JOURNAL OF BACTERIOLOGY, Sept. 1996, p. 5144–5152 Vol. 178, No. 17
0021-9193/96/$04.00+0

Plasmid-Amplified *comS* Enhances Genetic Competence and Suppresses *sinR* in *Bacillus subtilis*

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Received 11 April 1996/Accepted 28 June 1996

The establishment of genetic competence in *Bacillus subtilis* is controlled by a vast signal transduction network involving the products of genes that function in several postexponential-phase processes. Two of these proteins, SinR and DegU, serve as molecular switches that influence a cell's decision to undergo either sporulation or genetic competence development. In order to determine the roles of SinR and DegU in competence control, multicopy suppression experiments with plasmid-amplified *comS*, *sinR*, and *degU* genes were undertaken. Multicopy *comS* was found to elevate competence gene transcription and transformation efficiency in both wild-type and *sinR* mutant cells but not in *degU* mutant cells. Multicopy *degU* failed to suppress *comS* or *sinR* mutations. No suppression of *comS* or *degU* by multicopy *sinR* was observed. The expression of a *comS′::′lacZ* translational fusion and *srf-lacZ* operon fusion was examined in *sinR* cells and cells bearing plasmid-amplified *sinR*. The expression of *comS′::′lacZ* gene fusion was reduced by the *sinR* mutation, but both *comS′::′lacZ* and *srf-lacZ* were repressed by multicopy *sinR*. Cells bearing plasmid-amplified *sinR* were poorly competent. These results suggest that *sinR* is required for optimal *comS* expression but not transcription from the *srf* promoter and that SinR at high concentrations represses *srf* transcription initiation.

Several species of bacteria are able to bind and internalize DNA (32, 54). This property, known as genetic competence, can be a normal characteristic of growing and dividing bacteria or is induced in response to environmental changes. Possible explanations for why bacteria become competent range from the ability to acquire DNA conferring selective advantages to the recipient cell to a means by which bacteria can obtain an alternative source of carbon or nitrogen (32, 54). Competence is often observed as the end result of a developmental process that is induced by extracellular signals and that is in response to the nutritional state of the organism's environment. In *Bacillus subtilis*, this process is accomplished by the operation of a complex signal transduction network involving genes that function in many processes activated in cells encountering a growth-restricting environment (Fig. 1) (10, 12). One pathway to competence development is activated in response to high cell density and requires competence pheromone ComX (34). As ComX accumulates extracellularly to high concentrations, the membrane histidine protein kinase, ComP, is activated, presumably by binding ComX, and donates a phosphate to ComA, a homolog of response regulator proteins and ComP's two-component partner (12, 50, 62, 63). ComA, thus activated, stimulates the transcription of the *srf* operon (22, 41, 43, 45, 50) (also called *comL* [60]), which encodes the subunits of surfactin synthetase (4, 16, 17, 57, 61), a peptide synthetase complex that catalyzes the synthesis of the peptide portion of the lipopeptide antibiotic surfactin (2). Although transcription of *srf* is required for competence development, surfactin synthetase is not (57). Instead, a small gene, *comS*, nested within and out of frame with the *srfB* gene is necessary for the transcription of the late competence genes (9, 25) encoding the proteins that function in DNA binding and uptake. The expression of *comS* also depends on another signal transduction pathway involving the extracellular peptide factor known as competence-stimulating factor (CSF) and the genes that function in sporulation initiation (*spo0A*, *spo0H*, *abrB*, and *spo0K*) (53). Spo0A is the key response regulatory protein controlling many processes induced under growth-restricting conditions, and Spo0H is the minor RNA polymerase sigma subunit required for the transcription of some sporulation genes. Both are required for the production of CSF (53). *abrB*, the product of which is a transcriptional regulator (48, 55) that represses *spo0H* transcription (64), is repressed by phosphorylated Spo0A (56). Thus, *spo0A* is required for optimal *spo0H* expression. The gene(s) encoding CSF might require σ^H RNA polymerase for its transcription, although it is possible that another gene(s) required for CSF synthesis has yet to be identified. The oligopeptide permease complex coded for by the *spo0K* operon (47, 52) is required for the cell's ability to respond to CSF (53). The putative interaction between Spo0K and CSF is thought to affect ComA activity by stimulating another kinase or modulating the activity of a phosphatase which would tend to reduce the level of ComA-phosphate.

ComS, proposed to be a small, 46-amino-acid protein (8, 9, 25), somehow activates ComK, also known as competence transcription factor, which is required for late competence operon transcription (36, 58, 59). ComK is negatively controlled by the MecAB complex (13, 28, 38, 51) composed of Clp subunit homologs that are believed to sequester and thereby inhibit ComK (27). ComK activity also requires SinR (18) and unphosphorylated DegU (6, 12). Each of the proteins that control ComK activity and late competence operon transcription is a potential target of ComS.

SinR (35) and DegU can be viewed as molecular switches that influence a cell's decision to undergo competence development or to produce degradative enzymes and undergo sporulation. SinR, a transcriptional regulator (35), at high concentrations prevents sporulation (18). Its activity is modulated by SinI, a small protein that promotes sporulation by inhibiting SinR through direct interaction (3). Null mutations of *sinR* abolish competence development without adversely affecting

