JOURNAL OF BACTERIOLOGY, Mar. 1986, p. 663–670
0021-9193/86/030663-08$02.00/0

Vol. 165, No. 3

Molecular Cloning of *cis*-Acting Regulatory Alleles of the *Bacillus subtilis amyR* Region by Using Gene Conversion Transformation†

WAYNE L. NICHOLSON[1] AND GLENN H. CHAMBLISS[2]*

Laboratory of Genetics,[1] and Department of Bacteriology,[2] University of Wisconsin, Madison, Wisconsin 53706

Received 5 August 1985/Accepted 4 December 1985

Three *cis*-acting alleles (*gra-10*, *gra-5*, and *amyR2*) of the *Bacillus subtilis amyR* promoter locus each cause catabolite repression-resistance of *amyE*-encoded α-amylase synthesis. The *gra-10*, *gra-5*, and *amyR2* alleles were transferred from the chromosomes of their respective hosts to a plasmid carrying the *amyR1-amyE*$^+$ gene by the process of gene conversion which is carried out during transformation of competent *B. subtilis* by plasmid clones carrying homologous DNA. The cloned *amyR* promoter regions containing the *gra-10* and *gra-5* mutations were shown to confer catabolite repression-resistance in *cis* to the synthesis of chloramphenicol acetyltransferase encoded by the *cat-86* indicator gene when subcloned into the promoter-probe plasmid pPL603B. Implications concerning both the regulation of *amyR* utilization and the process of gene conversion in *B. subtilis* are discussed.

At the end of exponential growth in liquid medium, *Bacillus subtilis* activates a subset of genes, the expression of which normally silent during vegetative growth (for reviews, see references 20, 22, and 23). One of these temporally activated genes, *amyE*, encodes the extracellular enzyme α-amylase (α-1,4 glucan-4-glucanhydrolase, EC 3.2.1.1 [34, 39, 40]). Activation of *amyE* expression is thought to result from carbon source limitation (9) and can be repressed by the addition of readily metabolized carbon sources, particularly glucose, to a culture (9, 26).

In addition to presently unidentified host factors, a tightly linked *cis*-regulatory region designated *amyR* (33, 38), which has been postulated to be the promoter region of *amyE*, controls the modulation of *amyE* expression in *B. subtilis* 168. The *amyR* regulatory locus exists in at least three allelic forms: *amyR1* (from *B. subtilis* 168), *amyR2* (from *B. subtilis* subsp. *natto* [33]), and *amyR3* (from *B. subtilis* subsp. *amylosaccharitícus* [37]). The *amyR2* and *amyR3* loci can be introduced into *B. subtilis* 168-derived strains by DNA-mediated transformation, by which they are reported to confer an α-amylase hyperproducing phenotype on transformants (33, 37). In addition to α-amylase hyperproduction, the *amyR2* locus also confers catabolite repression-resistance to α-amylase synthesis in *B. subtilis* (unpublished data).

The synthesis of α-amylase in *B. subtilis* 168 was originally proposed to be regulated solely by carbon source catabolite repression (23). Temporal activation and glucose-mediated repression of α-amylase synthesis appear to be controlled by at least two distinct regulatory mechanisms, however, as mutants of *B. subtilis* 168 have been isolated which are specifically resistant to glucose-mediated repression of α-amylase synthesis, while postexponential activation of *amyE* remains unaffected (18). Two such mutations, *gra-10* (18) and *gra-5* (unpublished data), map tightly linked to the *amyE* structural gene, exert their catabolite repression-resistance effect in *cis*, and are likely additional alleles of the *amyR* locus.

The *amyE*$^+$ gene from *B. subtilis* 168 has been cloned (36), and the cloned *amyE*$^+$ gene has been shown to also contain a functionally intact *amyR1* *cis*-regulatory region (18). The nucleotide sequences of the *amyR1* (36) and *amyR2* (35) loci have been determined, and comparison of the two sequences reveals both similarities and differences (unpublished data). The specific relationship connecting the observed differences between *amyR1* and *amyR2* at the nucleotide sequence level and the different phenotypes conferred by these two regulatory loci has not yet been elucidated.

As part of an effort to define further the functional domains of the *amyR* region responsible for temporal activation and glucose-mediated repression of *amyE* expression, the *gra-10*, *gra-5*, and *amyR2* regulatory loci were cloned. As an alternative to more classical cloning strategies, we utilized the recently observed phenomenon of gene conversion during plasmid transformation of competent *B. subtilis* cells (7, 13, 29) to transfer rapidly the *gra-10*, *gra-5*, and *amyR2* alleles from the chromosomes of their respective hosts onto a plasmid-borne copy of the *amyR1-amyE*$^+$ gene. In this study we report the successful cloning of *gra-10*, *gra-5*, and *amyR2* using gene conversion transformation. We also offer physical evidence of the authenticity of the cloned *amyR2* allele by examination of a restriction enzyme site polymorphism which can be used to distinguish between *amyR1* and *amyR2*. Finally, we present evidence supporting a model according to which the *gra-10* mutation exerts its catabolite repression-resistance effect in *cis* on the expression of a heterologous plasmid-borne gene. Moreover, we confirm the symmetry of gene conversion, first observed by Chak et al. (7) and Iglesias and Trautner (13), by transferring the *gra-10* mutation from a plasmid back into the *B. subtilis* chromosome.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *B. subtilis* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. The following media were used: nutrient sporulation medium (24); A3 medium (antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.); LB medium (Luria-Bertani medium [16]); PBAB (peptone blood agar base; GIBCO Laboratories, Grand Island, N.Y.), and PBAB to which 1% starch and 2% glucose had been added. Kanamycin sulfate (Km), chloramphenicol (Cm), erythromy-

* Corresponding author.

