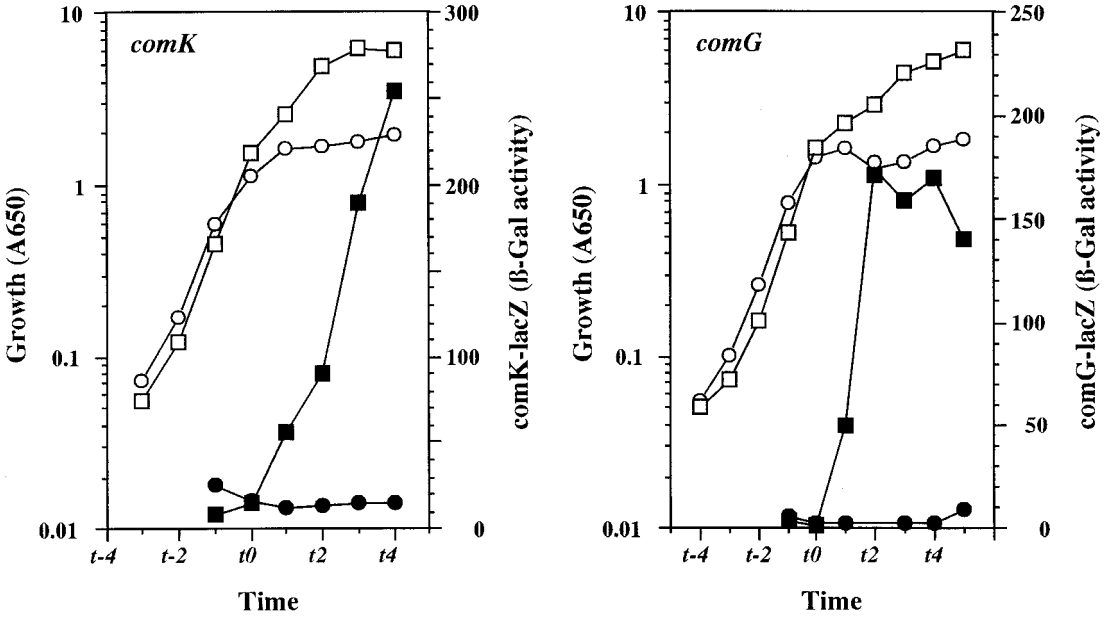

FIG. 2. Expression of competence genes *comK* and *comG* in the relaxed (*relA*) and stringent (*relA*$^+$) strains. A stringent strain (strain 61884 [squares]) and relaxed strain (strain 61883 [circles]) carrying either the *comK-lacZ* or *comG-lacZ* transcriptional fusion gene at the *amyE* locus were grown in TF medium containing 20 mM aspartate. Culture samples were taken at the indicated times, and culture density (A_{650} [open symbols]) and β-galactosidase (β-Gal) activity (closed symbols) were measured.

(by 40-fold) than that of the stringent strain (Fig. 1). Although the final cell mass of the *relA* strain was only one-third that of the stringent strain (Fig. 1), the difference in the transformation frequency is apparently much greater than the difference in the number of total viable cells. Although a *B. subtilis relA* null mutant requires valine, isoleucine, leucine, and methionine for growth in synthetic medium (29), the addition of these amino acids at a concentration of 50 μg/ml each did not restore the abilities to grow and to be transformed in strain 61883 (data not shown).

Extensive studies on the development of competence in *B. subtilis* have shown that the ComK protein can induce transcription of all the late competence genes that play a role in the uptake of exogenous DNA (2, 4, 7). To determine whether the ComK protein is produced in the relaxed strain, we monitored the expression of representative competence genes, *comK* and *comG*, using a transcriptional fusion of the target gene promoter to *lacZ* at the *amyE* locus. While both fusion genes were expressed at high levels in the stringent (*rel*$^+$) strain, expression in the *relA* strain was 20-fold lower (*comK*) or was substantially undetectable (*comG*) compared to that in the stringent strain (Fig. 2). These results indicate that the level of ComK protein is dramatically reduced in the *relA* strain (61883).

