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Cloning and Characterization of a Cluster of Linked *Bacillus subtilis* Late Competence Mutations

MARK ALBANO† AND DAVID A. DUBNAU*

Department of Microbiology, The Public Health Research Institute, New York, New York 10016

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We characterized a segment of chromosomal DNA from *Bacillus subtilis* that was required for the development of genetic competence. The chromosomal DNA was cloned from a group of genetically linked and phenotypically similar Tn*917lac* insertion mutants deficient in competence. This cluster of mutations defined the *comG* locus. Chromosomal DNA flanking each of the six insertions was cloned. Restriction maps of the cloned plasmids revealed that their chromosomal inserts consisted of overlapping fragments. These data, together with Southern blots of chromosomal DNA from the *comG* mutants, showed that the six Tn*917lac comG* insertions occurred in the following order: *comG12*, *comG39*, *comG412*, *comG107*, *comG56*, and *comG210*. Expression of the *comG* Tn*917lac* insertions was from a promoter located upstream from the first insertion, *comG12*. This was determined genetically and by low-resolution S1 nuclease mapping of the 3′ terminus. The *comG* region spanned about 5 kilobase pairs, based on low-resolution S1 nuclease mapping of the transcription terminator and Northern blotting. The *comG12* mutation had a partial epistatic effect on the expression of one other *com* locus, *comE*, but none of the other *comG* mutations affected expression of this or any other *com* gene tested. Based on these conclusions, and on its size and phenotype, the *comG* locus must be organized as a polycistronic operon that is subject to competence-specific regulation.

Genetic competence in *Bacillus subtilis* is an example of a regulated global response to environmental conditions and developmental signals. The final products of this regulated response are also of interest, because they are presumably required for the binding, processing, and transport of DNA from the medium into the cytosolic compartment. Little is known about these products or the specific roles they play (for a review, see reference 8), and it is of interest to characterize them on the molecular level.

A group of competence-deficient Tn*917lac* insertion mutants of *B. subtilis* was previously isolated by Hahn et al. (15). The mutations in this group (group VI) were shown to be linked genetically to *aroD* and *lys*. About half (six) of the *com* mutants in this group expressed β-galactosidase, presumably because the promoterless *lacZ* gene on the transposon was placed under the control of a *com* promoter(s) (2). This group of linked insertions defines the *comG* locus and consists of the mutations *comG12*, *comG39*, *comG412*, *comG107*, *comG56*, and *comG210*. The *comG* insertions confer a deficiency in the ability to bind exogenous DNA. Only one, *comG12*, prevents separation on Renografin (E. R. Squibb & Sons, Princeton, N.J.) gradients into the two cell density fractions characteristic of competent *B. subtilis* cultures (14, 16).

Expression of all six transcriptional fusions is dependent on growth in competence medium and increases sharply at about the time of transition from the exponential to the stationary growth phase (T_0) (2). The *comG* insertions thus define late competence functions, in contrast with early competence genes, which express their products at maximal or near maximal rates during exponential growth and in all growth media.

The Tn*917lac* insertional mutagen and its cloning derivatives constructed by Perkins, Youngman, and colleagues (29, 30, 32) provided us with powerful and convenient tools for cloning *comG* DNA from *B. subtilis*. We were able to clone Tn*917lac* insertion proximal chromosomal DNA from each of the six insertion mutants, obtaining in all about 6.5 kilobase pairs (kbp) of *B. subtilis* chromosomal DNA. We were also able to clone an intact region of *comG* DNA that was devoid of insertional disruptions and that consisted of about 8.5 kbp. We analyzed the cloned fragments by restriction mapping and determined the linear order of the six insertion positions in the *comG* region by Southern blotting. The epistatic relationships between *comG* and other competence genes were examined.

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MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from several sources (Boehringer Mannheim Biochemicals, Indianapolis, Ind.; New England BioLabs, Inc., Beverly, Mass.; and Bethesda Research Laboratories, Gaithersburg, Md.) and were used as specified by the manufacturers. T4 DNA ligase was obtained from Amersham Corp. (Arlington Heights, Ill.), S1 nuclease was from Boehringer Mannheim, and RQ1 DNase was from Promega Biotech. ^{32}P-labeled nucleotides were from Dupont, NEN Research Products (Boston, Mass.).

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1, and the plasmids and bacteriophages used in this study are listed in Table 2. All *B. subtilis* strains were isogenic with BD630 and were *his leu metB5* auxotrophs. In Table 1, strains listed as Campbell recombinants were constructed by transformation of strain BD630 with clones containing insertion-proximal chromosomal DNA by selecting for chloramphenicol resistance (Cm^r). *Escherichia coli* HB101 was used as the host for all in vitro-generated recombinant plasmids by using the pBR322

* Corresponding author.
† Present address: Laboratoire de Genetique Microbienne, Institute de Biotechnologie, Institut National de la Recherche Agronomique Domaine de Vilvert, 78350 Jouy en Josas, France.