† Paper no. 2822 from the Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI 53706.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference[a]
B. subtilis		
168	*trpC2*	Laboratory stock
168GR10	*trpC2 gra-10*	NTG mutant of 168 (18)
168GR5	*trpC2 gra-5*	NTG mutant of 168 (18)
1A46	*thr-5 trpC2 recE4*	BGSC
1A289	*amyE aroI906 metB5 sacA321*	BGSC
WLN-2	*metB5 sacA321*	1A46 ⇆ 1A289; Aro$^+$/Amy$^+$
WLN-6	*metB5 recE4 sacA321*	1A46 ⇆ 1A289; Aro$^+$/Amy$^+$ Rec$^-$ by congression
WLN-7	*amyE aroI906 sacA321*	1A46 ⇆ 1A289 (18)
WLN-11	*gra-10 sacA321*	168GR10 ⇆ WLN-7; Aro$^+$/Glu^r Amy$^+$ (18)
WLN-14	*gra-5, sacA321*	168GR5 ⇆ WLN-7; Aro$^+$/Glu^r Amy$^+$
1A412(NA64)	*amyR2 metB5 purB6*	BGSC
WLN-15	*amyR2 sacA321*	1A412 ⇆ WLN-7; Aro$^+$/Glu^r Amy$^+$
coli		
HB101	F$^-$, *hsd-20 hsdR* hsdM *recA13 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$	Laboratory stock (3).
Plasmids		
pAMY10[b]	Cm^r *amyR1-amyE$^+$*	D. Henner (1,36; see Fig. 2)
pE194 *cop-6*[c]	Em^r	B. Weisblum (31)
pAT153[d]	Ap^r Tc^r	F. Johnston (30)
pEAT-A63[b]	Em^r Tc^r (*E. coli*), Em^r (*B. subtilis*)	This study (Fig. 2)
pEATα1[b]	Em^r *amyR1-amyE$^+$*	This study (Fig. 2)
pEATα1W11-1[b]	Em^r *gra-10-amyE$^+$*	This study
pEATα1W11-4[b]	Em^r *amyR1-amyE$^+$*	This study
pEATα1W14-1[b]	Em^r *gra-5-amyE$^+$*	This study
pEATα1W15-1[b]	Em^r *amyR2-amyE$^+$*	This study
pPL603B[c]	Km^r *promoterless cat-86*	D. Rothstein (32)
p5′αB10[c]	Km^r *amyR1-cat-86*	Laboratory stock (18)
p5′αGR10[c]	Km^r *gra-10-cat-86*	This study (Fig. 3)
p5′αGR5[c]	Km^r *gra-5-cat-86*	This study

[a] Abbreviations: NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; BGSC, Bacillus Genetic Stock Center; tf, transformation.
[b] Replicates in either *E. coli* or *B. subtilis*.
[c] Relicates in *B. subtilis*.
[d] Replicates in *E. coli*.

cin (Em), ampicillin (Ap), and tetracycline (Tc) were purchased from Sigma Chemical Co., St. Louis, Mo. Selective antibiotic media contained 5 μg of Km per ml, 10 μg of Cm per ml, or 10 μg of Em per ml for *B. subtilis* and 50 μg of Ap per ml, 50 μg of Tc per ml, 10 μg of Cm per ml, or 300 μg of Em per ml for *E. coli*. Growth in liquid culture was monitored with a Klett-Summerson colorimeter fitted with a no. 66 red filter. Liquid cultures were incubated with vigorous aeration, and all growth, unless otherwise indicated, was at 37°C.

Plasmid transformation. Competent cells of *B. subtilis* were prepared by the method of Boylan et al. (4). Mixtures of purified plasmid DNA (1.0 to 3.0 μg of DNA in 0.1 ml of 10 mM Tris hydrochloride [pH 8.0], 1.0 mM disodium EDTA; TE buffer) and 0.9 ml of competent cells were incubated at 37°C with shaking for 30 min and then were plated directly, selecting for antibiotic-resistant transformants by a drug underlay method (10). Drug-resistant transformants were picked onto selective PBAB media containing 1% starch and 2% glucose and were scored for α-amylase production as described previously (18). Alternatively, transformation mixtures were diluted 10-fold into A3 medium containing a subinhibitory concentration of Em (0.1 μg of Em per ml) and were incubated with shaking for 3 h to overcome phenotypic lag and to preinduce expression of erythromycin resistance (31). These cultures were inoculated into 1 liter of A3 medium containing 10 μg of Em per ml, incubated overnight, and harvested the following day for preparation of plasmid DNA.

Introduction of plasmid DNA into calcium chloride-treated cells of *E. coli* was performed by standard procedures (8, 15).

Plasmid DNA extraction. Purification of plasmid DNA from 1-liter *B. subtilis* or *E. coli* cultures was performed by a cleared lysate procedure (2) as described previously (18). Rapid isolation of plasmid DNA from 5 ml of *E. coli* cultures, grown overnight in LB medium containing the appropriate selective antibiotic, was performed by the rapid boiling method of Holmes and Quigley (11).

Enzymatic assays. Assays of extracellular α-amylase and intracellular chloramphenicol acetyltransferase (CAT) activities were performed as described previously (18, 27). Specific activity of enzymes was defined as units of enzymatic activity per milligram of cellular protein, as determined by the protein assay of Lowry et al. (14) using bovine serum albumin as a standard.

Restriction endonucleases and T4 DNA ligase were purchased from commercial sources and were used in accordance with the recommendations of the manufacturer. Plasmid DNA and restriction endonuclease cleavage products were analyzed after electrophoresis through 1 or 1.5% horizontal agarose gels as described previously (18).

RESULTS

Cloning *gra-10* by gene conversion. To detect gene conversion of *gra-10* from the *B. subtilis* chromosome to a plasmid carrying the wild-type amylase gene, a two-step transformation strategy was used (Fig. 1). In the first step, plasmid pEATα1 (Fig. 2) was transferred into WLN-11, a *B. subtilis* strain harboring the *gra-10* mutation (Fig. 1A). During the course of transformation, some plasmids were expected to acquire the *gra-10* mutation from the host chromosome as a result of gene conversion (7, 13, 29). Because both the plasmid and the host produced amylase, it was necessary to purify plasmid DNA from this primary transformation mixture and to introduce this mixed population of plasmids into an amylase-negative indicator strain, WLN-7, to detect which plasmids had acquired from WLN-11 the glucose-resistant amylase-producing characteristic of the *gra-10* mutation (Fig. 1B).

Plasmid pEATα1 bearing *amyR1-amyE$^+$* (Fig. 2) was introduced into competent cells of *B. subtilis* WLN-11 (*gra-10-amyE$^+$*). As controls, pEATα1 was also introduced into *B. subtilis* WLN-2, carrying the wild-type *amyR1-amyE$^+$* locus, and an isogenic recombination-deficient strain, WLN-6 (*amyR1-amyE$^+$ recE4*). After allowing 3 h for expression of the Em^r trait of pEATα1, each of the three transformation mixtures was used to inoculate 1 liter of A3 medium containing 10 μg of Em per ml. The 1-liter culture of