Aspartate is responsible for competence efficiency. *B. subtilis* strain 168 (*asp*$^+$ *relA*$^+$ strain) and its isogenic *relA* strain, K2-007 (*asp*$^+$ *relA*), were transformed at similar efficiencies when grown in TF medium without aspartate, although the development of competence was delayed 1 h in K2-007 cells. The *asp*$^+$ transformant of strain 61883 was also shown to be transformed as efficiently as strain 168 (data not shown). To explain these observations, we suggested two possibilities: (i) the combination of *aspB* and *relA* mutations is responsible for the decreased efficiency of competence, or (ii) the presence of an excess amount (20 mM) of aspartate in the culture medium for strain 61883 is responsible for decreased efficiency. The latter possibility was verified to be correct. Strain K2-007 (*asp*$^+$ *relA*) revealed a 30- to 50-fold decrease in competence efficiency when cells were grown in TF medium supplemented with high concentrations (10 to 20 mM) of aspartate. Thus, the presence of aspartate, rather than the *aspB* mutation, is responsible for the decreased competence ability of the *relA* mutant 61883. Similar to the effect of adding aspartate, the transformation frequency of *asp*$^+$ *relA* mutant K2-007 was also reduced by 30-fold by adding 20 mM glutamate to the TF medium (data not shown). Arginine had no such effect.

Effect of decoyinine on *comG-lacZ* expression. Previous studies have demonstrated that a reduction of GTP can induce spore formation in *B. subtilis* and that the RelA protein is involved in the reduction of GTP by synthesizing (p)ppGpp, which potently inhibits IMP dehydrogenase (11, 12, 17, 18). To determine whether a forced reduction in the GTP pool size could lead to an increase in the amount of active ComK in the *relA* strain, we added decoyinine (a GMP synthetase inhibitor) at *t0* into the culture of strain 61883 grown in a high concentration (20 mM) of aspartate. Strikingly, decoyinine (2 mM) completely restored *comG* expression in the *relA* strain (61883) to the same level as that in the stringent strain (61884) (Fig. 3). When decoyinine was added at different growth phases (early, middle, or late exponential growth phase), the *comG-lacZ* expression was initiated at the same time (*t2*), irrespective of the addition time (data not shown). The addition of guanosine (2 mM) together with decoyinine abolished the decoyinine effect on *comG* expression (Fig. 3). The effect of guanosine on *comG* expression was no longer detected when guanosine was added 30 min after the addition of decoyinine (data not shown),

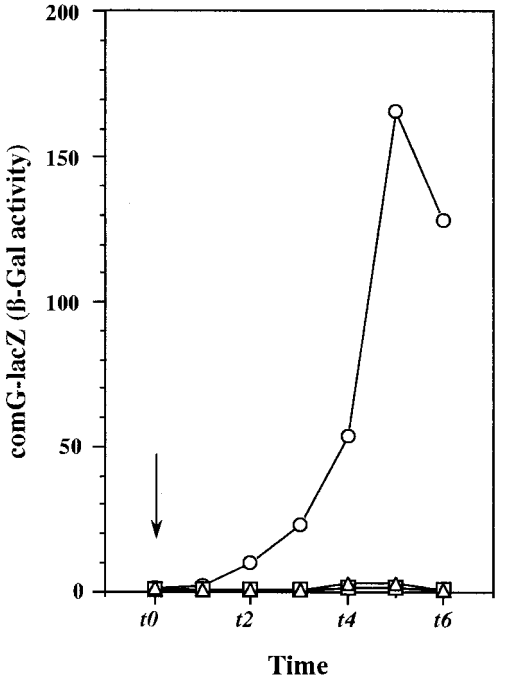

FIG. 3. Effect of decoyinine on *comG-lacZ* transcription. Strain 61883G carrying the *comG-lacZ* fusion gene was grown in TF medium containing 20 mM aspartate. Decoyinine (2 mM) (circles) or decoyinine plus guanosine (2 mM each) (triangles) were added to the culture at *t0* as indicated by the arrow. Culture samples were taken at the indicated times, and β-galactosidase (β-Gal) activity was measured. Untreated culture (squares) is shown as a control.