TABLE 1. *B. subtilis* strains used in this study

Strain	Chromosomal genotype	Comment	Reference
BD630	*hisH2 leu met*		I. Smith
BD1248	*comG12*		15
BD1254	*comG39*		15
BD1256	*comG412*		15
BD1260	*comG107*		15
BD1258	*comG56*		15
BD1252	*comG210*		15
BD1200	*comG12*(pTV21Δ2)		This work
BD1319	*comG39*(pTV21Δ2)		This work
BD1320	*comG412*(pTV21Δ2)		This work
BD1617	*comG107*(pTV21Δ2)		This work
BD1618	*comG56*(pTV21Δ2)		This work
BD1619	*comG210*(pTV21Δ2)		This work
BD1237	*comC530*		15
BD1245	*comD413*		15
BD1246	*comE510*		15
BD1247	*comE518*		15
BD1243	*comA124*		15
BD1241	*comB138*		15
BD1512	[*comG12-Bgl*II clone][a]	Campbell recombinant[b]	This work
BD1607	[*comG12-Hin*dIII clone]	Campbell recombinant	This work
BD1516	[*comG12-Sph*I clone]	Campbell recombinant	This work
BD1517	[*comG39-Hin*dIII clone]	Campbell recombinant	This work
BD1518	[*comG39-Sph*I clone]	Campbell recombinant	This work
BD1519	[*comG39-Bgl*II clone]	Campbell recombinant	This work
BD1520	[*comG412-Bgl*II clone]	Campbell recombinant	This work
BD1521	[*comG412-Hin*dIII clone]	Campbell recombinant	This work
BD1522	[*comG412-Sph*I clone]	Campbell recombinant	This work
BD1523	[*comG107-Bgl*II clone]	Campbell recombinant	This work
BD1524	[*comG107-Hin*dIII clone]	Campbell recombinant	This work
BD1525	[*comG107-Sph*I clone]	Campbell recombinant	This work
BD1526	[*comG56-Bgl*II clone]	Campbell recombinant	This work
BD1527	[*comG56-Hin*dII clone]	Campbell recombinant	This work
BD1528	[*comG56-Sph*I clone]	Campbell recombinant	This work
BD1529	[*comG210-Bgl*II clone]	Campbell recombinant	This work
BD1530	[*comG210-Hin*dIII clone]	Campbell recombinant	This work
BD1531	[*comG210-Sph*I clone]	Campbell recombinant	This work
BD1508	[*comG* operon *Sst*I clone]	Campbell recombinant	This work
BD1509	[M13 *Eco*RI-*Cla*I clone]	Campbell recombinant	This work
BD1510	[M13 *Eco*RI-*Sph*I clone]	Campbell recombinant	This work
BD1511	[M13 *Bgl*II-*Bam*HI clone]	Campbell recombinant	This work
BD1590	*comG12* Lac$^+$		This work
BD1591	*comG39* Lac$^-$		This work
BD1593	*comC530* Lac$^+$ *comG12* Lac$^-$		This work
BD1594	*comD413* Lac$^+$ *comG12* Lac$^-$		This work
BD1595	*comE510* Lac$^+$ *comG12* Lac$^-$		This work
BD1596	*comE518* Lac$^+$ *comG12* Lac$^-$		This work
BD1598	*comD413* Lac$^+$ *comG39* Lac$^-$		This work
BD1599	*comE510* Lac$^+$ *comG39* Lac$^-$		This work
BD1600	*comE518* Lac$^+$ *comG39* Lac$^-$		This work
BD1605	*comA124* Lac$^+$ *comG12* Lac$^-$		This work
BD1606	*comB138* Lac$^+$ *comG12* Lac$^-$		This work

[a] Brackets indicate an integrated plasmid molecule. The *comG* and M13 clones were integrated in the *comG* regions.
[b] Campbell recombinant refers to a strain that was made by transformation with a circular molecule and subsequent chromosomal integration by Campbell-like recombination.

vector. pTV55Δ2 was derived from pTV55 (31) by N. Guillen (unpublished data). It was constructed by partial digestion of pTV55 DNA with *Hpa*I and complete digestion with *Sma*I, followed by self-ligation to delete part of the *lacZ* gene.

Media. Solid media for *B. subtilis* were tryptose blood agar base (Difco Laboratories, Detroit, Mich.) and minimal medium (3) with appropriate growth supplements. Chloramphenicol or erythromycin (5 μg/ml) was added as required. The liquid media used for *B. subtilis* were VY medium (25 g of veal infusion [Difco], 5 g of yeast extract [Difco], and 1,000 ml of water) for the growth of phage and recipient cultures in transduction experiments and competence medium (7) for growth to competence. When added as supplements, amino acids were used at concentrations of 50 μg/ml. Liquid media used for *E. coli* experiments were Luria broth (LB) or 2× yeast tryptone (22). Solid medium consisted of LB agar supplemented with antibiotics (ampicillin, 100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 μg/ml), as required.

Purification of DNA. Rapid plasmid preparations in *E. coli* were made by using the alkaline lysis protocol (5). Large-scale preparations of *E. coli* plasmid DNA were made by

TABLE 2. Plasmids and bacteriophages used in this study

Plasmid or bacteriophage	Genotype	Comments	Reference
Plasmids			
pTV21Δ21	Cm^r Ap^r	pE194 replicon	29
pTV55	Cm^r Tet^r	pE194 replicon	31
pTV55Δ2	Cm^r Tet^r	pE194 replicon	N. Guillen, personal communication
pBR322	Ap^r Tc^r		6
pUC18	Ap^r *lacZ'* with multiple cloning sites		28
Bacteriophages			
PBS1			25
M13mp19			21
M13mp19*cat*			13
M13mp19*cat-comG-Eco*RI-*Cla*I fragment			This work
M13mp19*cat-comG-Eco*RI-*Sph*I fragment			This work
M13mp19*cat-comG-Bgl*II-*Bam*HI fragment			This work

using essentially the same alkaline lysis procedure followed by purification in CsCl-ethidium bromide density gradients, as described by Maniatis et al. (19). Transforming chromosomal DNA was isolated from *B. subtilis* strains as described previously (10).

Transduction and transformation. Preparation of phage PBS1 transducing lysates and transduction of *B. subtilis* strains were carried out as described previously (9). For studies of transformation frequency, *B. subtilis* strains were made competent and transformed as described previously (10). For some purposes, as noted in the text, the one-step competence procedure was used (2). Plate transformation was carried out as described elsewhere (15). Competent *E. coli* cells were prepared as described by Mandel and Higa (18) and Maniatis et al. (19).

Preparation of radiolabeled probe DNA. For Southern blotting, ^{32}P-labeled plasmid DNA probes were prepared by nick translation (23) with either [α-^{32}P]dATP or [α-^{32}P]dCTP (specific activities, 3,000 Ci/mmol; Dupont, NEN Research Products). The specific activities obtained were 5×10^7 to 5×10^8 cpm/μg of DNA. Labeled DNA was separated from unincorporated nucleotides by passage through Sephadex G50 columns (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with TES buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 100 mM NaCl). For S1 nuclease mapping, 3'-labeled probe was made by using [α-^{32}P]dATP and [α-^{32}P]dCTP in a fill-in reaction with the Klenow fragment of DNA polymerase as described previously (19).

Southern blotting. DNA samples that were electrophoresed through 0.8% agarose slab gels were transferred to nitrocellulose membranes (BA85; Schleicher & Schuell, Inc., Keene, N.H.) by a Southern blotting method (24) that has been modified for the transfer of large fragments of DNA (27). Filters were hybridized as described previously (17). Autoradiograms were exposed at −70°C with Cronex Lightning Plus (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) intensifying screens for variable periods of time.