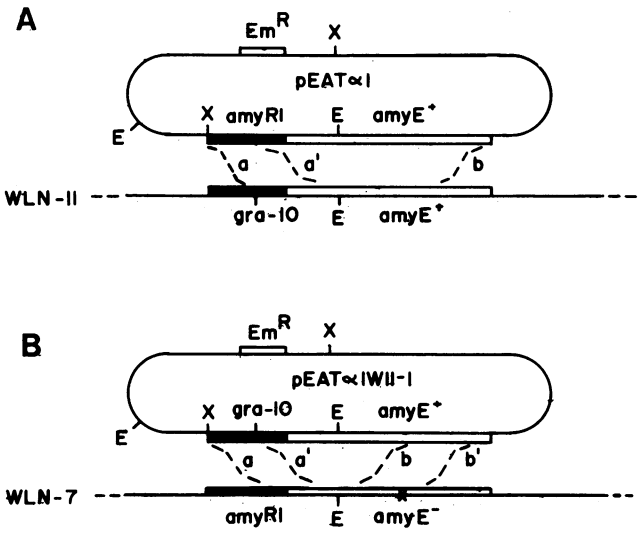

FIG. 1. Schematic diagram depicting hypothetical gene conversion events leading to observed plasmid genotypes. Marker transfer only in the direction of chromosome to plasmid is indicated. Restriction sites are abbreviated as described in the legend to Fig. 2. Thickened bars indicate regions of DNA homology. (A) Transformation of strain WLN-11 (*gra-10-amyE*$^+$) by plasmid pEATα1 (*amyR1-amyE*$^+$). Resulting plasmid genotypes are as follows: event ab, *gra-10-amyE*$^+$ (plasmid pEATα1W11-1, for example); event a'b, *amyR1-amyE*$^+$ (plasmid pEATα1W11-4, for example). (B) Transformation of strain WLN-7 (*amyR1-amyE*$^-$) by plasmid pEATα1W11-1 (*gra-10-amyE*$^+$). Resulting plasmid genotypes: event ab, *amyR1-amyE*$^+$; event a'b, *gra-10-amyE*$^+$; event ab', *amyR1-amyE*$^-$; event a'b', *gra-10-amyE*$^-$.

WLN-6 treated with pEATα1 failed to grow after overnight incubation under selective conditions, probably because of the dependence of plasmid transformation on recombination-proficiency in the recipient strain when a competence-inducing regime is used (6). The other two cultures grew well, and plasmid DNA was purified from each (WLN-2 and WLN-11 containing pEATα1). The purified plasmid DNA from each was used as donor DNA to transform competent cells of the indicator strain WLN-7 (*amyR1-amyE*) to Em^r. Transformant colonies were picked onto PBAB containing 10 μg of Em per ml, 1% soluble starch, and 2% glucose to test whether passage of pEATα1 through strain WLN-11 had resulted in transfer of the *gra-10* mutation from the chromosome of WLN-11 to the plasmid.

When pEATα1 DNA prepared from WLN-11 was used as donor DNA, three distinct amylase phenotypes were observed among the WLN-7 transformants (Table 2). The majority of Em^r transformants were also Amy$^+$. A small proportion (1.3%) of the Em^r Amy$^+$ transformants were also resistant to glucose-mediated repression of α-amylase synthesis (Glu^r; Table 2), which is consistent with gene conversion of *gra-10* from the chromosome of WLN-11 to pEATα1 during the initial transformational passage. In addition, a significant proportion of Em^r transformants of WLN-7 were Amy$^-$ (Table 2). This class of transformants may have arisen by gene conversion of the *amyE* structural gene mutation from the chromosome of WLN-7 to pEATα1 during the introduction of pEATα1 into WLN-7. When a similar experiment was performed, using as donor DNA pEATα1 passed through strain WLN-2 (*amyR1-amyE*$^+$), only two classes of Em^r transformants of WLN-7 were observed: Amy$^-$ and Amy$^+$ Glu^s (Table 2).

The proportion of Em^r Amy$^-$ transformants obtained when pEATα1, passaged through WLN-11, was introduced into competent WLN-7 was greater than the proportion of Glu^r Amy$^+$ transformants that were obtained (Table 2). This result may be due to the fact that the *B. subtilis* insert DNA in pEATα1 that is allelic to *gra-10* is situated closer to the vector-insert junction than is the insert DNA that is allelic to the *amyE* structural gene mutation, resulting in less efficient gene conversion of *gra-10* because of the more truncated region of homology (Fig. 1B).

Plasmid DNA was purified from two representative transformants of WLN-7 obtained in the experiment described above (Table 2) and were reintroduced into competent cells of WLN-7, selecting for Em^r (Table 2). When plasmid pEATα1W11-4, isolated from an Em^r Glu^s Amy$^+$ transformant, was used as donor DNA, all Em^r Amy$^+$ transformants obtained were Glu^s (Table 2), as was expected, because both the WLN-7 chromosome and plasmid pEATα1W11-4 contained the wild-type *amyR1* allele. When plasmid pEATα1W11-1, isolated from an Em^r Glu^r Amy$^+$ transformant of WLN-7, was reintroduced into competent WLN-7, greater than 90% of the resultant Em^r transformants of WLN-7 demonstrated a Glu^r Amy$^+$ phenotype (Table 2). In addition, 2.2% of the Em^r Amy$^+$ transformants of WLN-7 that had received pEATα1W11-1 DNA demonstrated a Glu^s Amy$^+$ phenotype, indicating that the wild-type (*amyR1*) *cis*-regulatory allele had been transferred from the WLN-7 chromosome onto pEATα1W11-1. Thus, it appears that either *gra-10* or *amyR1* can be transferred with roughly equivalent efficiency from the host chromosome to an incoming plasmid. Using an identical strategy, the *gra-5* and *amyR2* *cis*-regulatory alleles were cloned onto plasmid pEATα1 by gene conversion transformation from the chromosomes of strains WLN-14 and WLN-15, respectively,

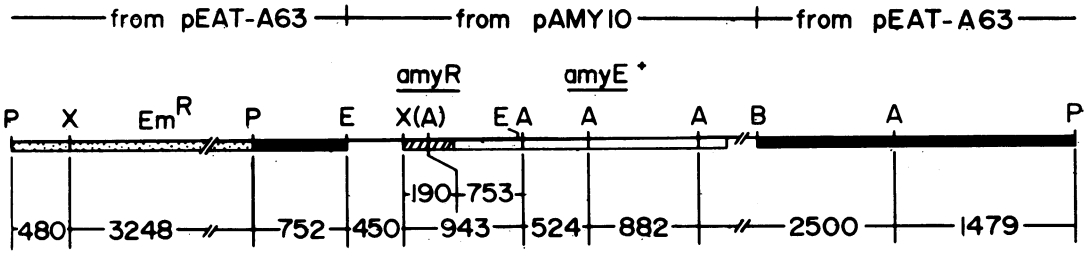

FIG. 2. Restriction map of plasmid pEATα1 linearized with *Pst*I. Positions of restriction sites (A, *Ava*I; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; X, *Xba*I) and sizes (in base pairs) are estimated from the restriction maps of pE194*cop6* (stippled bar [12]), pAT153 (solid bar [28, 30]), *amyR1* (hatched bar [36]), *amyE*$^+$ (open bar [36]). The thin line represents vector DNA from pAMY10. The *Ava*I site surrounded by parentheses indicates its predicted location in *amyR2*, inferred from the *amyR2* nucleotide sequence (35).