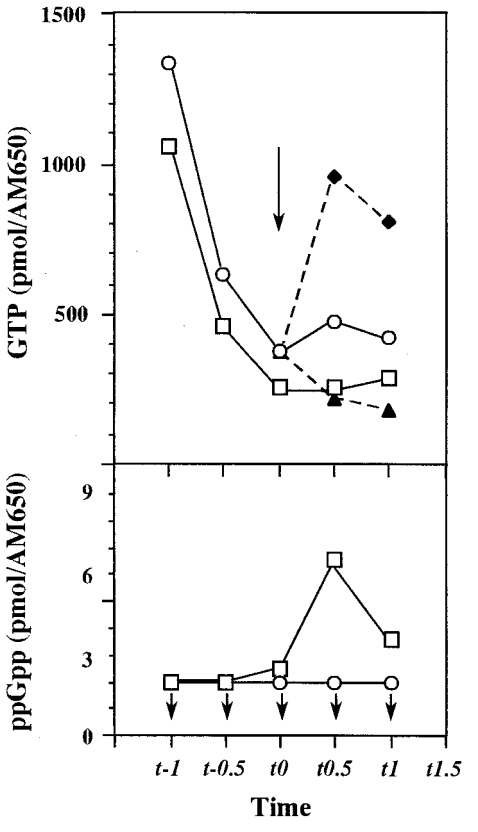

FIG. 4. Changes in the intracellular GTP and ppGpp concentrations during growth. The stringent (*relA*$^+$) strain (61884) (squares) and the relaxed (*relA*) strain (61883) (circles) were grown in TF medium containing 20 mM aspartate. Decoyinine (2 mM) (closed triangles) or decoyinine plus guanosine (2 mM each) (closed diamonds) were added to the culture at *t0*, as indicated by the long black arrow in the top panel. Samples were taken at the indicated times to determine culture density (A_{650}) and GTP and ppGpp concentrations. The short black arrows under the symbols in the bottom panel indicate that the values determined were below the detection limit by high-performance liquid chromatography (2 pmol/AM_{650} for ppGpp).

indicating that a short time (30 min or less) is sufficient to commit the cells to produce ComG protein by decoyinine treatment.

Changes in the intracellular GTP level during growth. Next, we assumed that the impaired ability of the *relA* strain for competence development may result from insufficient reduction of intracellular GTP level. To assess this possibility, we monitored the level of intracellular GTP during growth of strains 61883 and 61884 in transformation medium (TF medium). The GTP level in the stringent strain (61884) decreased rapidly at *t0* to approximately 200 pmol/AM_{650}, accompanied by a transient increase in ppGpp. However, the GTP level in the *relA* strain (61883) remained at 400 pmol/AM_{650}, and no increase in ppGpp was detected (Fig. 4). Similar results were reported previously by transferring cells from amino acid-sufficient to amino acid-limited conditions (17). When the relaxed mutant cells were treated with 2 mM decoyinine at *t0*, there was a significant reduction of GTP within 30 min, reaching 200 pmol/AM_{650} (Fig. 4). The addition of guanosine together with decoyinine resulted in a sharp increase of GTP to a level equivalent to that of exponentially growing cells (Fig. 4). Thus, it is reasonable to conclude that the inability to sufficiently reduce the level of GTP in the *relA* strain is a major cause for the inability of this strain to initiate genetic competence and sporulation (16–18). The observed intracellular concentrations of GTP during the transition phase (*t0* to *t1*) were estimated to be 1.5 to 2.5 mM (for the stringent strain) and 3.5 to 4.5 mM (for the relaxed strain) by the method of Ochi (15).

We further examined the effect of aspartate on the level of GTP using an *asp*$^+$ strain. Strain K2-007 (*asp*$^+$ *relA*) growing in TF medium without aspartate revealed GTP levels ranging from 450 to 220 pmol/AM_{650} during the transition phase (*t0* to *t1*), while GTP levels ranged from 620 to 350 pmol/AM_{650} when cells were grown in TF medium supplemented with 20 mM aspartate, indicating that the intracellular GTP level is critically affected by the presence of aspartate even in the *asp*$^+$ genetic background. These results, together with the results from strains 61883 and 61884 (see above), indicate a close relationship between the intracellular GTP level and acquisition of ability for competence.