S1 nuclease mapping. RNA was prepared as described by Ulmanen et al. (26) from strains that were grown through the one-step competence regimen. S1 nuclease mapping was carried out as described previously (4, 11). Protected fragments were analyzed by using 6% polyacrylamide–8 M urea gels (length, 23 cm) (20).

Determination of β-galactosidase activity. For the determination of β-galactosidase activity in competence medium, cells were grown in the one-step competence regimen. Samples (0.5 ml) were taken at various times during growth, and the β-galactosidase activity was determined as described previously by Gryczan et al. (12). β-Galactosidase specific activity was expressed as units per milligram of protein. Protein was determined from standard curves by relating the turbidity (in Klett units) and the protein concentration in the bacterial cultures. Protein was measured with a reagent (Bio-Rad Laboratories, Richmond, Calif.), as specified by the manufacturer.

RESULTS

Use of Tn*917* to clone insertion-proximal DNA. A collection of *B. subtilis* competence mutants was previously isolated by using Tn*917lac* as a mutagen (15). Six mutations (*comG12*, *comG39*, *comG412*, *comG107*, *comG56*, and *comG210*) that were genetically linked to *aroD* and *lys* and phenotypically similar to one another served to define the *comG* region. Tn*917* derivatives of the *E. coli* plasmid pBR322 were used to clone chromosomal DNA flanking the *comG* insertions (30, 32) (Fig. 1). In this procedure the resident Tn*917* element was replaced with one that carried a pBR322 replicon, which permitted the flanking chromosomal DNA to be excised and cloned in *E. coli*. This replacement is usually carried out by transformation. However, in this study the recipient strains were completely competence deficient. Therefore, strains carrying the Tn*917lac* mutations were transduced by PBS1 carrying pTV21Δ2. Selection for Cm^r produced transformants in which replacement of the recipient macrolide-lincosamide-streptogramin B resistance (MLS^r) transposon by the donor plasmid occurred. The loss of the MLS^r marker showed that integration of the pTV21Δ2 element occurred by replacement and not by Campbell-like recombination. The resulting strains are listed in Table 1.

Chromosomal DNA from each of these six recombinant strains was completely and separately digested with three restriction enzymes, *Bgl*II, *Hin*dIII, and *Sph*I. Each digested DNA was self-ligated and used to transform *E. coli* to ampicillin resistance. The structures of the resulting plasmids were confirmed by restriction site mapping.

The plasmid obtained in this manner by *Bgl*II digestion of DNA from the *comG12* insertion mutant (*comG12-Bgl*II) was used to clone the intact *comG* region as follows (Fig. 2). *comG12-Bgl*II plasmid DNA was used to transform strain BD630 by Campbell-like integration. Cm^r clones were selected, and 25 of these were assayed for transformability. All