TABLE 2. Cloning *gra-10* by gene conversion transformation[a]

Plasmid (source)	No. of Em^r transformants per 0.1 μg of DNA	No. of Amy$^+$/total (%)	No. of Glu^r/total (%)	No. of Glu^s/total (%)	No. of Amy$^-$/total (%)
A. pEATα1 (WLN-11)	4.42×10^3	406/456 (89.0)	6/456 (1.3)	400/456 (87.7)	50/456 (11.0)
pEATα1 (WLN-2)	3.55×10^3	377/456 (82.7)	0/456 (<0.2)	377/456 (82.7)	79/456 (17.3)
B. pEATα1W11-1 (WLN-7)[b]	1.8×10^4	424/456 (93.0)	414/456 (90.8)	10/456 (2.2)	32/456 (7.0)
pEATαW11-4 (WLN-7)[c]	1.43×10^4	433/456 (95.0)	0/456 (<0.2)	433/456 (95.0)	23/456 (5.0)

[a] Plasmid DNA prepared from transformation of pEATα1 into competent cells of WLN-11 (*gra-10*) or WLN-2 (*amyR1*) was introduced into competent WLN-7 (*amyR1-amyE*$^-$). Em^r transformants were scored for Glu^r Amy$^+$ or Glu^s Amy$^+$ on PBAB plates containing 10 μg of Em per ml, 1% starch, and 2% glucose (part A). An Em^r Glu^r Amy$^+$ transformant, strain WLN-7 (pEATα1W11-1), and an Em^r Glu^s Amy$^+$ transformant, strain WLN-7 (pEATα1W11-4), were chosen. Plasmid DNA was prepared from the two transformants and was again introduced into competent WLN-7 (part B).

[b] Em^r Glu^r Amy$^+$ transformant from part A.

[c] Em^r Glu^s Amy$^+$ transformant from part A.

creating plasmids pEATα1W14-1 and pEATα1W15-1, respectively (Table 1; data not shown).

The Glu^r Amy$^+$ phenotype of pEATα1W11-1 is plasmid borne. To test whether the observed Glu^r Amy$^+$ phenotype of strain WLN-7(pEATα1W11-1) was due to cloning of *gra-10* by gene conversion, a plasmid curing experiment was undertaken. It was predicted that the loss of plasmid DNA from WLN-7(pEATα1W11-1), as judged by the loss of the Em^r phenotype, would result in the simultaneous loss of the Glu^r Amy$^+$ phenotype, if *gra-10-amyE*$^+$ were indeed plasmid borne.

Plasmid pEATα1 is a derivative of plasmid pEAT-A63, a *B. subtilis-E. coli* shuttle vector constructed by fusion of the *Staphylococcus aureus* plasmid pE194*cop-6* (31) with the *E. coli* plasmid pAT153 (30) at their unique *Pst*I sites (Fig. 2). Replication of pEAT-A63 derivatives in *B. subtilis* results from utilization of the replication origin derived from the pE194*cop-6* moiety of the vector. This replication origin is temperature sensitive (25). High-efficiency plasmid curing can therefore be accomplished when *B. subtilis* strains carrying pEAT-A63 or its derivatives are cultured at a temperature restrictive for plasmid replication in the absence of selective antibiotic.

Curing of plasmids pEATα1W11-1 (*gra-10-amyE*$^+$) or pEATαW11-4 (*amyR1-amyE*$^+$) from strain WLN-7 in nearly all cases resulted in the simultaneous loss of α-amylase production, indicating that the Glu^r Amy$^+$ and Glu^s Amy$^+$ phenotypes of these two transformants were indeed plasmid borne (Table 3). At a low frequency, colonies arose from this treatment which retained an Em^r Amy$^-$, an Em^s Amy$^+$, or an Em^r Amy$^+$ phenotype. These classes of colonies may have arisen by recombination between the chromosomal *amyE*$^-$ allele of WLN-7 and the plasmid-borne *amyE*$^+$ allele during the course of the curing experiment, insertion of pEATα1W11-1 or pEATα1W11-4 into the WLN-7 chromosome by a Campbell-type recombination event (5), or failure to completely cure all cells with plasmid.

Construction of p5′ αGR10. In *B. subtilis* 168, synthesis of *amyE*$^+$-encoded α-amylase is modulated by a tightly linked regulatory locus designated *amyR1* (for a review, see reference 22). In a previous report (18) it was demonstrated that the *amyR1 cis*-regulatory locus was contained on the 5′ end of the *amyE*$^+$ gene cloned from *B. subtilis* 168 by Yang et al. (36). This was accomplished in part by subcloning a 1.35-kilobase-pair (kbp) *Eco*RI fragment from plasmid pAMY10 (Fig. 2), containing *amyE*$^+$ DNA coding for the NH_2-terminus of α-amylase plus approximately 400 base pairs of upstream *B. subtilis* DNA, into the *Eco*RI site preceding the promoterless *cat-86* structural gene in the promoter-probe plasmid pPL603B (18, 32). The resulting plasmid construct, designated p5′ αB10, encoded *amyR1*-directed CAT synthesis which was expressed in a manner that mimicked the regulation of α-amylase expression, both in terms of temporal activation and glucose-mediated repression (18).

In a manner directly analogous to that described above, the 1.35-kbp *Eco*RI fragment containing the 5′ end of *amyE*$^+$ was excised from plasmid pEATα1W11-1 (*gra-10-amyE*$^+$) and was inserted by ligation into the unique *Eco*RI site of the promoter-probe plasmid pPL603B (Fig. 3). The ligation mixture was introduced into competent cells of strain 168. Restriction analysis of plasmid DNA prepared from a typical Km^r Cm^r transformant confirmed that the 1.35-kbp *Eco*RI fragment from pEATα1W11-1 had been inserted in the proper orientation into the *Eco*RI site of pPL603B (data not shown). This plasmid, designated p5′ αGR10, was reintroduced into competent cells of strain 168, in which it was observed to confer glucose repression-resistance to the synthesis of CAT (Fig. 4B). Hence, the *gra-10* mutation was localized to the 5′ end of the *amyE*$^+$ structural gene.

Gene conversion of *gra-10* from plasmid to chromosome. Chak et al. (7) and Iglesias and Trautner (13) reported that allele conversion during plasmid transformation can operate symmetrically, i.e., in the direction from plasmid to chromosome as well as from chromosome to plasmid. We observed that both the *gra-10* and *amyR1* loci were transferred with roughly equal efficiencies from the *B. subtilis* chromosome onto an incoming plasmid containing DNA homologous to the *amyR1-amyE*$^+$ region (Table 2). Construction of plasmids p5′ αB10 (18) and p5′ αGR10 (Fig. 3) resulted in placement of *cat-86*, a selectable marker, under control of the *amyRl* and *gra-10 cis*-regulatory regions, respectively. These constructions allowed a rapid, sensitive means of testing for gene conversion of *amyR1* or *gra-10* from plasmid to chromosomal DNA because of the linkage of these *cis*-regulatory DNAs to two distinct enzymatic activities, plasmid-encoded CAT and chromosomally encoded α-amylase.