Decoyinine fully induces competence in the *relA* strain. We next studied the effect of decoyinine on transformation to assess the causal relationship between the level of GTP and the ability to be transformed. As expected from the results described above, competence of the *relA* strain (61883) was fully induced by decoyinine treatment. The transformation efficiency increased 100-fold with the addition of decoyinine at *t0* (Fig. 5), reaching a level comparable to that of the stringent strain (Fig. 1). Apparently, the addition of decoyinine could fully induce a set of genes for competence. It was striking that

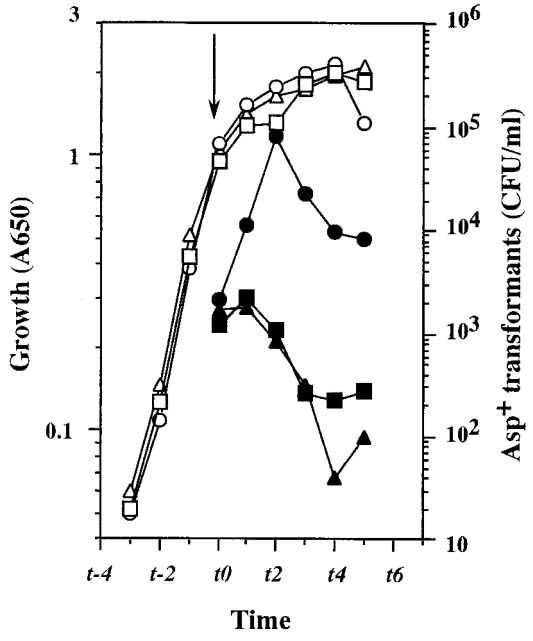

FIG. 5. Effect of decoyinine on transformation efficiency in the relaxed (*relA*) strain. The relaxed strain (61883) was grown in TF medium containing 20 mM aspartate. Decoyinine alone (2 mM) (circles) or with guanosine (2 mM each) (triangles) was added to the culture at *t0* as indicated by the arrow. Samples were taken to determine culture density (A_{650} [open symbols]) and transformation efficiency (closed symbols). Transformation was performed as described in the legend to Fig. 1. Untreated culture (squares) is shown as a control.

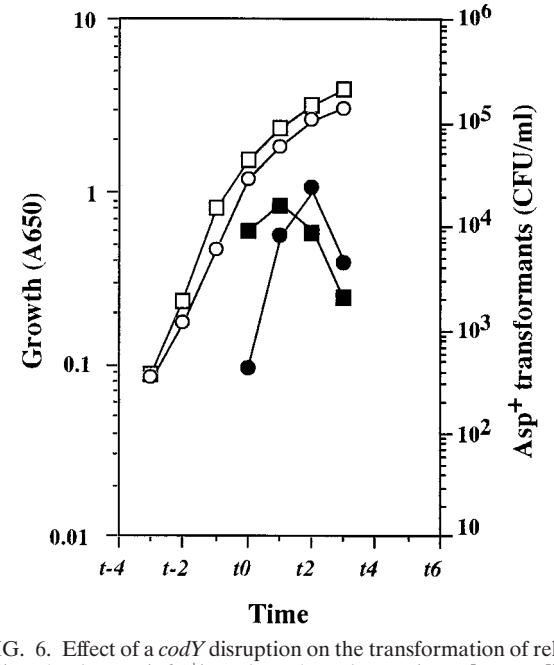

FIG. 6. Effect of a *codY* disruption on the transformation of relaxed (*relA*) and stringent (*relA*$^+$) strains. The stringent (TI70 [squares]) and relaxed (TI71 [circles]) strains were grown in TF medium containing 20 mM aspartate. Samples were taken to determine culture density (A_{650} [open symbols]) and transformation efficiency (closed symbols). Transformation was performed as described in the legend to Fig. 1.

the addition of guanosine together with decoyinine completely abolished the observed effect for decoyinine (Fig. 5).

Unlike the case for the *relA* strain, decoyinine addition was not effective in enhancing the frequency of transformation in wild-type (*rel*$^+$) cells, apparently because *rel*$^+$ cells can decrease GTP sufficiently without decoyinine (Fig. 4).

Effect of CodY on competence development. Serror and Sonenshein (21) demonstrated that CodY protein binds directly to the promoter region of the *comK* gene. More recently, Ratnayake-Lecamwasam et al. (20) working in the laboratory of A. L. Sonenshein have also demonstrated that the activity of the CodY protein is controlled by the level of intracellular GTP. These results raise the possibility that *comK* expression can be regulated by varying the level of intracellular GTP through CodY-mediated repression, eventually leading to full induction of genetic competence. To assess this possibility, we constructed *codY*-disruptant strains from the relaxed and stringent strains by inserting the *neo* gene as described in Materials and Methods. As expected, a disruption of the *codY* gene completely restored the competence ability in the *relA* strain (Fig. 6). In contrast, the disruption of *codY* did not give rise to any increase in competence ability when stringent strain 61884 was subjected to the gene disruption (compare Fig. 6 with Fig. 1), in agreement with the results from decoyinine addition (see above).