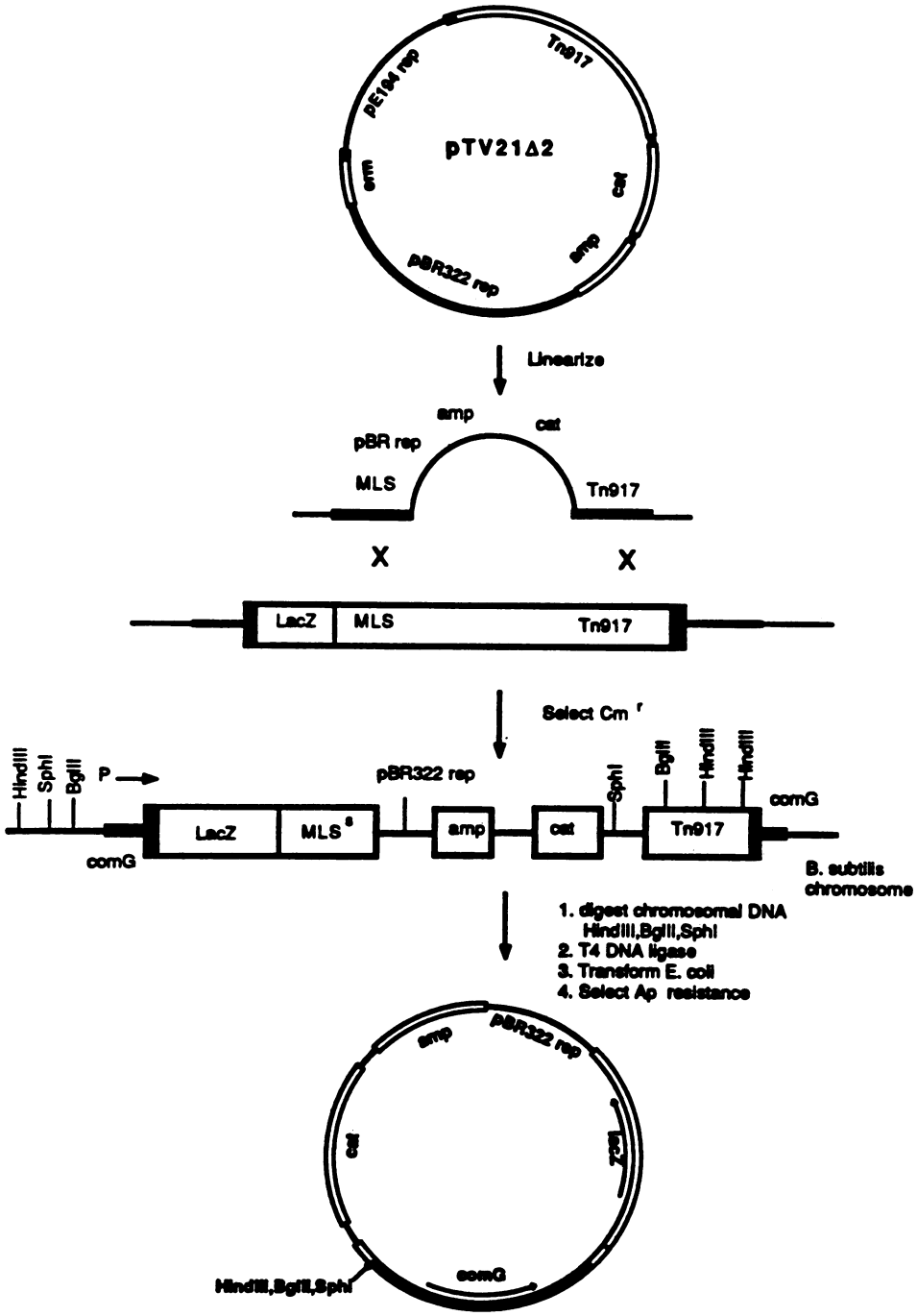

FIG. 1. Strategy described by Youngman et al. (29) for cloning Tn*917lacZ* insertion-proximal sequences adapted to *com* genes. A PBS1 phage lysate containing pTV21Δ2 (29) was used to transduce *comG* mutants that contained integrated chromosomal copies of Tn*917lac*. Homologous replacement recombination occurred between the integrated Tn*917lac* sequence and pTV21Δ2. The pTV21Δ2 moiety contained sequences for replication in *E. coli* as well as for resistance to ampicillin and chloramphenicol. The replacement recombination disrupted the Tn*917* MLS^r gene and resulted in a MLS^s phenotype. Three restriction sites that were used to clone insertion-proximal sequences are shown. An additional chromosomal *Sph*I site that was used to clone some of the insertion-proximal sequences is not indicated but occurred to the right of the *Bgl*II site (see text). Chromosomal DNA from strains containing the replacement pTV21Δ2 construct was digested with each of the three restriction enzymes. The digestion mixtures were self-ligated and used to transform *E. coli* by selecting for Ap^r. Plasmid preparations from the resistant colonies yielded insertion-proximal clones, as indicated.

were transformable at the wild-type frequency, indicating that *comG12-Bgl*II contains the *comG* upstream sequences that are required for transcription (see below). Two clones were assayed for β-galactosidase activity. They produced the same activity as the original *comG12* mutant strain, which further confirmed the presence of the sequences essential for transcription. Chromosomal DNA from one of these strains was separately digested to completion with the

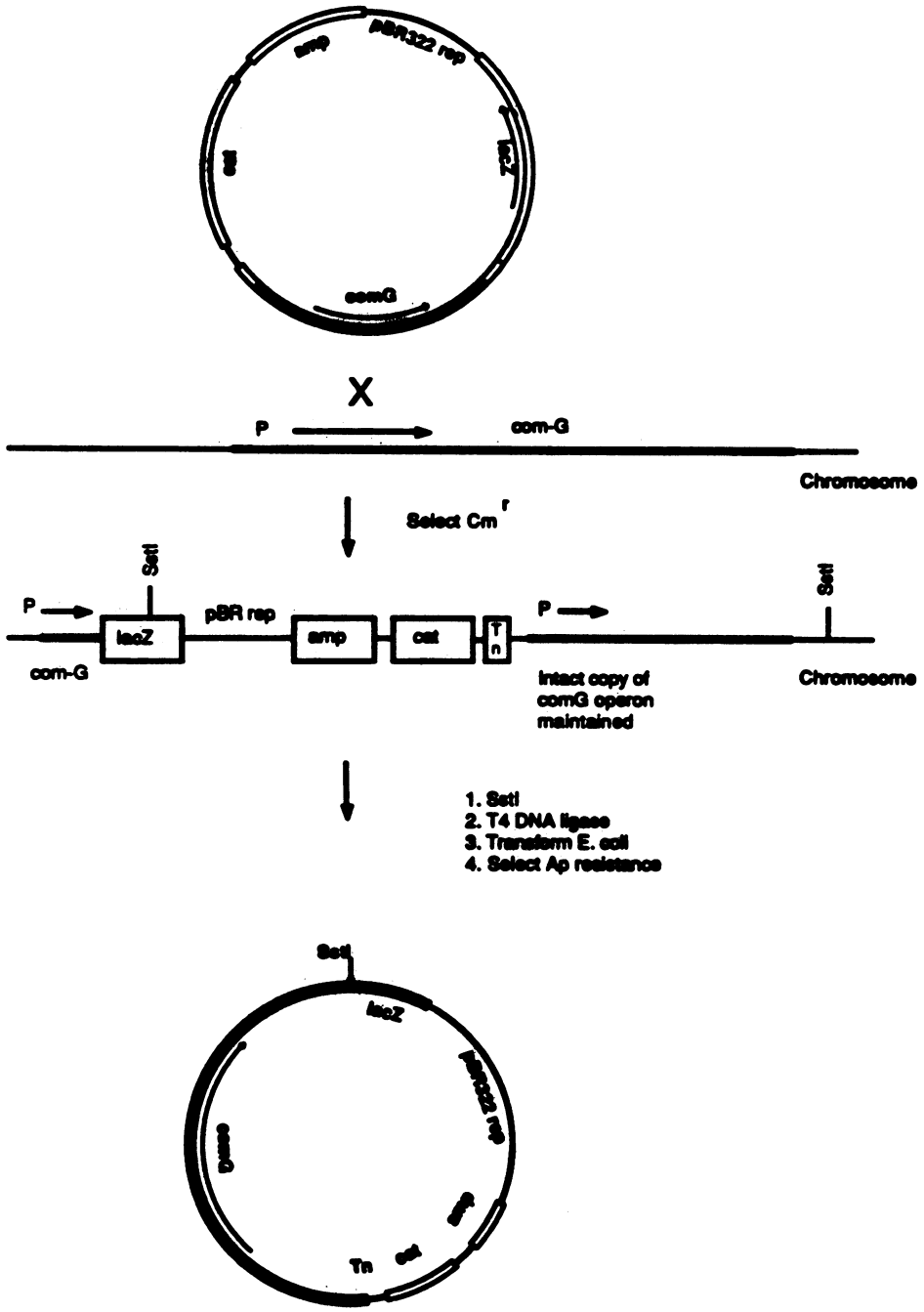

FIG. 2. Strategy for cloning the intact *comG* region. The insertion-proximal clone *comG12-Bgl*II, which was derived as shown in Fig. 1, contained the intact *comG* transcription border (shown genetically and by S1 nuclease promoter mapping; see text). This plasmid was used to transform the wild-type Com$^+$ strain BD630, yielding Cm^r transformants. The plasmid was integrated into homologous *comG* chromosomal sequences by Campbell-like recombination, producing one disrupted and one intact copy of the *comG* region. Chromosomal DNA from this Cm^r strain was then digested with *Sst*I, self-ligated, and used to transform *E. coli* by selecting for Ap^r. Plasmid preparations from a transformant produced the intact *comG* region clone.

restriction enzymes *Bal*I, *Hpa*I, and *Sst*I. The digestion mixtures were self-ligated and transformed into *E. coli* with selection for ampicillin resistance. Only the DNA that was cut and ligated with *Sst*I gave transformants. These carried a plasmid containing about 7 kb of *B. subtilis* chromosomal DNA, in addition to the vector (Tn*917lac* Cm^r Ap^r pBR322) sequences.