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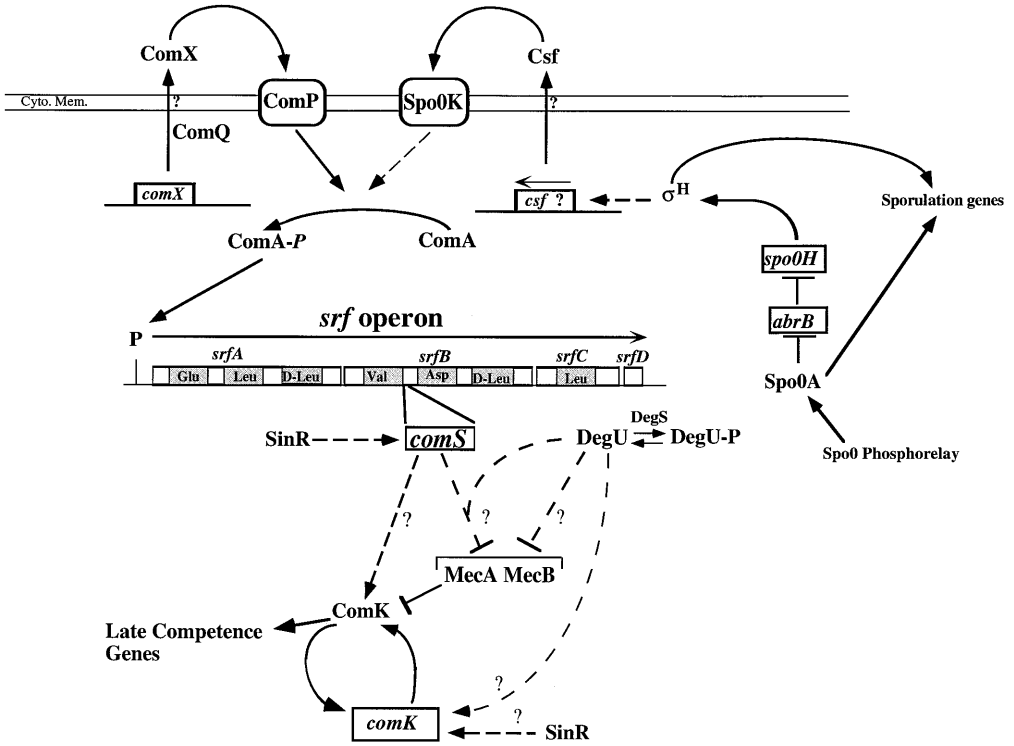

FIG. 1. A diagram summarizing the regulatory network controlling competence gene expression. Shown are some of the relevant interactions described in the text. Solid arrows indicate positive control, and perpendiculars indicate negative control. The dashed arrows indicate hypothetical interactions. The shaded boxes in the diagram of the *srf* operon indicate the DNA sequences encoding the amino acid-activating domains of the enzyme surfactin synthetase. They are labeled according to their amino acid substrate. Cyto. Mem., cell membrane.

sporulation (19). DegU, like ComA, is a member of the response regulator class of two-component regulatory proteins (37). It is encoded by an operon that also contains the gene specifying its two-component partner, the histidine protein kinase DegS. In its phosphorylated form, DegU tends to promote sporulation and degradative enzyme production, while unphosphorylated DegU is required for competence development (6). Thus, the availability of active SinR and the phosphorylated state of DegU profoundly influence sporulation and competence.

The location of the operation of DegU and SinR in the competence developmental network has not been firmly established, and the targets of the two regulators have not been determined. In this report, evidence that a *sinR* null mutation affects the expression of *comS* but not the transcription initiation of the *srf* operon is presented. DegU appears to be in a separate, parallel pathway controlling competence development. We also show that multicopy *comS* does not enhance competence gene expression above that observed in a *mecA* mutant, suggesting that the target of ComS function is the Mec-dependent inhibition of ComK, as has been proposed (8, 23).

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 contains a list of strains used in the studies described herein. All strains constructed during this study are derivatives of *B. subtilis* JH642 (from J. A. Hoch). Routine propagation of plasmid constructs was carried out with *Escherichia coli* AG1574 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galUK* r^- m^+ *strA recA56 srl*] (from A. Grossman) or MV1190 [Δ(*lac proAB*)*thi supE* Δ(*srl-recA*)*306*::Tn*10* (Tet^r)(F′ *traD36 proAB lacI*^{q}*lacZ*Δ*M15*)]. Strain LAB2129 bears a *comG-lacZ* fusion which does not disrupt the *comG* operon (1). The fusion construct bears a spectinomycin resistance determinant from pJL62 (obtained from J. Ledeaux and A. Grossman) which was used to replace the fusion-associated *cat* gene (26). The *sinR* mutation Δ*sinR*::$Phleo^r$ was transferred by transformation to LAB2129 to create LAB2241. DNA from cells bearing the *degU* mutation Δ*degU*::Neo^r (obtained from E. Ferrari) was used to transform LAB2129 competent cells with selection for neomycin resistance to create strain LAB2242.

Plasmid pSHC1 (39) is a pUB110 derivative bearing the *degU* gene. Plasmid pIS74 (19) is a derivative of pUB110 which contains the *sinR* gene (obtained from the Bacillus Genetic Stock Center). Plasmid pIS90 (19) is a derivative of pIS74, but the *sinR* gene has undergone a deletion mutation. Plasmid pMMN284 is a pBD64 (21) derivative bearing the *comS* gene fused to the promoter region of *srf*. The allele of *comS* used in this construction bears an ATG start codon instead of TTG, a mutation generated by oligonucleotide-mediated site-directed mutagenesis according to published protocols (29). The mutant *comS* gene was situated in a 760-bp *Sma*I-*Hin*dIII fragment and propagated on a pUC18 plasmid replicon. The fragment was inserted into pMMN283, a pUC8-pBD64 shuttle plasmid containing the *srf* promoter region. pMMN283 was constructed by deleting part of the *neo* marker of the pBD64 moiety within pMMN94 (43). This construction was accomplished by cleaving with *Apa*I and *Bgl*II, treating with mung bean nuclease, and then intramolecular ligation. pMMN283 was cleaved at a *Sal*I site immediately downstream of the *srf* promoter, treated with Klenow fragment and deoxynucleotides to fill in the 5′ protruding *Sal*I ends, and then cleaved with *Hin*dIII. The cleaved plasmid was ligated with the *Sma*I-*Hin*dIII fragment bearing the mutant $comS_{ATG}$ allele, thus giving rise to pMMN284.

Media and culture conditions. *B. subtilis* cells were routinely cultivated in YT (2X) medium (42) for the purpose of obtaining cells for the preparation of DNA or inducing antibiotic resistance in transformed cells. Competent *B. subtilis* cells were prepared as previously described (46). One-step competence medium (CM) (11, 44) was used to culture cells for assays of *lacZ* fusion-encoded β-galactosidase activity. TSS medium (15) was used to screen for auxotrophy and to select competent cells in assays for genetic competence. *E. coli* cells were propagated in YT (2X) medium to obtain cells for plasmid isolation. The antibiotic concentrations used for the selection of drug-resistant organisms were as follows: chloramphenicol, 5 μg/ml; erythromycin, in combination with lincomycin, 1 μg/ml and 25 μg/ml, respectively; neomycin, 5 μg/ml; phleomycin, 0.1 μg/ml; spectinomycin, 75 μg/ml; and ampicillin, 25 μg/ml. The antibiotic concentrations used to induce drug resistance were as follows: chloramphenicol, 0.5 μg/ml; and erythromycin, 0.1 μg/ml.