Competent cells of strains 168 (*amyR1-amyE*$^+$) or 168GR10 (*gra-10-amyE*$^+$) were treated with either p5′ αB10 (*amyR1-cat-86*) or p5′ αGR10 (*gra-10-cat-86*) DNAs and plated onto PBAB containing 5 μg of Km per ml and 10 μg of Cm per ml (final concentration) using a drug underlay method (10). Km^r Cm^r transformants were picked onto selective media containing 1% starch and 2% glucose to score their α-amylase regulatory phenotypes. Both the *gra-10* and *amyR1* alleles can be transferred from the donor plasmid to the recipient chromosome, providing evidence that supports the observation that gene conversion can

TABLE 3. Plasmid curing experiment[a]

Strain	No. of Em^r Amy$^+$/total (%)	No. of Em^r Amy$^-$/total (%)	No. of Em^s Amy$^+$/total (%)	No. of Em^s Amy$^-$/total (%)
WLN-7(pEATα1W11-1)	1/152 (0.7)	0/152 (<0.7)	1/152 (0.7)	150/152 (98.6)
WLN-7(pEATα1W11-4)	1/76 (1.3)	1/76 (1.3)	2/76 (2.6)	72/76 (94.7)

[a] Indicated strains were incubated in A3 medium containing 10 μg of Em per ml at 37°C. At late exponential phase, cultures were diluted 100-fold into A3 medium without Em and incubated at 48°C overnight. Appropriate dilutions of the cultures were spread onto PBAB without Em. Single colonies were picked onto PBAB containing either 10 μg of Em per ml or 1% starch.

operate symmetrically (7, 13) (Table 4). Note also that when p5′ αGR10 (*gra-10-cat-86*) was introduced into strain 168GR10 (*gra-10-amyE*$^+$) no Glu^s Amy$^+$ transformants were observed, indicating that the *gra-10* allele cloned from WLN-11 and fused to *cat-86* in plasmid p5′ αGR10 is either an identical copy of the *gra-10* mutation in the chromosome of 168GR10 or is too close to the chromosomal *gra-10* mutation in strain 168GR10 to be separated by recombination in the number of colonies tested.

The *gra-10* mutation exerts its glucose resistance effect in *cis*. Previous data suggested that the *gra-10* mutation, in chromosomal linkage to *amyE*$^+$, exerted its effect in *cis* only on glucose-mediated repression of α-amylase synthesis (18). To further test the validity of this interpretation, it was desirable to perform the *cis-trans* experiment in reverse, i.e., to monitor the effect of a plasmid-borne copy of *gra-10*, fused to *cat-86* in plasmid p5′ αGR10, on the simultaneous regulation of plasmid-encoded CAT and chromosomally encoded α-amylase synthesis. Plasmid p5′ αGR10 was introduced into competent individuals of strains 168 and 168GR10. Strain 168 harboring p5′ αGR10 expressed chromosomally encoded α-amylase activity in a temporally activated and glucose-repressible manner (Fig. 4A). Simultaneous expression of CAT activity encoded by p5′ αGR10 was resistant to glucose-mediated repression, but activation of CAT expression at the end of exponential growth appeared unaffected (Fig. 4B). Thus, when the *gra-10* mutation was plasmid borne, it was unable to confer glucose repression-resistance in *trans* to chromosomally encoded α-amylase synthesis, although it could confer glucose repression-resistance in *cis* to plasmid-encoded CAT synthesis.

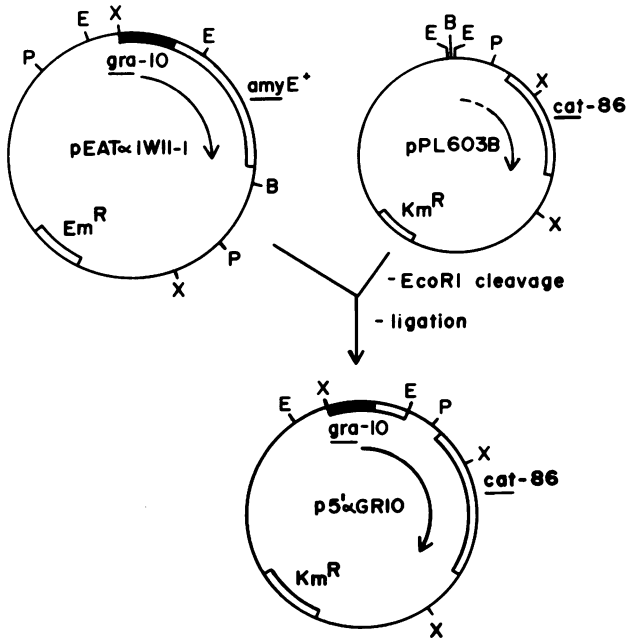

FIG. 3. Construction of plasmid p5′αGR10. Restriction sites are abbreviated as described in the legend to Fig. 2. See text for details.

Only when the *gra-10* mutation was situated in the *cis* configuration with respect to both the chromosomal *amyE*$^+$ and the plasmid-borne *cat-86* genes, as in strain 168GR10 (p5′αGR10), were the syntheses of both α-amylase (Fig. 5A) and CAT (Fig. 5B) resistant to glucose-mediated repression. The observation that neither CAT nor α-amylase synthesis were activated until the end of exponential growth in 168GR10(p5′αGR10) also indicated that the *gra-10* mutation did not affect the temporal activation inherent to the *amyR* locus. Results of experiments similar to those described above indicated that the *gra-5* mutation also exerted its catabolite repression-resistance effect only in *cis* (data not shown).

Physical evidence for cloning *amyR2*. Examination of the published nucleotide sequences of *amyR1*, cloned from *B. subtilis* 168 (36), and *amyR2*, cloned from *B. subtilis* NA64 (35), revealed an *Ava*I restriction enzyme site polymorphism that was present in the *amyR2* sequence and absent in the wild-type *amyR1* sequence. This *Ava*I site in *amyR2* is due to a T→C transition at nucleotide 291 of the published *amyR1* nucleotide sequence, as defined by Yang et al. (36). From this observation it was predicted that the *amyR2* locus cloned by gene coversion transformation onto plasmid pEATα1W15-1 should also contain an *Ava*I restriction site in the DNA corresponding to the *amyR2* locus (Fig. 2). To test this prediction, plasmid pEATα1 (*amyR1-amyE*$^+$) and plasmid pEATα1W15-1 (*amyR2-amyE*$^+$) were digested with the restriction enzymes *Xba*I and *Ava*I, and their restriction patterns were analyzed after electrophoresis through a 1.5% agarose gel. Plasmid pEATα1W15-1 contains an extra *Ava*I site at the location predicted from the *amyR2* nucleotide sequence, providing physical evidence that gene conversion transformation resulted in the cloning of DNA from strain WLN-15 which encodes a functional *amyR2* allele and which also contains a predicted structural feature that distinguishes it from *amyR1* (Fig. 6).

