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Identification of Conserved Genetic Functions in *Bacillus* by Use of Temperature-sensitive Mutants

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INTRODUCTION

Related organisms contain regions of homologous base sequences in their deoxyribonucleic acid (DNA) molecules. In microorganisms, a measurement of the extent of these common base sequences serves to determine the degree of relatedness (*for review, see* 24). In this context, genetic conservation refers to those base sequences that are evolutionarily conserved so that measurable genetic exchange can occur between two related but different organisms. The genus *Bacillus* provides a unique group of organisms in which to study genetic relatedness. Within this group, there is a wide divergence in the base composition of genetic material, ranging from 33 to 35% guanine plus cytosine (GC) for *B. cereus* (2) to 52 to 53% GC for *B. macerans* (23, 32). In addition, the transformability and transducibility of some species, for example, *B. subtilis* (35, 37) and *B. licheniformis* (20, 43), allow for a measure of the functional exchange of genetic information within the genus. Currently, a detailed genetic map for *B. subtilis* is emerging (10, 30, 46). This permits consideration of the effects of gene conservation on the organizational features of the genetic material.

Measurement of conserved nucleotide sequence homology, by hybridization of informational macromolecules in *Bacillus*, has shown that 16*S* and 23*S* ribosomal ribonucleic acid (RNA) and 4*S* transfer RNA are conserved (9, 12). This conservation is considerable even when the DNA base composition differs by as much as 10% GC. At this level of difference, few cistrons would be expected to be homologous in their nucleotide sequence (37). This expectation is reflected in the results with messenger RNA (12). Conservation is then appreciably reduced when the DNA base compositions of *Bacillus* strains differ by as little as 2% GC. Thus, the nucleotide sequence of those cistrons involving at least part of the translational processes of the cell have been conserved to a greater extent than other cistrons. With a difference in DNA base composition of 2% GC from the reference organism *B. subtilis*, approximately 20% of all cistrons retain significant nucleotide sequence homology as measured by hybridization of messenger RNA with DNA (12). Only a small part of this sizeable number of conserved cistrons can be accounted for by those involved in the translational process (11, 27, 28). To illustrate this point, simple numerology may be useful if one realizes that many assumptions underlie its determination. For example, the molecular weight for the *B. subtilis* genome has

been determined to be 3.9×10^9 daltons (13). The size of an average cistron, expressed in terms of its molecular weight, is 7.7×10^5 daltons (21). Thus, the genome of *B. subtilis* could contain 5,000 cistrons, 1,000 of which would be conserved at this level of GC difference. About 100 of these conserved cistrons would be accounted for by those involved in the translational process. That leaves 900 other conserved cistrons unidentified.

Interspecific transformation reveals that the nucleotide sequences determining nutritional characteristics are not strongly conserved (12, 16, 18, 25, 36). In fact, recombinant formation drops below the level of detectability with slight differences in GC base composition of the exchanged DNA molecules. However, drug-resistance characteristics exhibit measurable recombinant formation at this level (12, 16). Mode of action studies on the drugs involved indicate that they affect ribosomes (4, 6, 7, 44). It remains to be determined what other kinds of functions are genetically conserved.

Mapping of the ribosomal RNA and transfer RNA places the majority of these cistrons in a small region of the *B. subtilis* genetic map, closely associated with the drug-resistance markers (11, 27, 28). This position lies near the origin of replication. Recently, more refined techniques have indicated that some of the cistrons are located distally on the map (33). It is of interest to know whether other regions are genetically conserved.

To provide additional information as to the identity of genetically conserved functions and their location on the map of *B. subtilis*, a novel class of mutants has been employed. They are temperature-sensitive mutants (15, 17, 19). Their mutant phenotype is expressed at an elevated temperature, and little or no expression of the mutant phenotype occurs at lower temperatures. The use of temperature-sensitive mutants permits isolation of strains having altered functions not observable by classical methods because the alteration would be lethal. When isolated to exclude nutritional types, they provide access to genetic functions vital to the cell. It is the purpose of this report to demonstrate the utility of temperature-sensitive mutants in *B. subtilis* for determining the kinds of genetic functions that remain conserved and their location on the genetic map.

MATERIALS AND METHODS

Strain

Table 1 lists all strains used in this study with their genotype and source.