Construction of multicopy plasmid-bearing strains. To create *sinR* and *degU* mutant strains bearing multicopy *comS*, plasmid pMMN284 DNA in combination with DNA from strain LAB2274 (Δ*sinR*::$Phleo^r$) or LAB2275 (*degU*::Neo^r) DNA was used to transform competent LAB2129 cells with selection for chlor-

TABLE 1. Strains used in this work

Strain	Genotype	Source or reference
JH642	*pheA trpC2*	J. Hoch
BD2102	*his leu met* Δ*mecA*::Kan^r *comG-lacZ*	A. Grossman
BD1512	*hisA1 leuA8 metB5 comG-lacZ* Cm^r	1
IS432	Δ*sinR*::Cm^r *leuA8 metB5 hisA1*	34
LAB844	*pheA trpC2 sfp*° Δ*srf*::Phleo^r	7
LAB1874	*pheA trpC2 sfp*° *srfB*::pCD103 (*comS′*::*′lacZ*) Cm^r	8
LAB1906	*pheA sfp srfB*::pCD108 (*srfB′*::*′lacZ*) Cm^r	8
LAB2129	*pheA trpC2 sfp*° *comG-lacZ* Spc^r	This study
LAB2240	*pheA trpC2 sfp*° *comG-lacZ* Spc^r pMMN284 (multi-*comS*, Cm^r)	This study
LAB2241	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*sinR*::Phleo^r	This study
LAB2242	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*degU*::Neo^r	This study
LAB2243	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*sinR*::Phleo^r pMMN284	This study
LAB2244	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*degU*::Neo^r pMMN284	This study
LAB2245	*pheA trpC2 sfp*° *comG-lacZ* Spc^r pMMN283 (P*srf*)	This study
LAB2246	*pheA trpC2 sfp*° *comG-lacZ* Spc^r pMMN283 (P*srf*) Δ*sinR*::Phleo^r	This study
LAB2247	*pheA trpC2 sfp*° *comG-lacZ* Spc^r pMMN283 (P*srf*) Δ*degU*::Neo^r	This study
LAB2271	*pheA trpC2 sfp*° *comG-lacZ* Spc^r pIS74 (multi-*sinR*, Cm^r)	This study
LAB2272	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*comS*::Phleo^r pIS74	This study
LAB2273	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*degU*::Neo^r pIS74	This study
LAB2274	*pheA trpC2 sfp*° Δ*sinR*::Phleo^r	This study
LAB2275	*pheA trpC2 sfp*° Δ*degU*::Neo^r	This study
LAB2280	*pheA trpC2 sfp*° *comS′*::*′lacZ* Cm^r	This study
LAB2281	*pheA trpC2 sfp*° *comS′*::*′lacZ* Cm^r Δ*sinR*::Phleo^r	This study
LAB2282	*pheA trpC2 sfp*° *comS′*::*′lacZ* Cm^r Δ*degU*::Neo^r	This study
LAB2283	*pheA trpC2 sfp*° *srfB′*::*′lacZ* Cm^r	This study
LAB2284	*pheA trpC2 sfp*° *srfB′*::*′lacZ* Cm^r Δ*sinR*::Phleo^r	This study
LAB2285	*pheA trpC2 sfp*° *srfB′*::*′lacZ* Cm^r Δ*degU*::Neo^r	This study
LAB2295	*pheA sfp*° Δ*srf*::Cm^r	This study
LAB2301	*pheA trpC2 sfp*° *comS′*::*′lacZ* Spc^r pIS74	This study
LAB2302	*pheA trpC2 sfp*° *comG-lacZ* Spc^r pSHC1 (multi-*degU*, Neo^r)	This study
LAB2303	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*sinR*::Phleo^r pSHC1	This study
LAB2304	*pheA trpC2 sfp*° *comG-lacZ* Spc^r Δ*comS*::Cm^r pSHC1	This study
LAB2435	*pheA trpC2 sfp*° *comG-lacZ* Δ*mecA*::Kan^r	This study
LAB2436	*pheA trpC2 sfp*° *comG-lacZ* Δ*mecA*::Kan^r pMMN284	This study
LAB2483	*pheA trpC2 sfp*° SPβ*c2del2*::Tn*917*::pXL5 (*srf-lacZ*)	This study
LAB2484	*pheA trpC2 sfp*° SPβ*c2del2*::Tn*917*::pXL5 (*srf-lacZ*) Δ*sinR*::Phleo^r	This study
LAB2485	*pheA trpC2 sfp*° SPβ*c2del2*::Tn*917*::pXL5 (*srf-lacZ*) pIS74 (*sinR*$^+$)	This study
LAB2486	*pheA trpC2 sfp*° SPβ*c2del2*::Tn*917*::pXL5 (*srf-lacZ*) pIS90 (Δ*sinR*)	This study

amphenicol resistance (Cm^r, conferred by pMMN284) and screen for either phleomycin resistance (Phleo^r) or neomycin resistance (Nm^r). Retention of spectinomycin resistance (Spc^r, conferred by the *comG-lacZ* fusion construct) by the transformants was also examined, as was the characteristic colony phenotype of the *sinR* mutants (19). Plasmid pSHC1-bearing Δ*srf*::Cm^r *comG-lacZ* and Δ*sinR*::Cm^r *comG-lacZ* strains (LAB2304 and LAB2303, respectively) were constructed in a similar way with pSHC1 and chromosomal DNA from either strain IS432 (Δ*sinR*::Cm^r, from I. Smith) or strain LAB2295 (Δ*srf*::Cm^r) to transform competent cells of strain LAB2129 with selection for plasmid-encoded Neo^r and Phleo^r and screening for Cm^r and Spc^r. The presence of the multicopy *degU* plasmid was verified by examining protease overproduction on milk-soft agar plates. Strains bearing multicopy *sinR* plasmid pIS74, *comG-lacZ*, and the Δ*srf*::Phleo^r or Δ*degU*::Neo^r mutations (LAB2272 and LAB2273, respectively) were constructed as described above, with strain LAB2129 being used as a recipient and the plasmid along with DNA from LAB844 (Δ*srf*::Phleo^r) or LAB2275 (Δ*degU*::Neo^r) being used for the transformations.