DISCUSSION

This report describes the use of gene conversion transformation (7, 13, 29) as a rapid method for cloning mutations either in the *amyE* structural gene or in the tightly linked *cis*-regulatory *amyR* DNA sequences controlling the expression of *amyE*. In particular, in this study we describe the cloning and further characterization of *gra-10*, *gra-5*, and *amyR2*, three *cis*-acting mutations that specifically confer catabolite repression-resistance to α-amylase synthesis in *B. subtilis* 168 (18; unpublished data). In addition, we offer physical evidence of the fidelity of gene conversion by demonstrating that a predicted *Ava*I restriction site polymorphism present in *amyR2* (35) was cloned by gene conversion

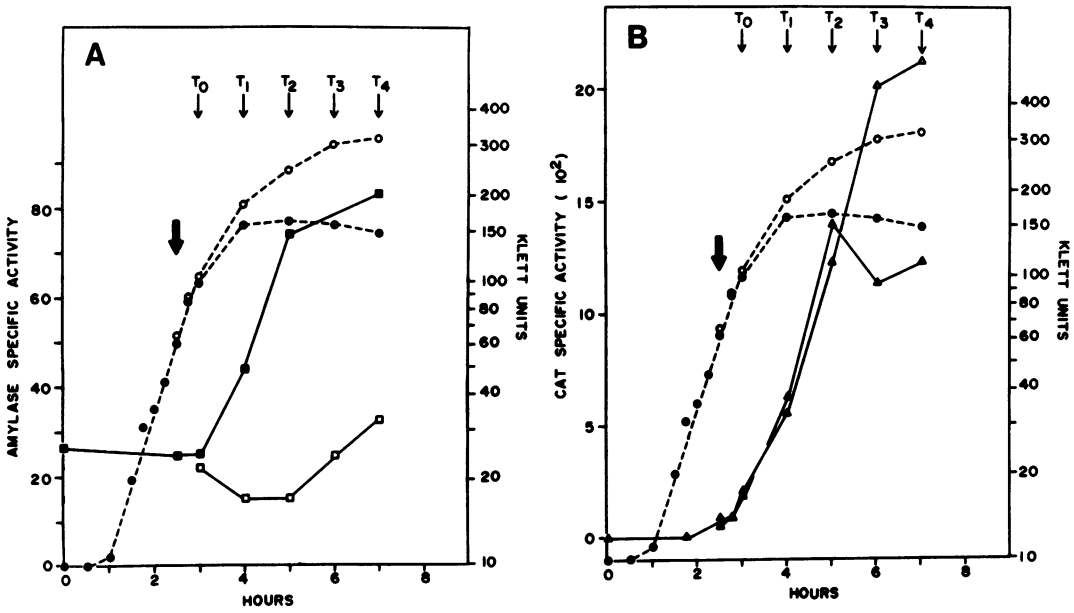

FIG. 4. Simultaneous regulation of α-amylase (A; squares) and CAT (B; triangles) synthesis during growth (circles) of strain 168(p5′αGR10). Cultures were grown in nutrient sporulation medium containing 5 μg of Km per ml with (open symbols) or without (closed symbols) added glucose. At midexponential phase (heavy arrow) the culture was split, and one-half received glucose to a 1% final concentration. At the indicated times, samples were removed from cultures and assayed for extracellular α-amylase activity and intracellular CAT activity as described in the text. T_0, through T_4 denote the time (in hours) following the end of exponential growth.

onto plasmid pEATα1W15-1 concomitant with cloning of the DNA responsible for the *amyR2* phenotype (Fig. 6).

It was originally observed that the *gra-10* mutation allowed normal temporal activation of *amyE* expression even in the presence of an otherwise inhibitory concentration of glucose (18). This observation was interpreted as an indication that two distinct mechanisms, temporal activation and glucose-mediated repression, governed the regulation of α-amylase synthesis in *B. subtilis* 168. Furthermore, *gra-10* was unable to confer catabolite repression-resistance in *trans* to *amyR1*-driven *cat-86* expression encoded by plasmid p5′αB10, indicating that *gra-10* is a *cis*-acting regulatory mutation (18).

The glucose repression-resistant character of CAT synthesis encoded by plasmid p5′αGR10 (Fig. 4B and 5B) derives from the regulatory DNA 5′ to the *amyE* gene from plasmid pEATα1W11-1 (*gra-10-amyE*$^+$) which is linked in *cis* to the *cat-86* indicator gene of pPL603B. The resultant phenotype of plasmid p5′αGR10 supports earlier genetic mapping experiments which indicated that the *gra-10* mutation is situated at the 5′ end of the *amyE* structural gene (18). Linkage of *gra-10* in *cis* to *cat-86* in plasmid p5′αGR10 also resulted in normal temporal activation of plasmid-encoded CAT synthesis in strain 168(p5′αGR10), even in the presence of 1% glucose (Fig. 4B), while chromosomal *amyR1*-directed *amyE* expression remained glucose repressible (Fig. 4A). Based on these results, it appears that *gra-10* exerts its glucose resistance effect only on the expression of genes to which it is associated in the *cis* configuration, thus providing further evidence that *gra-10* is a *cis*-regulatory mutation.

These results are consistent with the hypothesis that temporal activation and catabolite repression of α-amylase synthesis in *B. subtilis* are distinct regulatory phenomena which can be separated by mutation (18). The observed pattern of *gra-10*-driven *cat-86* expression in strain 168(p5′αGR10) suggests that the *gra-10* mutation defines a target site presumably upstream from *amyE* which is no longer recognized by a *trans*-acting regulatory factor responsible for the repression of *amyR1* utilization in the presence of more readily metabolized carbon sources. Because strain 168 possesses the necessary *trans*-acting factors for effecting repression of *amyR1*-directed *amyE* expression (Fig. 4A), or *amyR1*-directed CAT synthesis encoded by plasmid p5′αB10 (18), but is unable to repress *gra-10*-directed CAT synthesis

TABLE 4. Transferring *amyR1* and *gra10* from plasmid to chromosomal DNA[a]

Donor DNA (source)	Recipient	No. Km^r Cm^r transformants per μg of DNA	No. of Glu^r Amy$^+$/total (%)	No. of Glu^s Amy$^+$/total (%)	No. of Amy$^-$/total (%)
p5′ αGR10 (168)	168	4.0×10^2	22/400 (5.5)	378/400 (94.5)	0/400 (<0.25)
p5′ αB10 (168)	168	1.42×10^2	0/142 (<0.7)	142/142 (100.0)	0/142 (<0.7)
p5′ αGR10 (168)	168GR10	5.2×10^2	524/524 (100.0)	0/524 (<0.2)	0/524 (<0.2)
p5′ αB10 (168)	168GR10	1.7×10^3	224/228 (98.2)	4/228 (1.8)	0/228 (<0.4)

[a] Transformants were selected on PBAB containing 5 μg of Km per ml and 10 μg of Cm per ml. The transformants were screened on PBAB containing 1% starch and 2% glucose for glucose-resistant or glucose-sensitive α-amylase synthesis.