Media and Growth Conditions

Minimal medium consisted of mineral salts [0.6% (w/v) KH_2PO_4, 1.4% K_2HPO_4, 0.1% sodium citrate $\cdot 2H_2O$, 0.2% $(NH_4)_2SO_4$, and 0.01% $MgSO_4$ added aseptically) and 0.5% glucose supplemented with 50 μg of L-glutamic acid per ml and 50 μg of L-asparagine per ml. Other amino acids and adenine were added at a final

TABLE 1. *List of strains*

Strain	Other designation[a]	Genotype[b]	Source
B. subtilis 168			
BC 26		*phe-12 argA3 ery-1*	D. Dubnau
BC 27	SB202 (25)	*aro-2 trp-2 hisB2 tyr-1*	E. Nester
BC 28	C14	*cysA14*	I. Takahashi
BC 29	168-2 (12)	*trp-2 leu-2*	J. Pene
BC 30		*trp-2 ery-1*	
BC 31	SB19SEM (12)	*str-1 ery-1 mic-1*	
BC 50		*purA16 leu-8 metB5 nia-38*	
BC 51		*purA15 leu-8 metB5 lys-21*	
BC 100	Mu5u8u16 (21)	*purA16 leu-8 metB5*	N. Sueoka
BC 101		*purA16 leu-8 metB5 tms-12*	
BC 102		*purA16 leu-8 metB5 tms-26*	
B. niger	ATCC 6554 (12)	*ery-3 mic-3 str-3*	
B. licheniformis	ATCC 9945	*mic-4 str-4*	C. Thorne
B. licheniformis	ATCC 8480	Indicator strain for PBS1	B. Reilly

[a] Some of these strains have been designated differently (10). Where possible, these names and references are indicated.

[b] Gene symbols indicate mutations in cistrons leading to requirements for, sensitivity or resistance to the following: *arg* (arginine), *aro* (aromatic amino acids), *cys* (cysteine), *ery* (erythromycin), *his* (histidine), *leu* (leucine) *lys* (lysine), *met* (methionine), *mic* (micrococcin), *nia* (niacin), *phe* (phenylalanine), *pur* (adenine or guanine), *str* (streptomycin), *tms* (temperature-sensitive), *trp* (tryptophan), and *try* (tyrosine).

concentration of 50 μg/ml when required. The liquid medium was enriched by the addition of 0.1% yeast extract (Difco) and 0.02% Casamino Acids (Difco). The complete medium used was Brain Heart Infusion (BHI; Difco). Solid media were made by the addition of 2% agar (Difco). Streptomycin (Nutritional Biochemicals Corp. Cleveland, Ohio) was added to the complete medium at a final concentration of 1 mg/ml and erythromycin (Abbott Laboratories, Franklin Park, Ill.) was added at 1 μg/ml.

The temperature-sensitive mutants were stored as spores. The spores were spread onto BHI agar to make stock plates. From these plates, single colonies were picked to inoculate liquid cultures. These stock plates were renewed weekly to avoid growing cultures with high revertant frequencies, which apparently are caused by the loss in viability of the temperature-sensitive mutants during storage.

Overnight (ON) cultures consisted of 16- to 18-hr-old liquid cultures incubated at 34 C. Log-phase cultures were made by diluting an ON culture 50- to 100-fold into fresh medium and by following growth with a Klett colorimeter until logarithmic increase in Klett units was obtained.

Mutagenesis

Temperature-sensitive mutants were selected in the following way. An ON broth culture was diluted 1:100 into fresh complete medium and incubated for 3 hr at 34 C. A sample was centrifuged and resuspended in an equal volume of mineral salts solution without glucose. To this was added 0.2 ml of ethylmethane sulfonate (EMS; Eastman, Rochester, N.Y.) or 100 μg of freshly made *N* - methyl - *N'* - nitro - *N* - nitrosoguanidine (NTG; Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml. After incubation for 30 min at 34 C, the cells were diluted and spread onto BHI plates, which were incubated at 34 C overnight. Survivors of the mutagenesis treatment were picked onto BHI master plates. These were subsequently replicated onto selective minimal agar plates and BHI plates incubated at 34 and 48 C. Provisional temperature-sensitive mutants with the proper growth requirements were streaked out for single-colony isolates, and these were tested again before inclusion in the mutant collection. Mutation *tms-12* was selected after EMS treatment and *tms-26* after NTG treatment.