To construct the *comG-lacZ* fusion-bearing *mecA* mutant, competent cells of LAB2129 were transformed with DNA from strain BD2102 (Δ*mecA*::Kan^r [28]). The resulting transformant, LAB2435, was purified, made competent, and then transformed with plasmid pMMN284 with selection for Cm^r to yield strain LAB2436.

Construction of *comS*- and *srf-lacZ* fusion-bearing strains. To create a *comS′*::*′lacZ* Δ*sinR* strain, DNA from LAB1874 (*comS′*::*′lacZ* Cm^r) and LAB2274 (Δ*sinR* Phleo^r) was used to transform JH642 cells with selection for Cm^r and screening for Phleo^r. To verify that the *comS′*::*′lacZ* integration had occurred in the *srf* operon, cells of the selected transformant were lysogenized with SPβ*sfp* (40). Lysogens of JH642 would be Srf$^+$ (surfactin producing) unless the *srf* operon was disrupted by the integration of plasmid pCD103 bearing *comS′*::*′lacZ* (6). The same congression strategy was used to create *comS′*::*′lacZ* Δ*degU*::Neo^r, *srfB*::*lacZ*(pCD108 [8]) Δ*sinR*::Phleo^r, and *srfB′*::*′lacZ* Δ*degU*::Neo^r strains. The *comS′*::*′lacZ* fusion contains the first six codons of *comS* fused in frame to the 16th codon of *lacZ* (8). The *srfB′*::*′lacZ* fusion contains codon 1051 of *srfB* joined in frame to the 16th codon of *lacZ* (8).

To create a *comS′*::*′lacZ* strain bearing plasmid pIS74, the Cm^r marker associated with *comS′*::*′lacZ* was first replaced with a Spc^r marker of pJL62 by transformation. The *comS′*::*′lacZ* strain was poorly competent, as it was a *comS* mutant, but Spc^r transformants that were Cm^s appeared at a low frequency. Competent cells of the Spc^r transformant were prepared and transformed with pIS74 with selection for Cm^r, yielding strain LAB2301. Strains LAB2485 and LAB2486 were constructed by first transforming competent JH642 cells with plasmid pIS74 or pIS90. The plasmid-bearing cells were then lysogenized with SPβ*c2del2*::Tn*917*::pXL5 (43). pXL5 contains a 3.5-kb *Eco*RV-*Sal*I fragment of the promoter region and the 5′ end of the *srfA* gene. LAB2483 (SPβ*c2del2*::Tn*917*::pXL5) was transformed with DNA bearing *sinR*::Phleo^r with selection for phleomycin resistance, resulting in strain LAB2484.

Assay of genetic competence. Competent cells of strains to be tested were prepared as previously described (7). After incubation in GM2 (11), 1-ml samples were collected for total cell count, for minus DNA control, and for incubation with transforming DNA (from strain ZB307 [prototroph]). All strains tested were derivatives of JH642 and were Phe$^-$. Selection for Phe$^+$ on TSS agar medium was carried out in all assays to determine the number of cells that have undergone transformation.

RESULTS

Plasmid-amplified *comS*$_{ATG}$ confers a high level of *comG-lacZ* expression and transformation efficiency. In order to further define the roles of *comS*, *sinR*, and *degU* in the development of genetic competence, the effect of plasmid-amplified *comS* on competence and late competence operon expression

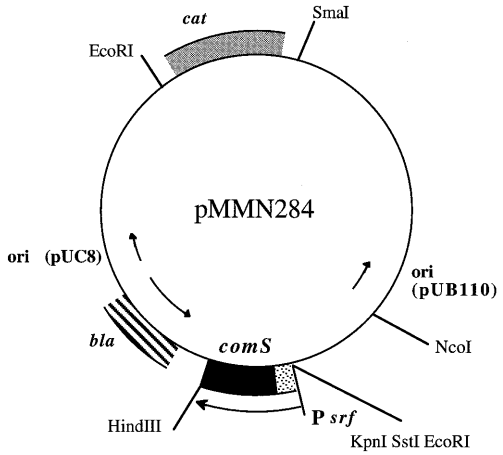

FIG. 2. Diagram of plasmid pMMN284. Shown are the locations of the fragment carrying the *comS* gene, the promoter of the *srf* operon (P*srf*), the gene encoding β-lactamase (*bla*) of pUC18, and the *cat* gene encoding chloramphenicol acetyltransferase of pBD64.

was examined in wild-type, *sinR*, and *degU* mutant cells. Plasmid pMMN94 (43) is a pUC8-pBD64 hybrid plasmid able to replicate in both *B. subtilis* and *E. coli*. It also contains the *srf* operon promoter region. The Neo^r marker within the pBD64 moiety was deleted, as described in Materials and Methods, to yield plasmid pMMN283. A *comS* allele bearing a mutation which changes TTG to an ATG start codon ($comS_{ATG}$) was inserted into pMMN283 downstream of the *srf* promoter to create pMMN284 (Fig. 2). The plasmid was introduced into cells of LAB2129 (*trpC2 pheA comG-lacZ* Spc^r) by transformation with selection for Cm^r. The expression of *comG-lacZ* was examined in the resulting transformant (LAB2240) and in LAB2129. The strain bearing plasmid-amplified $comS_{ATG}$ exhibited a higher level of *comG-lacZ* and transformation efficiency compared with the cells lacking plasmids (Fig. 3A; Table 2).

As a control, the effect of pMMN283 (lacking $comS_{ATG}$) propagation on the expression of *comG-lacZ* was examined. We had previously reported that the plasmid amplification of the *srf* promoter caused a twofold reduction in *srf-lacZ* expression, presumably because of the titration of the positive regulator, ComA-phosphate (43). This titration effect appears to affect competence gene expression significantly. The presence of pMMN283 led to a dramatic reduction in *comG-lacZ* expression in cells of strain LAB2245 (Fig. 3C). The experiments discussed above show that the elevated levels of *comG-lacZ* expression and transformation frequency are due to plasmid-amplified $comS_{ATG}$.