Effect of CodY on sporulation. As demonstrated by Ratnayake-Lecamwasam et al. (20) and Slack et al. (22) working in the laboratory of A. L. Sonenshein, CodY plays a crucial role in sporulation in *B. subtilis*. On the other hand, the *relA* mutation has a deleterious effect on sporulation (17). Likewise, the deleterious effect of the *relA* mutation was evident, as demonstrated by cultivating strains 61884 (*rel*$^+$) and 61883 (*relA*) in TF medium supplemented with excess (20 mM) aspartate, displaying a 60-fold reduction in spore titers (Table 2). It was striking that the spore titer of a *codY*-disrupted relaxed strain TI71 was even greater than that of *rel*$^+$ strain 61884 (Table 2). Moreover, strain TI71 formed heat-resistant spores earlier than strain 61884 did. The impaired sporulation ability of *relA* strain 61883 was also completely restored (2.0×10^8

TABLE 2. Sporulation of *B. subtilis* strains grown in modified TF medium[a]

Strain	Time (h)	Total no. of viable cells (CFU/ml)	No. of heat-resistant spores (CFU/ml)[b]	Frequency of sporulation (%)
61884 (*rel*$^+$)	12	6.0×10^8	2.5×10^4	0.004
	18	1.7×10^8	2.8×10^4	0.016
	24	2.0×10^8	1.6×10^6	0.8
	36	6.8×10^8	4.2×10^7	6
61883 (*relA1*)	12	5.6×10^8	2.1×10^5	0.04
	18	3.2×10^8	3.7×10^5	0.1
	24	7.3×10^8	7.2×10^5	0.1
	36	7.3×10^8	7.0×10^5	0.1
TI71 (*relA1* Δ*codY*)	12	3.1×10^8	1.8×10^2	<0.001
	18	6.7×10^8	1.0×10^7	1
	24	7.8×10^8	6.4×10^7	8
	36	5.4×10^8	1.7×10^8	31

[a] TF medium (see Materials and Methods) was supplemented with 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 mM $MnCl_2 \cdot 4H_2O$, and 1 μM $FeSO_4 \cdot 7H_2O$.

[b] Heat-resistant spores were determined using L agar plate after heating the cultured broth at 80°C for 15 min.