Isotope Incorporation

To individual samples of log-phase cells, grown in enriched salts medium, were added the ^{3}H-labeled nucleic acid or protein precursors (Nuclear-Chicago Corp., Des Plaines, Ill.). The final concentrations of each were as follows: thymidine, 5 μg/ml (4 μc/μg); uridine, 125 μg/ml (0.02 μc/μg); and phenylalanine, 12 μg/ml (0.4 μc/μg). To facilitate thymidine incorporation in thymine prototrophic strains, 250 μg of deoxyadenosine (P-L Biochemicals Inc., Milwaukee, Wis.) per ml was added to those samples (3). The cells were prelabeled for 30 min at 34 C before the experiment was started. A portion was then transferred to 48 C, and 0.1- or 0.2-ml samples were taken consecutively at 10-min intervals for a total of 60 min. The samples were added to 5.0 ml of iced 5% trichloroacetic acid. The trichloroacetic acid-precipitable counts were then collected on nitrocellulose filters (Matheson-Higgens Co., Cambridge, Mass.). The filters were dried, added to a toluene scintillator [0.1 g of bis 2-(5-phenyloxazolyl)-benzene and 4 g of 2,5-diphenyloxazole per liter of toluene], and counted in a liquid scintillation counter.

Transformation and DNA Isolation

Transforming DNA was isolated according to the method of Marmur (22). Molecular weight was determined by sedimentation analysis in a Spinco ultracentrifuge, model E (14). The molecular weights were as follows: *B. subtilis* DNA, 2.2×10^7 daltons, *B. niger* DNA, 1.1×10^7 daltons, and *B. licheniformis* DNA, 1.2×10^7 daltons. The DNA base compositions were calculated from the buoyant densities in CsCl with *B. subtilis* phage 2C DNA (density, 1.742 g/ml) used as the reference standard (32). All densities have been related to that of *Escherichia coli* DNA taken to be 1.710 g/ml. Competent cultures were made by essentially the method of Anagnostopoulos and Spizizen (1). Competent cells were quick-frozen and stored at −90 C until used (10). Transformations were carried out with a saturating concentration of DNA (2 μg/ml). Cells were incubated with the DNA at 34 C for 30 min, chilled, and then diluted and plated on appropriate media.

Transductions

General transducing phage PBS1 (39) was used for all transductions. The procedure given below was adapted from the methology used for P1kc phage on *E. coli* (Curtiss, *personal communication*).

To prepare phage lysates, log-phase cultures of motile strains of *B. subtilis* were grown in BHI broth at 34 C to a cell density measured as 150 Klett units (blue filter no. 42). They were diluted 1:10 into BHI broth to which 2.5×10^{-3} M $CaCl_2$ had been freshly added and were incubated for an additional 90 min. At this time, viable-cell titers

ranged between 10^7 and 4×10^7 cells/ml. To a sample of cells, phage were added at a multiplicity of infection (MOI) of about 0.5. This adsorption mixture was incubated standing at 34 C for 20 min. An 0.2-ml sample of this mixture was added to 3.0 ml of BHI soft agar (0.5%) containing 2.5×10^{-3} M $CaCl_2$ and was maintained at 45 C. The soft agar was overlaid onto regular BHI agar plates which were incubated overnight at 34 C. To each plate was added 4.0 ml of sterile BHI broth, and the plates were incubated at 4 C for 1 hr. The soft agar layer was broken up with a glass spreader, and this slurry was centrifuged to remove agar, cells, and debris. The supernatant fluid was carefully drawn off into a sterile tube, and pancreatic deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.) was added at a concentration of 10 μg/ml with $MgCl_2$ at 10^{-2} M. After incubation for 60 min at 34 C, a few drops of $CHCl_3$ was added to maintain a cell-free lysate. Titers of 10^{10} to 4×10^{10} plaque-forming phages/ml are readily obtained with this method. Usually, the phage have multiplied about 10^3 or more. Transducing phage are present at a frequency of about 10^{-6} per viable phage. Transducing lysates were passed serially three times on the same donor strain.

PBS1 was titered on the indicator strain *B. licheniformis* ATCC 8480 by this same procedure. All phage dilutions were made in BHI broth with 2.5×10^{-3} M $CaCl_2$ added.

Transductions were performed in a similar way. Phage were added at an MOI of about 5, based on viable-phage and viable-cell numbers. After the 20-min preadsorption incubation, the cells were centrifuged and washed once with an equal volume of mineral salts solutions and finally were concentrated 10 times in it before they were plated on appropriate selective agar. Control cells were treated in the same manner. All transductants were purified by single-colony isolation on the same medium from which they were originally selected before characterization of their genotype by replica plating.