Plasmid-amplified $comS_{ATG}$ suppresses *sinR* but not *degU*. LAB2129 derivatives bearing pMMN284 and either *sinR* or *degU* mutations were constructed by congression (Materials and Methods). In LAB2243 cells [Δ*sinR*::Phleo^r *comG-lacZ*-(pMMN284)], the level of *comG*-directed β-galactosidase activity (Fig. 3A) was similar to that observed in LAB2240 [*sinR*$^+$ (pMMN284)], while little *comG-lacZ* activity was observed in LAB2241 (Δ*sinR*::Phleo^r *comG-lacZ*) (Fig. 3A) or LAB2246 [Δ*sinR*::Phleo^r *comG-lacZ*(pMMN283)] (Fig. 3C) cells. Likewise, LAB2243 cells exhibited a level of competence above that observed for wild-type cells, whereas LAB2241 cells were poorly competent (Table 2). Unlike, *sinR*, *degU* was not suppressed by the multicopy $comS_{ATG}$ plasmid. LAB2244 cells [Δ*degU*::Neo^r *comG-lacZ*(pMMN284)], like those of the plasmidless *degU* mutant LAB2242 (Δ*degU*::Neo^r *comG-lacZ*) and

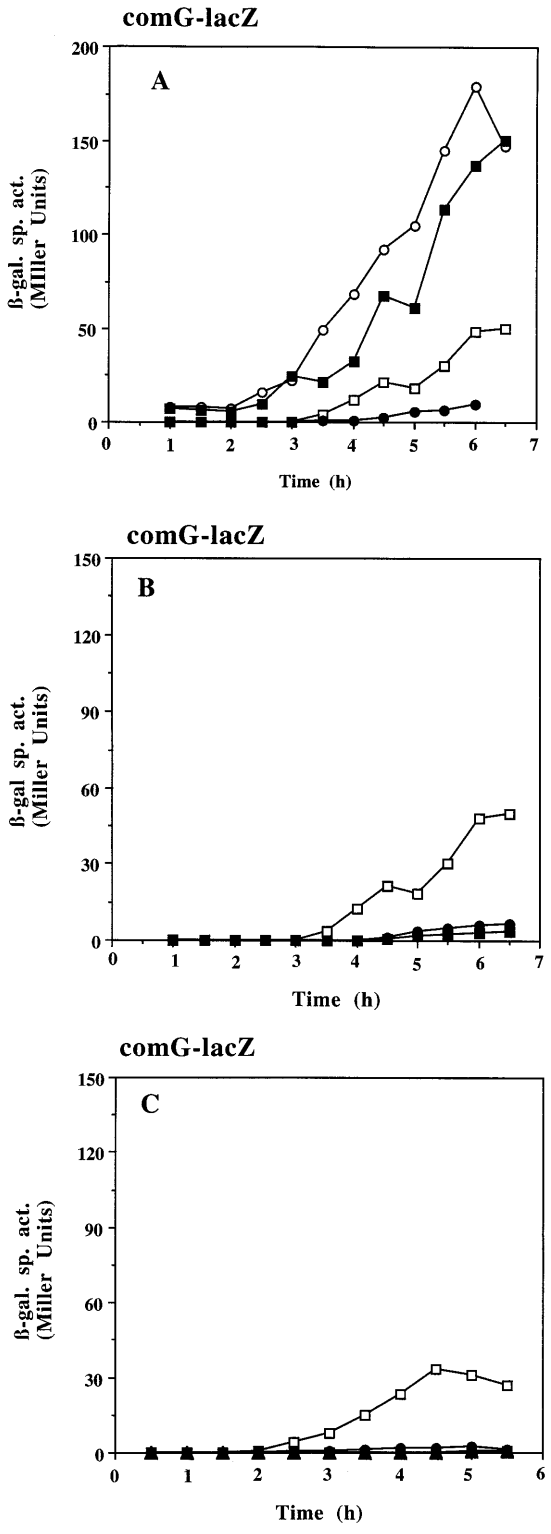

FIG. 3. A graph showing the level of *comG*-directed β-galactosidase (β-gal.) specific activity (sp. act.) over time in strains bearing either pMMN284 (P*srf-comS*) or pMMN283 (P*srf*). Cultures were grown in CM, and 1-ml samples were collected at 30-min intervals. β-Galactosidase specific activity was determined as described previously (41). (A) LAB2129 *comG-lacZ*, □; LAB2240 *comG-lacZ*-(pMMN284), ○; LAB2241 Δ*sinR comG-lacZ*, ●; LAB2243 Δ*sinR*(pMMN284), ■. (B) LAB2129, □; LAB2242 *comG-lacZ* Δ*degU*, ●; LAB2244 *comG-lacZ* Δ*degU*(pMMN284), ▲. (C) LAB2129, □; LAB2245 *comG-lacZ*(pMMN283), ●; LAB2246 *comG-lacZ* Δ*sinR*(pMMN283), ■; LAB2247 *comG-lacZ* Δ*degU* (pMMN283), ▲.

TABLE 2. Expression of *comG*::*lacZ* and transformation frequency in various strains

Strain	Relevant genotype	Maximum β-galactosidase sp act (Miller units)	% Transformation[a]
LAB 2129	Wild type	85.3	5.3
LAB 2240	Multi-*comS*	147.6	47
LAB 2241	Δ*sinR*	9.7	<0.0003
LAB 2243	Δ*sinR*, multi-*comS*	151	13
LAB 2242	Δ*degU*	6.3	<0.00012
LAB 2244	Δ*degU*, multi-*comS*	3.6	<0.0069
LAB 2271	Multi-*sinR*	0.5	0.25
LAB 2272	Multi-*sinR*, Δ*comS*	0.046	<0.014
LAB 2273	Multi-*sinR*, Δ*degU*	0.257	<0.00005
LAB 2302	Multi-*degU*	21.9	2.1
LAB 2303	Multi-*degU*, Δ*sinR*	1.1	<0.0002
LAB 2304	Multi-*degU*, Δ*comS*	0.14	<0.0005

[a] The number of transformant CFU as a percentage of the total number of CFU in the CM culture multiplied by 10^2.

pMMN283-bearing *degU* mutant (LAB2247), exhibited low-level *comG*-directed β-galactosidase activity (Fig. 3B) and transformation efficiency (data not shown).