Each of the plasmids containing insertion-proximal sequences and the one containing the presumptive intact *comG* region were analyzed by restriction digestion (Fig. 3). The plasmid that was presumed to carry the intact *comG* region contained the same restriction sites that were found in the largest insertion-proximal clone (*comG210-Bgl*II). Additional sites occurred which corresponded to an additional 2 kb of DNA downstream from the *comG210* insertion. It was clear from this study that the six insertions are related and

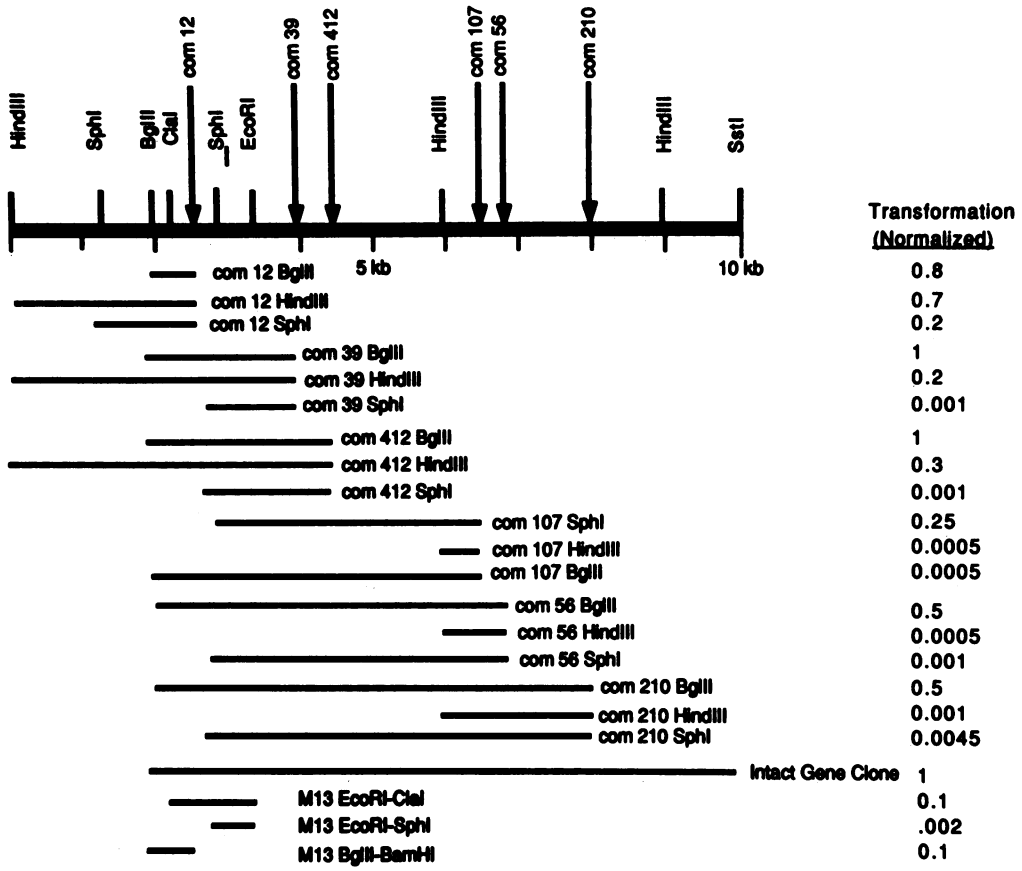

FIG. 3. Linear map of the *comG* region. A 10-kb region of *B. subtilis* chromosomal DNA contained the *comG* sequences that are required for competence. Relevant restriction sites are indicated. Some sites occurred more than once but are not indicated. The approximate locations of the Tn*917lac* insertions are shown. The lower half of the figure shows various clones that were made from the *comG* region by using the strategies presented in Fig. 1 and 2. Each clone was aligned to the 10-kb region, indicating the fragment of *comG* DNA cloned in each case. Each clone is labeled with the insertion mutation number and the restriction enzyme that was used to derive the clone, except in the last four cases. The M13 clones contained fragments of *comG* DNA that were subcloned into M13mp19*cat* (13). Each clone was used to transform the *com*$^+$ strain (BD630) by Campbell-like integration of the cloned fragment. The resulting transformants were tested for transformability, with the results given on the right. These values were normalized to the transformation frequency of the *com*$^+$ strain.

that the largest cloned plasmid possibly contains the intact *comG* region.

Southern blotting. To confirm the spatial relationships of the six Tn*917lac* insertion mutants, a number of Southern blotting experiments were performed (24). Figure 4 shows the results of a typical experiment. Chromosomal DNA from each of the mutant strains was digested to completion with the restriction enzyme *Bgl*II. The digested DNA was resolved by agarose gel electrophoresis and blotted onto a nitrocellulose filter. The blot was then probed with ^{32}P-labeled, nick-translated DNA from the *comG12-Hin*dIII plasmid (Fig. 3). This probe contained sequences upstream from the unique *Bgl*II *comG* site plus sequences downstream to the point of insertion of Tn*917lac* in *comG12*. The Southern blot in Fig. 4 shows two bands that hybridized to the probe in the mutant and wild-type strains. The lower bands were identical in size and corresponded to a *Bgl*II fragment that hybridized to sequences upstream of the unique *Bgl*II *comG* site. The other bands were the result of hybridization to another *Bgl*II fragment whose original size was estimated from the lane containing BD630 (Fig. 4, lane 8). This 8-kb BD630 fragment represents the native fragment into which the insertions occurred. The lanes containing mutants (Fig. 4, lanes 2 to 7) show hybridizing fragments of different sizes. This is a result of Tn*917lac* insertions at different sites downstream from the unique *Bgl*II site, since the Tn*917lac* element contained a single *Bgl*II site about 5.5 kb from its left terminus. The *comG12* mutation (smallest fragment) appeared to be the closest to the *Bgl*II site, while the *comG210* mutation (largest fragment) was the farthest away.

Additional Southern blotting experiments were performed. Chromosomal DNA from each of the insertion mutants was digested with the appropriate restriction enzymes and probed with other nick-translated insertion-proximal clones. These experiments generated a consistent linear map (Fig. 3).