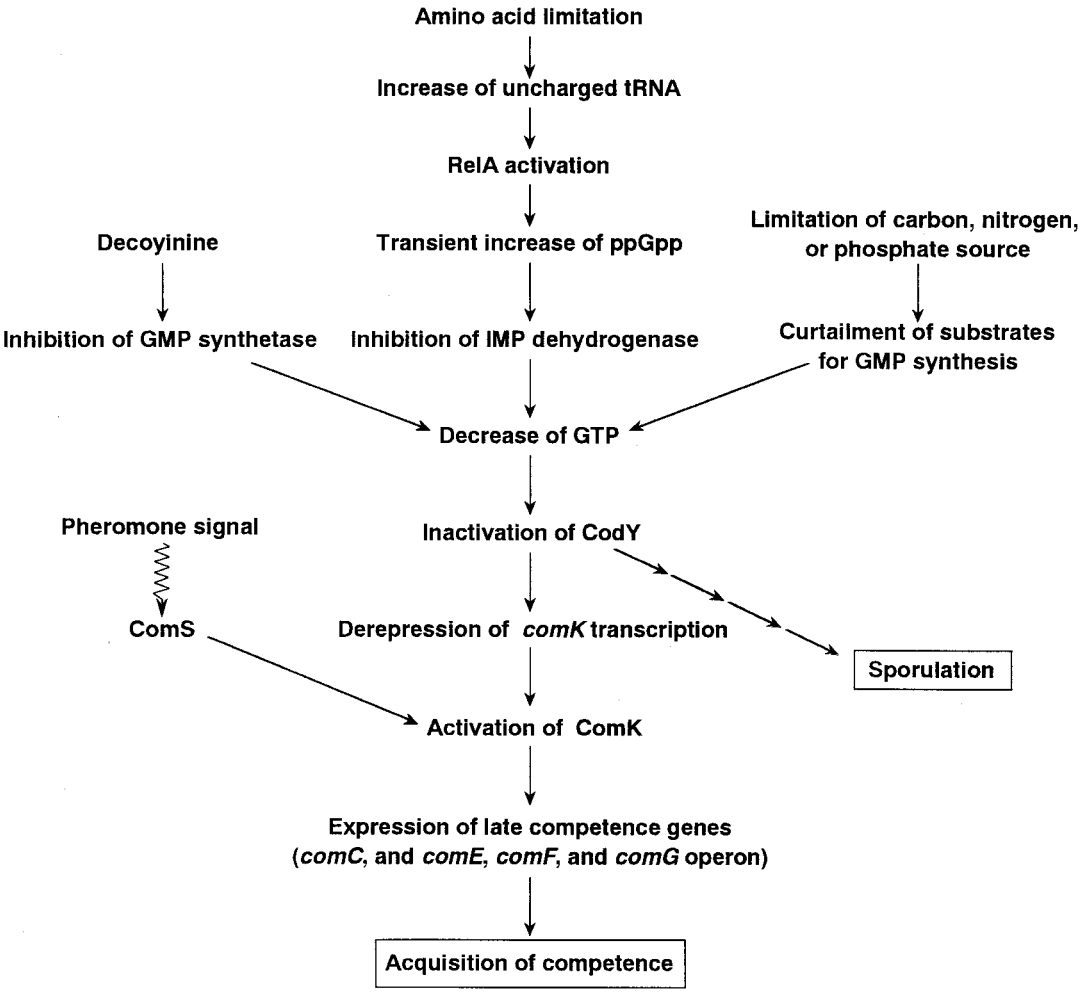

FIG. 7. Schematic showing the signal transduction pathway starting from nutrient limitation to the acquisition of competence. This schematic was constructed on the basis of our present work and previously reported research in *B. subtilis* (2, 4, 7, 17, 18, 20, 21), including relevant work with *Streptomyces griseus* (15).

spores produced per ml at 24 h) by adding 2 mM decoyinine at *t0* (data not shown). Thus, under excess aspartate conditions, the stringent response plays a role in restoring not only competence development but also sporulation by reducing the intracellular GTP level sufficiently (Fig. 4), which eventually leads to inactivation of CodY as illustrated in Fig. 7.

DISCUSSION

In this paper, we described the significance of the RelA protein in competence development, although the RelA protein is required for complete competence development only in the *aspB relA* mutant. As discussed by Dworkin and Losick (3), one of the most intriguing challenges in biology is to elucidate the mechanisms by which cells sense and respond to changes in extracellular nutritional conditions. However, the challenge of linking nutrient availability to alterations in gene expression has been met with success in only a few instances. Among prokaryotes, *B. subtilis* offers one of the best systems for studying such mechanisms, displaying a wide range of adaptations to nutrient limitations, including production and excretion of antibiotics and enzymes, entry into a state of genetic competence, elaboration of systems for motility and chemotaxis, and formation of endospores as an extreme response to nutrient limitation (3). In this light, recent work by Ratnayake-Lecamwasam et al. (20) and Slack et al.(22) in A. L. Sonenshein's laboratory should be highlighted, since they successfully linked nutritional status to sporulation, focusing mainly on CodY for sensing and responding to nutrient limitation. Our principal findings in this study were that (i) the RelA protein is essential for full induction of competence ability when cells were grown in the presence of high concentrations of aspartate (or glutamate), (ii) decoyinine treatment can elicit the competence ability, and (iii) aspartate significantly affects the nutritional status of *B. subtilis*. Thus, we successfully demonstrated that the development of competence and entry into the sporulation processes are tightly linked with nutrient limitations. This was verified by monitoring the changes in intracellular GTP and ppGpp levels and subsequent analysis of the expression of early (*comK*) and late (*comG*) competence genes as well as of the master protein CodY that controls both sporulation and competence. Thus,