SinR is required for optimal *comS*::*lacZ* gene fusion expression but not *srf-lacZ* operon fusion expression. The finding that multicopy $comS_{ATG}$ could suppress *sinR* suggested that *sinR* acts upstream of *comS* and may be required for *comS* expression. It has been reported that neither *degU* nor *sinR* is required for *srf* operon transcription initiation (22), but recently reported evidence indicated that *srf* expression was controlled at the posttranscriptional level by PnpA, a polynucleotide phosphorylase homolog (33). Hence, we reexamined *comS* expression and *srf* transcription in cells harboring either a *sinR* or *degU* deletion allele. *comS*-directed β-galactosidase activity was examined in cells of strains LAB2280 (*srfB*::pCD103 [*comS'*::*'lacZ*]), LAB2281 (*srfB*::pCD103 [*comS'*::*'lacZ*] Δ*sinR*::Phleo^r), and LAB2282 (*srfB*::pCD103 [*comS'*::*'lacZ*] *degU*::Neo^r). A *degU* deletion mutation had no effect on *comS'*::*'lacZ* expression, but a 47% reduction in *comS*-directed β-galactosidase activity was observed in the *sinR* deletion mutant (Fig. 4A and B) (the 47% value is based on four independent experi-

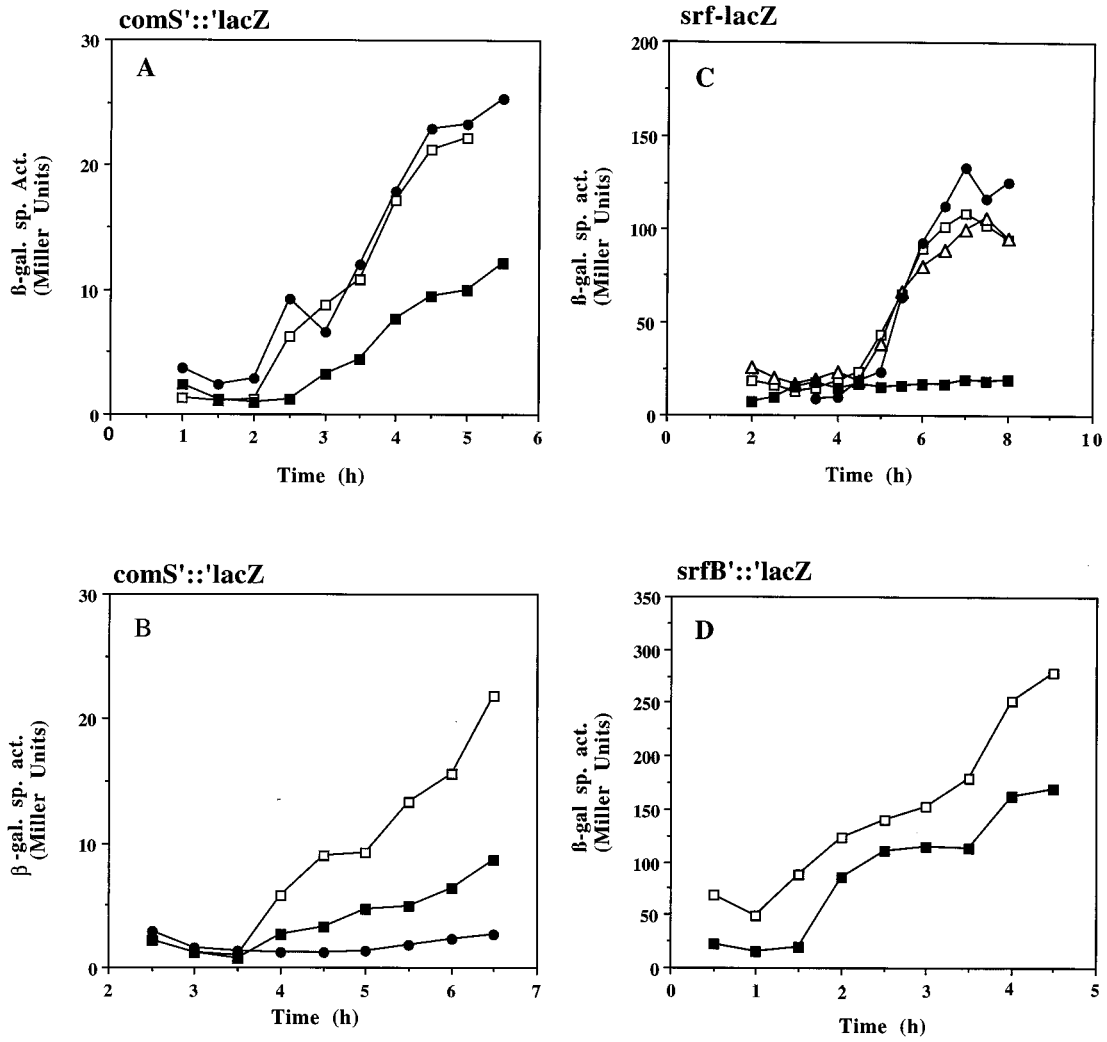

FIG. 4. The effect of *sinR*, *degU*, and plasmid-amplified *sinR* mutations on *comS'*::*'lacZ* and *srf-lacZ* expression. β-Galactosidase (β-gal.) specific activity (sp. act.) in 1-ml samples collected at 30-min intervals from cultures grown in CM was measured. (A) LAB2280 *comS'*::*'lacZ*, □; LAB2281 *comS'*::*'lacZ* Δ*sinR*, ■; LAB2282 *comS'*::*'lacZ* Δ*degU*, ●. (B) LAB2280 *comS'*::*'lacZ*, □; LAB2281 *comS'*::*'lacZ* Δ*sinR*, ■; LAB2301 *comS'*::*'lacZ*(pIS74), ●. (C) LAB2483 *srf-lacZ*, □; LAB2484 *srf-lacZ* Δ*sinR*, △; LAB2485 *srf-lacZ*(pIS74), ■; LAB2486 *srf-lacZ*(pIS90), ●. (D) LAB2283 *srfB'*::*'lacZ*, □; LAB2284 *srfB'*::*'lacZ* Δ*sinR*, ■.

ments). The effect of Δ*sinR* on *comS′*::*′lacZ* expression was not due to inhibition of *srf* transcription initiation. This conclusion was determined by examining the expression of a *srf-lacZ* transcriptional fusion (SPβ-borne pXL5 [43]) in wild-type (LAB2483) and Δ*sinR* (LAB2484) cells (Fig. 4C). No effect of the *sinR* mutation on the expression of *srf-lacZ* on pXL5 was observed after the plasmid was integrated at the *srf* locus (data not shown).