Promoter mapping. The six mutations which define the *comG* region express β-galactosidase similarly in competence medium (2). Each expressed it at about T_0 and in a cell-type-specific fashion. The latter refers to the expression of β-galactosidase activity only in the Renografin (Squibb) light fraction. An exception to this was the case of *comG12* strains, which failed to resolve in Renografin (see below). This largely common pattern of β-galactosidase expression suggests that all of the insertions may be transcribed from a single upstream promoter.

To test this we used the promoter mapping technique

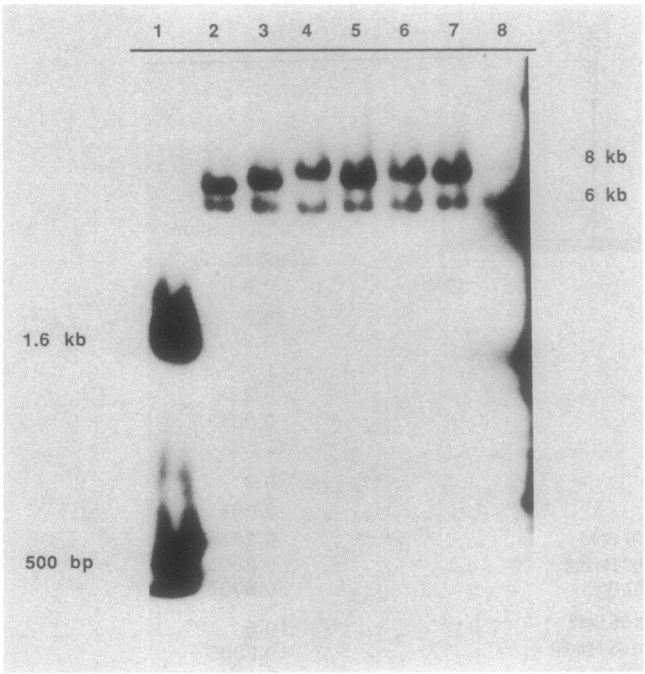

FIG. 4. Southern blot of chromosomal DNA from *comG* mutants. Chromosomal DNAs from *comG* Tn*917lacZ* insertion mutants and from BD630 (*com*$^+$) were digested with *Bgl*II and hybridized to a ^{32}P-labeled, nick-translated *comG12-Hin*dIII probe. Lane 1, ^{32}P-labeled, restriction enzyme-digested λ DNA size standard; lanes 2 through 7, DNA from *comG12*, *comG39*, *comG210*, *comG412*, *comG56*, and *comG107*, respectively; lane 8, DNA from BD630.

described by Youngman et al. (29). BD630 (Com$^+$) was transformed by Campbell-like recombination, with each of the insertion-proximal plasmid clones, with the intact gene clone, and with three M13mp19*cat-comG* bacteriophage DNAs (see alignment to the map of the *comG* region in Fig. 3). Integration in these cases only occurred by a Campbell-like event, since the selected marker (Cm^r) was not flanked by homologous chromosomal DNA in the normal orientation. Twelve Cm^r clones from each transformation were tested for competence by the plate transformation assay by using chromosomal DNA and by selecting for *leu* prototrophs. In all cases, the 12 isolates from a given transformation yielded identical results in this semiquantitative test. For confirmation, one isolate from each batch was also then assayed in the standard liquid competence regimen. The results are shown in Fig. 3 as the frequency of transformation normalized to that of the wild type. All of the strains made by Campbell-like transformation of BD630 with plasmid clones derived by *Bgl*II from the six *com* mutants had normal or near normal transformation frequencies. The transformation frequencies described as near normal probably represented the slight variation we encountered among competence cultures. However, there may also have been some unexplained variation because of the structure of the Campbell-like derivatives. These results indicate that the *Bgl*II clones all contained the sequences that are required for transcription and, hence, when integrated by Campbell-like (single reciprocal) recombination, yielded one disrupted and one intact copy of the *comG* transcriptional unit. Strains constructed by transformation with plasmid clones that did not contain the *Bgl*II site exhibited sharply reduced competence (Fig. 3), although in some cases the transformability (10^{-3} of the wild-type level) was 10^3-fold higher than that observed with the *comG* mutant strains (15).

In total, these results suggest that the *comG* promoter and regulatory sequences lie to the right of the *Bgl*II site (Fig. 3) and upstream from the *comG12* insertion (Fig. 3). To obtain an estimate of the size of the *comG* mRNA transcript, Northern transfers were performed. Total RNA was prepared from cultures of BD630 and from competence mutants (*comG12*, *comG39*, and *comG210*) as described in the Materials and Methods. RNAs from the mutants and BD630 were electrophoresed on 1% agarose gels containing formamide and were transferred to nitrocellulose filters. The filters were probed with nick-translated plasmids containing promoter-proximal sequences. The results of these Northern transfer studies indicated that the *comG* transcript is about 5 kb in size (data not shown), since BD630 RNA showed hybridization to one species of that size. This transcript was not observed in RNA from the *com* mutants. Instead, truncated hybridizing species were noted.

S1 nuclease mapping. The *comG210* insertion produced a Com$^-$ phenotype, and therefore, sequences downstream from this mutation are required for competence. Based on data from Northern transfers and complementation studies with the intact *comG* clone (see the accompanying paper [1]), the transcription termination site was presumed to be within about 2 kb of and downstream from the *comG210* insertion. This 2 kb of sequence was included in the plasmid which presumably carried the entire *comG* region. A low-resolution 3′ S1 nuclease RNA protection study was performed to determine the approximate location of the 3′ terminus of the *comG* mRNA. A 3-kb *Hin*dIII fragment (Fig. 3) from the right end of the *comG* region was cloned into the polylinker region of pUC18. The resulting plasmid was cut with *Cla*I and 3′-end-labeled with ^{32}P by a fill-in reaction with the Klenow fragment of DNA polymerase I (a *Cla*I site was located just downstream of the *comG210* insertion). This labeled DNA was secondarily cut with *Hin*dIII. The 1.5-kb *Cla*I-labeled probe should protect mRNA that terminates at a point 3′ to the *comG210* insertion. Total RNA from competent BD630 (obtained 1 h after the transition from the exponential to the stationary growth phase) was hybridized to this probe, producing one major protected bank of about 700 base pairs (bp) and one much weaker protected fragment of about 400 bp (Fig. 5). This places the probable major *comG* termination site about 700 bp downstream of the *comG210* insertion. In addition, a strong signal corresponding to a completely protected probe was visible, leaving open the possibility that a more distal termination site is also present.