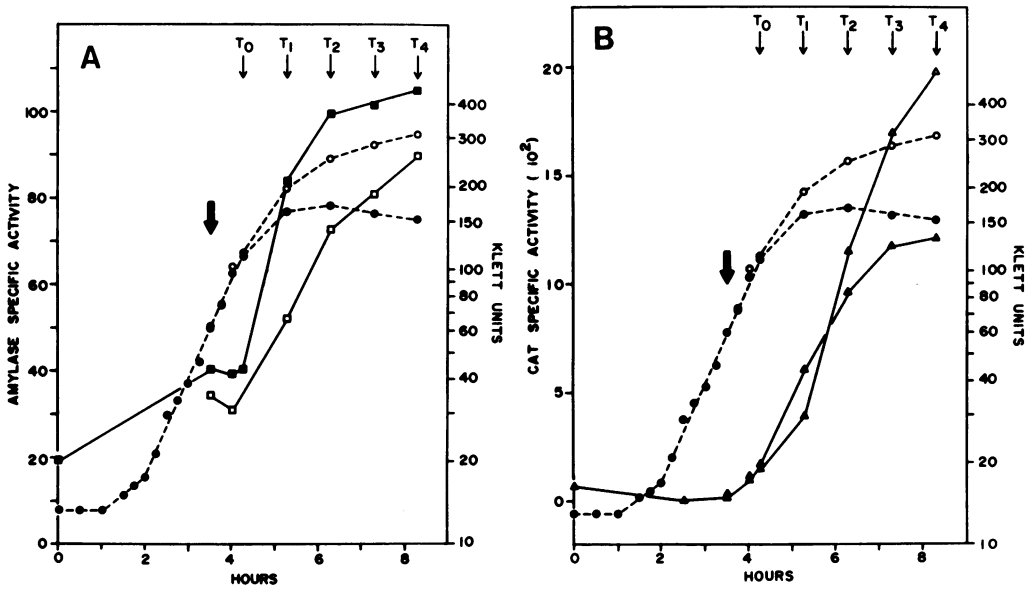

FIG. 5. Simultaneous regulation of α-amylase (A; squares) and CAT (B; triangles) synthesis during growth (circles) of strain 168GR10(p5′αGR10). Cultures were grown in nutrient sporulation medium containing 5 μg of Km per ml and were treated as described in the legend to Fig. 4.

in the presence of glucose (Fig. 4B), we tentatively conclude that carbon source catabolite repression of α-amylase synthesis in *B. subtilis* 168 is negatively controlled.

In addition, the data suggest that the mechanism responsible for the activation of *amyE* expression at the end of vegetative growth is naturally insensitive to carbon source catabolite repression. In the wild-type case, temporal activation of *amyR1-amyE* expression in the presence of 1% glucose is prevented by the glucose repression mechanism. The *gra-10* mutation circumvents this repression event, unmasking the fact that the *trans*-acting factors necessary for temporal activation of *amyE* expression are present and functional at the end of exponential growth, regardless of the presence or absence of glucose.

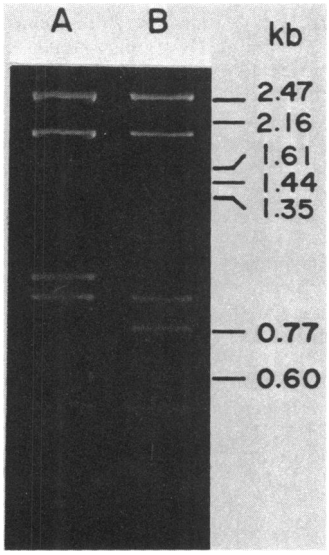

FIG. 6. Agarose-gel electrophoretic analysis of pEATα1 (lane A) and pEATα1W15-1 (lane B) DNAs digested with *Xba*I and *Ava*I. Size standards consisted of a mixture of lambda DNA digested with *Hin*dIII and pEAT-A63 DNA digested with *Taq*I. kb, Kilobases.

The similar phenotypes conferred on cells harboring the *gra-10* and *gra-5* mutations, their map locations, and their ability to be cloned by gene conversion onto a plasmid-borne *amyR1-amyE*$^+$ region suggest that these regulatory mutations are alleles of that portion of *amyR1* responsible in *cis* for catabolite repression of *amyE* expression. The *gra-10* mutation is contained on the 1.35-kbp *Eco*RI fragment of pEATα1W11-1 which consists of the 5′ end of the *amyE* coding sequence and spans the *amyR* region. In a previous study (19) it was demonstrated that a spontaneously arising deletion mutant of plasmid p5′αB10 (*amyR1-cat-86*), designated p5′αB10Δ1, lacking all of the 5′ end of the *amyE* coding sequence plus 114 base pairs of DNA preceding *amyE* (including all of the putative *amyE* ribosome binding sites), still retained temporally activated and glucose-repressible expression of the *cat-86* indicator gene. From these results it was concluded that the DNA sequences responsible for *amyR1* function are located 5′ to the leftward deletion endpoint in p5′αB10Δ1 and that both temporal activation and glucose-mediated repression of *amyR1* utilization operate at the transcriptional level, in agreement with earlier conclusions of Priest (21) and Nagata et al. (17). It is expected, therefore, that the *gra-10* and *gra-5* mutations also occur in the region of *amyR* 5′ to the DNA corresponding to the leftward deletion endpoint present in p5′αB10Δ1. The rapid cloning of these *cis*-regulatory mutations by the use of gene conversion transformation will greatly facilitate their precise localization by nucleotide sequencing, thus further defining at a high level of resolution the region of *amyR* which is involved in glucose-mediated repression of α-amylase synthesis.

ACKNOWLEDGMENTS

We thank the Bacillus Genetic Stock Center, Dennis Henner, Frank Johnston, David Rothstein, and Bernard Weisblum for providing strains and plasmids and Paula Bogdanow for technical assistance.