In order to determine if the activity of *sinR* was specific for *comS* or also affected the other genes of the *srf* operon, the expression of a *srfB′*::*′lacZ* gene fusion (*srfB*::pCD108 [8]) in the *sinR* mutant was also examined. Figure 4D shows that the *sinR* mutation in strain LAB2284 conferred a slight decrease in *srfB′*::*′lacZ* expression (69% of that of the wild type on the basis of four independent experiments), the significance of which is uncertain. It is possible that *sinR* exerts a mild influence on *srfB* expression.

Plasmid-amplified *sinR* does not suppress *degU* or *comS* but instead represses *srf* transcription. If our prediction that *sinR* operates upstream of *comS* in the competence regulatory pathway is correct, then plasmid-amplified *sinR* should not suppress mutations in *comS*. The expression of *comG-lacZ* was examined in cells bearing pIS74, a pUB110 derivative that harbors the *sinIR* operon and that inhibits sporulation after being propagated in *B. subtilis* cells, because of the overexpression of *sinR*. In LAB2272 cells [*comG-lacZ* Δ*srf*::Phleo^r(pIS74)], plasmid-amplified *sinR* did not result in an elevation of *comG*-directed β-galactosidase activity above the level observed in the plasmidless *comS* mutant (Fig. 3 and data not shown). Likewise, pIS74 does not suppress Δ*degU*::Neo^r with respect to *comG-lacZ* expression in LAB2273 cells. The β-galactosidase activity in LAB2301 [*comS′*::*′lacZ*(pIS74)] and LAB2485 [SPβ*srf-lacZ*(pIS74)] cells was next examined to assess the effect of *sinR* overexpression on *comS* expression and *srf* operon transcription. The expression of both fusions was dramatically reduced in the plasmid-bearing cells (Fig. 4B and C), whereas a deletion of *sinR* did not affect *srf-lacZ* expression. Thus, it is likely that multicopy *sinR* inhibits *comS* expression by repressing the transcription of the *srf* operon. This outcome is the result of plasmid-amplified *sinR*, because a derivative of pIS74, pIS90, bearing a deletion-mutated allele of *sinR* does not cause repression of *srf-lacZ* (LAB2486) (Fig. 4C).

The effect of plasmid-amplified *degU* on the expression of *comG-lacZ* in *comS* and *sinR* mutant cells was also examined. β-Galactosidase activity in LAB2302 [*comG-lacZ*(pSHC1)] cells was about fourfold lower than that observed in LAB2129 (*degU*$^+$ *comG-lacZ*) cells (Table 2). This result is probably due to the repression of *srf* transcription by *degU* overexpression, resulting in an increase in the concentration of DegU-phosphate, which has been reported to repress *srf* transcription (5). The effect of Δ*comS* and Δ*sinR* mutations on *comG-lacZ* expression and transformation efficiency was unaltered after *degU* was plasmid amplified (Table 2).

Plasmid amplification of *comS*$_{ATG}$ does not enhance competence gene expression in a *mecA* mutant. The *mecA* gene is the site of mutations that suppress *comS* (28, 51), and its product has been shown to bind directly to the ComK protein (27), thereby negatively regulating *comK* and late competence gene transcription. Hence, the *mecA* gene or its product is a candidate for the target of ComS activity. If this is the case, then one would predict that competence gene expression in a *mecA* mutant would not be enhanced further by amplification of *comS*. As can be seen from Fig. 5, the *comG*-directed β-galactosidase activity in a *mecA* mutant is high and nearly constitutive (compare Fig. 3A and 5). The presence of the *comS*-bearing multicopy plasmid, which causes a significant enhancement of *comG-lacZ* expression in wild-type cells, did not result in a further increase in *comG-lacZ* expression in the *mecA* mutant.

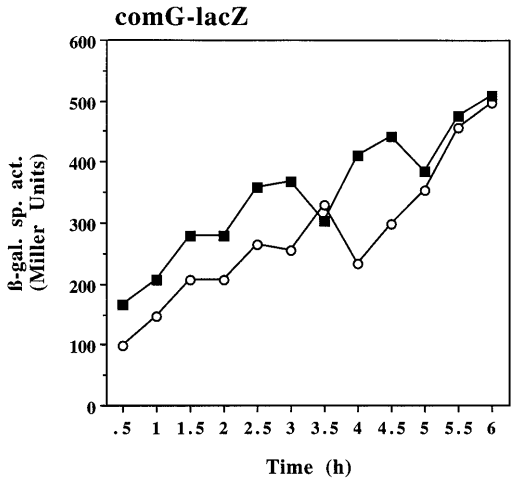

FIG. 5. The effect of plasmid-amplified *comS* on the expression of *comG-lacZ* in a *mecA* mutant. β-Galactosidase (β-gal.) specific activity (sp. act.) in 1-ml samples collected at 30-min intervals from cultures grown in CM was measured. LAB2435 *comG-lacZ* Δ*mecA*, ○; LAB2436 *comG-lacZ* Δ*mecA*(pMMN284), ■.

DISCUSSION

An investigation of the *sinR* and *degU* genes has been undertaken in order to understand their roles in the process of competence development and specifically their relationship to *comS*, which encodes a small peptide required for the activation of transcriptional regulator ComK. The ComS polypeptide is thought to directly or indirectly inactivate MecA and/or MecB, which negatively controls ComK. MecA and MecB show homology to, respectively, the catalytic and regulatory subunits of the ATP-dependent Clp proteases (27, 38). MecA probably inhibits the activity of the late competence gene activator, ComK, by a direct interaction (27). At present, we do not know the primary functions of SinR or DegU in the regulatory network that governs competence development, but they, like *comS*, are required for expression of *comK* (23). As reported here, the plasmid amplification of *comS*$_{ATG}$ results in suppression of a *sinR* deletion but not *degU*. This result suggests that *sinR* could act upstream of *comS* in one of the regulatory pathways controlling competence, whereas DegU is part of a parallel pathway also affecting Mec or ComK activity. In support of this view, data showing that *sinR* is required for the optimal expression of a *comS′*::*′lacZ* fusion are presented here. However, it is also possible that the *sinR* mutation blocks a step of competence development that lies downstream of *comS*, with this step also being directly influenced by the ComS peptide. For example, one could imagine that *sinR* might be required for the transcriptional repression of *mecB* or *mecA* or the activation of *comK* transcription, in keeping with its known function as a DNA-binding protein (20). The *mec* genes would be derepressed in a *sinR* mutant, resulting in the inhibition of ComK and competence gene transcription. This inhibition caused by *mec* gene derepression would be expected to be reversed by increasing the production of ComS, which would compensate for the elevation in Mec protein levels. Elevated ComS levels could also compensate for a low ComK concentration in a *sinR* mutant by severely reducing the activity of MecA and -B proteins. Hence, while the data presented here