Epistasis. The products of the sporulation genes *spo0A* and *spo0H*, as well as those of *comA*, *comB*, and *sin*, are required for the expression of several late-expressing *com* transcriptional units, including *comG* (2; Y. Weinrauch, unpublished data). It is possible that a further epistatic hierarchy exists among competence genes, in which some *com* gene products that are expressed at T_0 are required for the expression of other *com* genes. *comG* mutations were therefore assayed for their epistatic effects on the expression of other representative Tn*917lac* competence mutations.

Strains were constructed which contained some of the original *comG* mutations with their *lacZ* moieties deleted. This was accomplished by a recombination replacement scheme similar to the one shown in Fig. 1, except that pTV55Δ2 was used instead of pTV21Δ2. pTV55Δ2 was derived from pTV55 (31) following partial digestion with *Hpa*I and complete digestion with *Sma*I (N. Guillen, unpub-

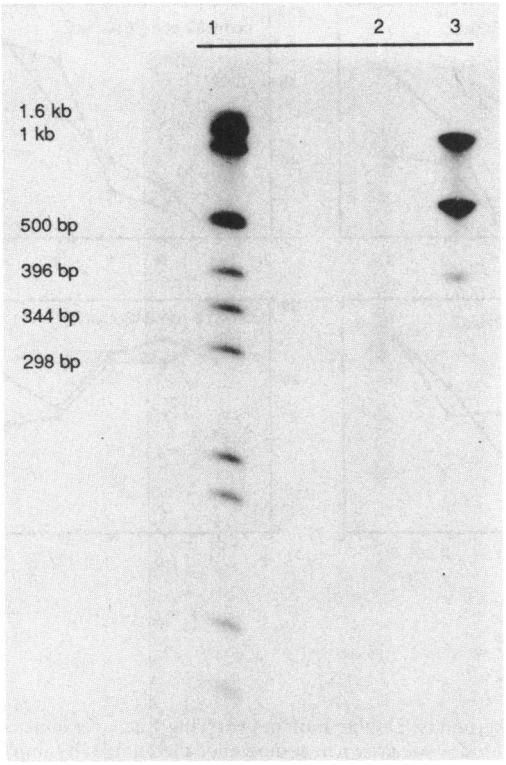

FIG. 5. Low-resolution S1 nuclease mapping of the *comG* transcription terminator. RNA isolated from the Com$^+$ strain BD630, which was grown in competence medium to 1 h after T_0 was hybridized to a 1.5-kb *Cla*I-*Hin*dIII fragment as described in the text. The fragment was labeled with ^{32}P at the 3′ end at the *Cla*I site. The hybridization mixtures were treated with S1 nuclease, denatured, and electrophoresed on an 8 M urea–6% polyacrylamide gel. An autoradiogram of the gel is shown. A 1-kb ladder (Bethesda Research Laboratories) labeled with ^{32}P at the 5′ end was run as a size standard (lane 1). Lane 2, S1 nuclease-treated, denatured probe; lane 3, probe hybridized to BD630 RNA 1 h after T_0 and treated with S1 nuclease; lane 3, 150 μg of RNA (one protected band of about 700 bp and one weak signal of about 400 bp can be seen).

lished data). This eliminated the *lacZ* gene, leaving homology to Tn*917*, and also leaving Ap^r, Cm^r, and both the pBR322- and pE194-specific replication activities. pTV55Δ2 was transduced into each of the *comG* mutant strains by selection for Cm^r at 50°C, to eliminate replication from the temperature-sensitive pE194 origin. Em^s and Lac$^-$ transductants were obtained.

Phage PBS1 lysates were prepared from these strains and were used to transduce the *lacZ* fusion competence mutants carrying *comA124*, *comB138*, *comC530*, *comD413*, *comE510*, and *comE518*. Cm^r transductants were purified and screened to confirm the presence of MLS^r. *comA124* and *comB138* were expressed early and throughout competence development, whereas the remaining fusion strains expressed β-galactosidase beginning at about T_0 in competence medium (2). The double mutant strains, which carried Lac$^-$ versions of *comG39* or *comG12* and Lac$^+$ fusions to other *com* genes, were grown through the one-step competence protocol; and samples were withdrawn for β-galactosidase determinations. The parent Lac$^+$ strains were grown and assayed concurrently as controls. Sample results from these experiments are shown in Fig. 6. The *comG39* mutation was not epistatic on expression of any of the *com* mutations tested. The *comG12* mutation was not epistatic on *comA124*, *comB138*, *comC530*, or *comD413*. However, *comG12* produced a two- to fourfold reduction in expression of the *comE510* and *comE518* fusions. This partial dependency was reproducible.

DISCUSSION

The six competence mutations which define the *comG* region of *B. subtilis* have been previously isolated and partially characterized with respect to genetic linkage and patterns of expression (2, 15). The genes defined by these mutations were expressed in competence medium and were expressed at very low rates in complex medium. Their expression increased sharply at about T_0, and expression was shown to be dependent on the products of the *spo0A* and *spo0H* loci. The powerful genetic tools associated with Tn*917* derivatives (29, 32) allowed the direct cloning of the *comG* region in *E. coli*. Thus, as shown in Fig. 3, insertion-proximal sequences from each of the six *comG* Tn*917lac* mutations were cloned with three different restriction endonucleases. Also, an *E. coli* clone which appeared to contain the intact *comG* region was obtained. The restriction maps showed that the *comG* DNA fragments cloned from each of the mutants are related, since the clones contained overlapping restriction sites. Based on this information, a rough map of the *comG* region could be produced in which the six Tn*917lac* insertions were arranged in a linear order (Fig. 3). This preliminary map was then verified by Southern blotting. The data placed the six Tn*917lac* insertions in the following order; *comG12*, *comG39*, *comG412*, *comG107*, *comG56*, and *comG210*. These insertions spanned a distance of approximately 5 kb. An additional 2 kb of DNA downstream of the *comG210* insertion was cloned in a plasmid that appeared to carry the intact *comG* region.

The promoter driving expression of the *comG* region was mapped by genetic methods. The results of these experiments indicated that the major *comG* promoter is located near the first insertion, *comG12*, and is responsible for expression of all of the downstream *lac* fusions. The location of this promoter has been confirmed by S1 nuclease and primer extension mapping experiments which place the probable *comG* transcription start site about 75 to 100 bp upstream from the *comG12* insertion (1).