This research was supported by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison and by Public Health Service grants GM 32199 and GM 34324 from the National Institutes of Health. W.L.N. was a National Institutes of Health predoctoral trainee (Public Health Service training grant GM 07133).

LITERATURE CITED

1. **Band, L., and D. J. Henner.** 1984. *Bacillus subtilis* requires a "stringent" Shine-Delgarno region for gene expression. DNA **3**:17–22.
2. **Bonamy, C., and J. Szulmajster.** 1982. Cloning and expression of *Bacillus subtilis* spore genes. Mol. Gen. Genet. **188**:202–210.
3. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
4. **Boylan, R. J., N. H. Mendelson, D. Brooks, and F. E. Young.** 1972. Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid. J. Bacteriol. **110**:281–290.
5. **Campbell, A.** 1962. Episomes. Adv. Genet. **11**:101–146.
6. **Canosi, U., A. Iglesias, and T. A. Trautner.** 1981. Plasmid transformation in *Bacillus subtilis*: effects of insertion of *Bacillus subtilis* DNA into plasmid pC194. Mol. Gen. Genet. **181**:434–440.
7. **Chak, K., H. deLencastre, H.-M. Liu, and P. Piggot.** 1982. Facile *in vivo* transfer of mutations between the *Bacillus subtilis* chromosome and a plasmid harbouring homologous DNA. J. Gen. Microbiol. **128**:2813–2816.
8. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *E. coli* cells. Gene **6**:23–28.
9. **Heineken, F., and R. O'Conner.** 1972. Continuous culture studies on the biosynthesis of alkaline protease, neutral protease, and α-amylase by *Bacillus subtilis* NRRL-B3411. J. Gen. Microbiol. **73**:35–44.
10. **Henkin, T. M., and G. H. Chambliss.** 1984. Genetic mapping of a mutation causing an alteration in *Bacillus subtilis* ribosomal protein S4. Mol. Gen. Genet. **193**:364–369.
11. **Holmes, D., and M. Quigley.** 1981. A rapid method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
12. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150**:804–814.
13. **Iglesias, A., and T. A. Trautner.** 1983. Plasmid transformation in *Bacillus subtilis*: symmetry of gene conversion in transformation with a hybrid plasmid containing chromosomal DNA. Mol. Gen. Genet. **189**:73–76.
14. **Lowry, O., N. Rosebrough, A. Farr, and R. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
16. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Nagata, Y., H. Yamauchi, and B. Maruo.** 1980. Assay of messenger activity of RNA from *Bacillus subtilis* in heterologous cell-free system and its relation to α-amylase productivity. Agric. Biol. Chem. **44**:1283–1289.
18. **Nicholson, W. L., and G. H. Chambliss.** 1985. Isolation and characterization of a *cis*-acting mutation conferring catabolite repression resistance to α-amylase synthesis in *Bacillus subtilis*. J. Bacteriol. **161**:875–881.
19. **Nicholson, W. L., G. H. Chambliss, L. Buckbinder, N. P. Ambulos, Jr., and P. S. Lovett.** 1985. Isolation and expression of a constitutive variant of the chloramphenicol-inducible plasmid gene *cat-86* under control of the *Bacillus subtilis* amylase promoter. Gene **35**:113–120.
20. **Piggot, P., and J. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40**:908–962.
21. **Priest, F.** 1975. Effect of glucose and cyclic nucleotides on the transcription of α-amylase mRNA in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **63**:606–610.
22. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41**:711–753.
23. **Schaeffer, P.** 1969. Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriol. Rev. **33**:48–71.
24. **Schaeffer, P., J. Millet, and J.-P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54**:704–711.
25. **Scheer-Abramowitz, J., T. Gryczan, and D. Dubnau.** 1981. Origin and mode of replication of plasmids pE194 and pUB110. Plasmid **6**:67–77.
26. **Sekiguchi, J., and H. Okada.** 1972. Regulation of α-amylase production in a *Bacillus subtilis* Marburg strain. I. Isolation of mutants which produce high levels of α-amylase and analysis of their enzymes. J. Ferment. Technol. **50**:801–809.
27. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43**: 737–755.
28. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
29. **Tanaka, T.** 1982. Transfer of a *Bacillus subtilis* chromosomal mutation to a homologous DNA region on a plasmid. Agric. Biol. Chem. **46**:1101–1102.
30. **Twigg, A., and D. Sherratt.** 1980. Trans-complementable copy-number mutants of plasmid ColE1. Nature (London) **283**: 216–218.
31. **Weisblum, B., M. Y. Graham, T. Gryczan, and D. Dubnau.** 1979. Plasmid copy number control: isolation and characterization of high-copy-number mutants of plasmid pE194. J. Bacteriol. **137**:635–643.
32. **Williams, D., E. Duvall, and P. Lovett.** 1981. Cloning restriction fragments that promote expression of a gene in *Bacillus subtilis*. J. Bacteriol. **146**:1162–1165.
33. **Yamaguchi, K., Y. Nagata, and B. Maruo.** 1974. Genetic control of α-amylase synthesis in *Bacillus subtilis*. J. Bacteriol. **119**:410–415.
34. **Yamaguchi, K., Y. Nagata, and B. Maruo.** 1974. Isolation of mutants defective in α-amylase from *Bacillus subtilis*: genetic analysis. J. Bacteriol. **119**:416–424.
35. **Yamazaki, H., K. Ohmura, A. Nakayama, Y. Takeichi, K. Otozai, M. Yamasaki, G. Tamura, and K. Yamane.** 1983. α-Amylase genes (*amyR2* and *amyE*$^+$) from an α-amylase hyperproducing *Bacillus subtilis* strain: molecular cloning and nucleotide sequence. J. Bacteriol. **156**:327–337.
36. **Yang, M., A. Galizzi, and D. Henner.** 1983. Nucleotide sequence of the amylase gene from *Bacillus subtilis*. Nucleic Acids Res. **11**:237–249.
37. **Yoneda, Y., K. Yamane, K. Yamaguchi, Y. Nagata, and B. Maruo.** 1974. Transformation of *Bacillus subtilis* in α-amylase productivity by deoxyribonucleic acid from *B. subtilis* var. *amylosacchariticus*. J. Bacteriol. **120**:1144–1150.
38. **Yuki, S.** 1968. On the gene controlling the rate of amylase production in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **31**:182–187.
39. **Yuki, S.** 1975. The chromosomal location of the structural gene for amylase in *Bacillus subtilis*. Jpn. J. Genet. **50**:155–157.
40. **Yuki, S., and Y. Ueda.** 1968. Fine mapping analysis of the amylase gene in *Bacillus subtilis* by transformation. Jpn. J. Genet. **43**:121–128.