we can draw a scheme (Fig. 7) to describe the signal transduction system (starting from nutrient limitations to the eventual acquisition of competence) on the basis of our present work and previous research in other laboratories (2, 4, 7, 20, 21). Previous studies have shown that the stringent response initiates sporulation by reducing the level of intracellular GTP in *B. subtilis* (11, 12, 17, 18). When *B. subtilis* cells encounter a depletion of amino acids, cells immediately start to synthesize ppGpp via the RelA protein from ATP and GDP. The *relA* mutant lacks the ability to synthesize ppGpp (Fig. 4). It was also shown that ppGpp inhibits IMP dehydrogenase, the first enzyme in the GMP synthesis pathway, resulting in a rapid reduction in the level of GTP (18). Treatment with decoyinine, which inhibits GMP synthetase, the second enzyme in the GMP synthesis pathway, or limitation of the substrate for GMP synthesis also can cause a decrease of GTP, even in the *relA* mutant. Results of our present work support previous findings (Fig. 4). We also successfully demonstrated that a sufficient decrease in GTP caused by decoyinine treatment can fully induce genetic competence and sporulation in a relaxed strain. Therefore, we concluded that the RelA protein contributes significantly to the induction of genetic competence by reducing the level of GTP caused by the inhibitory effect of ppGpp (Fig. 7).

CodY mediates the inhibitory effects of glucose and amino acids on stationary-phase gene expression (22) and is considered to be a GTP-sensing transcriptional regulator having a predicted GTP binding pocket (20). Ratnayake-Lecamwasam et al. (20) working in A. L. Sonenshein's laboratory originally reported that the capacity of CodY to block transcription depends on the GTP concentration, but GTP does not have a strong effect on binding of CodY to DNA, although CodY actually binds GTP. However, in more-recent experiments, it was found that the difference in affinity between CodY and DNA varies as much as 10-fold in environments with low (0.2 mM) and high (2 mM) concentrations of GTP (K. Tachikawa and A. L. Sonenshein, personal communication). It is important to point out that blocking of transcription by CodY requires physiological amounts (2.2 mM) of GTP, as examined for *dpp* transcription (20), and that our calculated GTP concentrations in transition-phase cells were 1.5 to 2.5 mM for the stringent strain and 3.5 to 4.5 mM for the relaxed strain. Therefore, at these calculated concentrations, the CodY protein can no longer repress transcription in the stringent strain but can repress transcription in the relaxed strain. A sufficient decrease in GTP can lead to the derepression of CodY-mediated genes including *comK* (21), which correlates well with the results from our present work (Fig. 2). It is of interest that the impaired growth of the *relA* strain (Fig. 1) was restored by the *codY* disruption (Fig. 6). These observations suggest that the deleterious effects caused by the relaxed mutation resulted from the CodY-mediated repression, implying a wide variety of functions for CodY in late growth phase as originally predicted by Slack et al. (22).

Our results were further corroborated with research using the aspartate-auxotrophic strains. Since the *asp*$^+$ relaxed strain could be transformed efficiently unless excess aspartate was present, the RelA protein is not thought to be crucial to the development of genetic competence. Nevertheless, it should be stressed that the effect of the RelA protein can be pronounced under specified physiological conditions, i.e., growth with abundant sources for carbon, nitrogen, and phosphate. Under such nutritional conditions, cells would not need to meet the severe requirements for curtailing the amount of substrates used in GMP synthesis. Therefore, modulation of the GTP level depends largely upon the stringent response (Fig. 7). The relaxed mutant cannot initiate the ppGpp system and therefore is dependent on the secondary pathway, i.e., curtailing the substrates used in GMP synthesis to reduce the level of GTP. Aspartate serves as a substrate for both purine (including GMP) and pyrimidine nucleotide biosynthesis as well as various other amino acids. Apparently, excess amounts of aspartate can block the consumption of substrates for GMP synthesis, resulting in an insufficient decrease in the GTP level in the relaxed mutants (see Results).

It is important to point out that although decoyinine addition was effective for competence development, the acquisition of competence was detected at the specified time (*t2*), irrespective of the time of decoyinine addition (see Results). Moreover, expression of *comK* and *comG* was not enhanced (but suppressed to some degree) when the *rel*$^+$ strain 61884 was subjected to tryptophan deprivation (accompanied by the stringent response) at mid-exponential growth phase (A_{650} = 0.5) (unpublished results). These observations suggest that factors besides the ppGpp-GTP-CodY signal transduction system (Fig. 7) could promote the development of competence. In the framework of this notion, the pheromone signal transduction system (13, 23) should be highlighted. Since quorum-sensing oligopeptide pheromones have been demonstrated to govern a ComS signal transduction system, the development of competence is thought to be regulated by these mutually independent systems, i.e., the GTP signal system and pheromone signal system, as illustrated in Fig. 7.

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