support a role for SinR in regulating *comS* expression, it is also possible that SinR could act downstream of *comS* and yet still be subject to *comS* multicopy suppression. Another potential effect of a *sinR* mutation on competence relates to its observed phenotype conferred on cells grown on solid medium. Mutant cells within colonies show enhanced cell-to-cell adherence (18, 19), due perhaps to alterations of the cell surface or cell wall. These alterations could potentially affect the later stages of competence, such as the assembly of the DNA uptake apparatus or DNA binding. This possibility might be why transformation efficiency, although elevated in *sinR*(pMMN284) (*comS*) cells with respect to that in the plasmidless wild type, is not as high as transformation efficiency in *sinR*$^+$(pMMN284) cells (Table 2).

Previous reports have described the role of *sinR* as a positive regulator of competence development and a negative regulator of sporulation (3, 19), that is, it acts as a molecular switch which influences a cell's decision to undergo either competence development or sporulation. One of its functions in competence appears to be the positive control of *comS* expression. The effect of a *sinR* mutation on *comS′::′lacZ* expression is modest, with it being about twofold. But if ComS is a limiting factor in the competence regulatory network, as appears to be the case, since *comS* plasmid amplification results in elevated transformation efficiency, then perhaps it is not surprising that a mild repression of *comS* would have such a drastic effect on competence, such as that observed in a *sinR* mutant. In support of this view is the observed effect of *srf* promoter amplification on competence gene expression. This effect is a drastic repression, even though the multicopy *srf* promoter has only a twofold effect on *srf* transcription (43). The multicopy *srf* promoter also causes a 10-fold decrease in transformation efficiency (data not shown). How might SinR function in *comS* expression? SinR is a DNA-binding protein that acts as a transcriptional repressor of such sporulation operons as *spoIIA*. So it is possible that SinR could be a transcriptional regulator of a gene whose product controls *comS* expression at the posttranscriptional level. For example, a potential target of SinR is the *pnp* gene (33), which is required for the posttranscription initiation control of *comS*. It is also true that certain DNA-binding proteins also bind to RNA. LacI, LexA, and lambda CI all have been shown to bind to 10Sa RNA (49). The homeodomain protein, *bcd* (bicoid), of *Drosophila melanogaster* has been shown to bind to a site on the control region of genes activated during larval development as well as the 5′ untranslated region of *cad* (caudal) mRNA (14) and thus is both a positive and negative regulator of larval development. It is possible that SinR itself could bind to *srf* RNA to somehow facilitate translation of the *comS* message.

Whereas it is true that *sinR* is required for competence, according to the data reported herein, SinR, if overproduced, can also inhibit competence development. Plasmid amplification of *sinR* was observed to cause severe repression of a *srf-lacZ* transcriptional fusion. This repression is caused by *sinR* because a deletion of the *sinR* coding sequence in the multicopy plasmid eliminates *srf* transcriptional repression. At this time, it is not known at what step prior to *srf* transcription initiation SinR overproduction causes a block in the competence regulatory network. Studies of AbrB have shown that this transition state regulator is required for competence development but that if it is overproduced, as is the case in cells bearing a mutation in *spo0A* encoding the repressor of *abrB*, it will inhibit competence (24). AbrB overproduction results in the repression of two genes required for optimal competence development, *spo0H* (64) encoding the minor form of the RNA polymerase sigma subunit σ^H, which is also required for sporulation, and *comK* (24). Like AbrB, SinR, within a narrow concentration range, is required for competence development.

The results presented herein suggest that DegU occupies a position within a pathway that is parallel to that in which *comS* and *sinR* function, since multicopy $comS_{ATG}$ does not suppress a *degU* mutation. Because plasmid-amplified *sinR* represses competence, presumably by repressing *srf* transcription initiation, we do not know if multicopy *sinR* can suppress *degU*. This question might be resolved if *srf* transcription could be placed under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter, such as P*spac* (65), and thus rendered insensitive to SinR-dependent repression. One could then overexpress *sinR* in a *degU* mutant and observe the effect on competence development.

DegU, like SinR, can also be regarded as a molecular switch influencing the competence-or-sporulation decision. DegU, a member of the response regulator class of two-component signal transduction proteins, promotes competence development if it is unphosphorylated, whereas the phosphorylated form favors sporulation and degradative enzyme production. Mutants of DegU that are hyperphosphorylated cause competence to be repressed and sporulation to initiate, even under conditions of high nutrient availability which normally repress spore formation (6, 30). The DegU protein may represent another target of ComS function if the two competence-regulating pathways, *comS* dependent and *degU* dependent, converge at the level of Mec activity.

The results summarized in Fig. 5 show that *comS* plasmid amplification, while increasing the level of competence in a wild-type background, does not elevate further competence gene expression in a *mecA* mutant. This result would be expected if the target of *comS* activity is the *mec*-dependent inhibition of ComK, since removing Mec activity would render inconsequential any increase in the ComS concentration. In fact, synthetic ComS peptide can interact with His6-tagged MecA protein, but not MecB, in vitro (31). These data suggest that the target of ComS in vivo is MecA, which would be an interaction that could affect the binding of MecA with either MecB or ComK.

ACKNOWLEDGMENTS

We thank D. Dubnau, A. Grossman, T. Tanaka, and I. Smith for strains and plasmids and D. Dubnau and A. Grossman for valuable discussions. C. D'Souza constructed the $comS_{ATG}$ allele.

Support for this work was provided by grant GM45898 from the National Institutes of Health and by the Center of Excellence in Cancer Research, Treatment and Education, LSUMC-S.

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