There also appeared to be minor *comG* promoter(s). As noted in the Results, strains into which insertion-proximal clones lacking the major *comG* promoter were transformed exhibited very low transformation frequencies. These frequencies were at least 1,000-fold lower than that of the wild-type strain, but also at least 1,000-fold higher than the frequencies obtained with the original *comG* mutants. Since these transformants lacked the *comG* promoter, they should have been as noncompetent as the original *com* mutants. This result was found with all of the insertion mutations that occurred downstream of the *comG12* mutation, suggesting the presence of at least one minor promoter somewhere between the *comG12* and *comG39* insertions. The *comG107*, *comG56*, and *comG210-Hin*dIII strains constructed by Campbell-like recombination also showed transformation frequencies higher than those of the original mutants, suggesting the presence of a second internal minor promoter. In a low-resolution S1 nuclease protection experiment (data not shown), a weak protected signal appeared which corresponded to a possible start site near the insertion point of *comG39*. The latter result suggested that the intermediate level of competence noted in the genetic experiments is not due to the readthrough of *comG* mRNA from a weak promoter in Tn*917lac*.

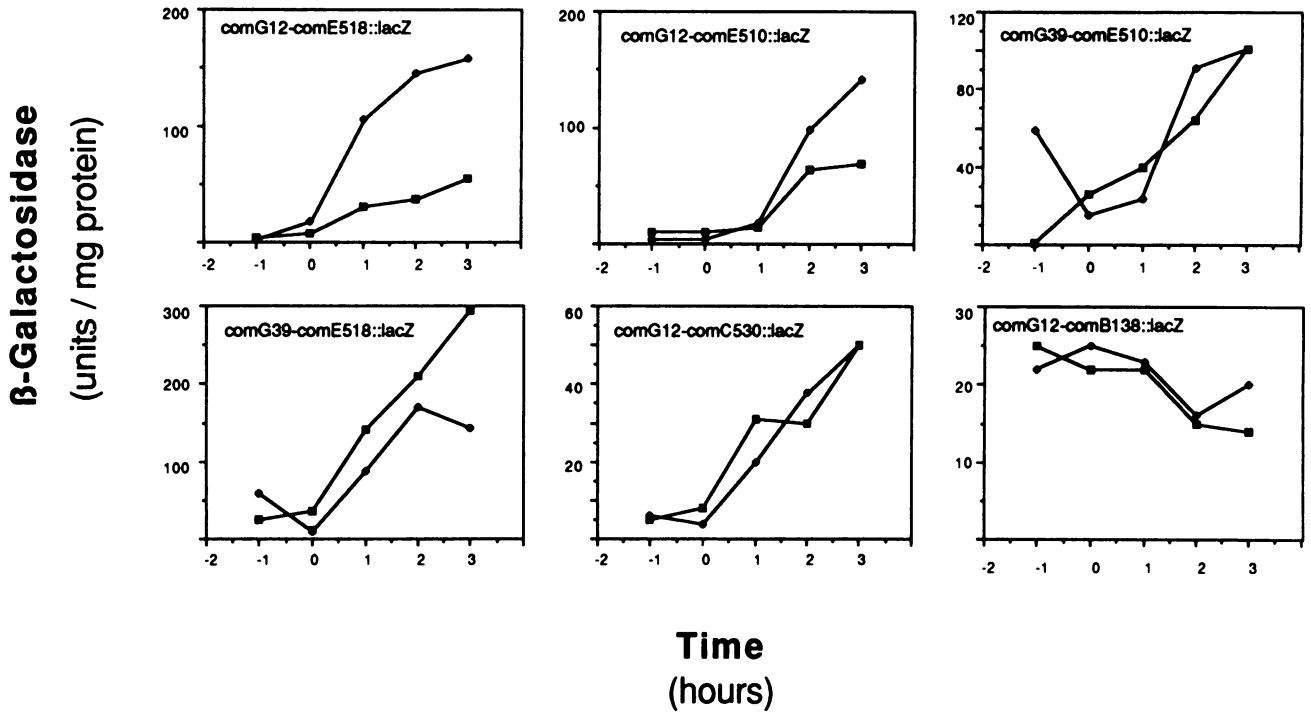

FIG. 6. Expression of *com-lacZ* fusions in *comG12* and *comG39* mutant backgrounds. Double mutants carrying Lac$^-$ versions of either *comG12* or *comG39*, together with transcriptional fusions of several *com* genes to *lacZ*, were grown in competence medium; and samples were withdrawn at various times for the determination of β-galactosidase specific activity (●). Control strains carried the *lacZ* fusions in *comG*$^+$ backgrounds (■). The numbers on the time scale refer to hours before or after T_0.

We also mapped the 3′ terminus of the *comG* transcript using S1 nuclease protection. Northern blotting (data not shown) indicated that the *comG* transcript consists of about 5 kb. Since the distance between the major promoter and the last (*comG210*) insertion was about 4 kb, this placed the termination site about 1 kb downstream from the *comG210* insertion. The low-resolution S1 nuclease protection experiment confirmed this by revealing one major protected fragment corresponding to a termination site about 700 bp beyond the *comG210* insertion. A minor termination site may also exist about 400 bp beyond this insertion. It is possible that these termini are generated by posttranscriptional processing.

The epistasis experiments showed that the *comG12* mutation appears to affect the *comE* locus by reducing its expression by about three- to fourfold. The two Tn*917lac* insertions used to determine this effect (*comE510* and *comE518*) have been shown by Southern blotting and DNA sequencing to be located in the same gene (J. Hahn and Y. Kozlov, unpublished data). This is a relatively small effect, but it was reproducible. The *comE* mutants were interesting in that they exhibited near normal binding of transforming DNA, but they exhibited little uptake. The *comG12* mutation occurred in an open reading frame whose gene product may be a regulatory protein (see the accompanying paper [1]), and its effect on the expression of *comE* may reflect that function.

We cloned a region of the *B. subtilis* chromosomal DNA carrying the *comG* locus and showed that it is required for the development of competence. The size of the *comG* region and the presence of a single major promoter suggest that this is a polycistronic operon. This suggestion has been confirmed (see the accompanying paper [1]). The *comG* promoter appears to be competence specific and controls transcription of the approximately 5-kb *comG* region. The following paper (1) presents the results of DNA sequencing and further characterization of the *comG* region.

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