Density Transfer Experiment

Spores of BC 30 were prepared according to Schaeffer et al. (31). Clean spores, free from cells and debris, were made by repeated washings in distilled water. The final spore suspension was heated at 75 C for 30 min before being stored at 4 C. These light spores were allowed to germinate in D_2O medium essentially as described by J. Mangan (*manuscript in preparation*). First, the spores were washed twice in an equal volume of sterile D_2O (>99% D_2O) and then suspended in D_2O. Spores were allowed to germinate at 34 C in mineral salts medium made up in D_2O. This medium contained the necessary requirements and 500 μg of L-alanine per ml, and was supplemented with deuterated algal extract (5), kindly supplied by H. Crespi. At appropriate intervals, samples were removed and iced with 10 mM sodium azide. Cells were collected by centrifugation at 0 C and washed with 10 ml of 0.05 M tris(hydroxymethyl)-aminomethane(Tris, *p*H 8.2)-SSC (0.15) M NaCl plus 0.015 M trisodium citrate). They were brought to a final volume of 2.0 ml in Tris-SSC containing 10 mM sodium azide and 500 μg of lysozyme per ml. Cells were lysed by incubation at 34 C for 60 min. To facilitate breakage of spore coats, the samples were frozen and thawed three times. Sarkosyl NL 30 (Geigy, Ardsley, N.Y.) was added at a final concentration of 1%, and the samples were incubated at 60 C for 10 min. They were then centrifuged to remove ungerminated spores and spore coats. The samples were added to CsCl, brought to a volume of 3.5 ml, and adjusted to a density of 1.723 g/ml. The samples were put into nitrocellulose tubes and centrifuged in an SW 39 rotor at 33,000 rev/min for about 60 hr at 25 C in a Spinco model L2-65B preparative ultracentrifuge. Centrifugation was stopped without braking, and fractions were collected dropwise from the bottom of the tubes. The fractions were diluted with 1.0 ml of SSC, and samples were used in an assay for transforming activity (30, 45, 47).

Microscopy and Staining

Morphology of cells grown at 34 and 48 C was observed with a Zeiss phase-contrast microscope. Nuclear-body staining was done by using a modification of the DeLamater method (8).

RESULTS

Growth Characteristics

Stationary-phase cells of wild-type (*wt*) BC 100, from which mutants were isolated, formed colonies at 48 C on complete BHI medium with about the same efficiency as at 34 C. The temperature-sensitive mutants were isolated by their inability to form colonies at the high temperature. Growth of two such mutants and the *wt* strain in enriched mineral salts medium is shown in Fig. 1. The *wt* cells grew at about the same rate and to the same cell mass at 48 C as at 34 C. The mutant *tms-12* grew equally well at both temperatures. The *tms-26* mutant grew less well at 48 C than at 34 C. This mutation resulted in a slower growth rate and an earlier stationary phase at a lower cell mass.

Table 2 gives the viable titer of these cultures and the ratio of cell viability at 48 C to that at 34 C after 4 hr of incubation. The mass-doubling

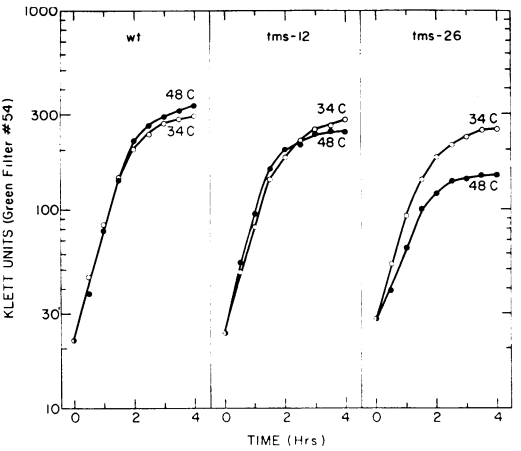

FIG. 1. *Growth curves of BC 100 (wt), BC 101 (tms-12), and BC 102 (tms-26) at 34 and 48 C. Cells were grown to log phase at 34 C in enriched salts medium before start of experiment.*

TABLE 2. *Growth characteristics of temperature-sensitive mutants*[a]

Strain	Time	Temp	Cells/ml	Ratio, 4 hr (48 C/34 C)	Mass-doubling time[b]
	hr	*C*			*min*
BC 100 (*wt*)	0	34	3.6×10^7	0.64	
	4	34	1.5×10^9		35
	4	48	9.6×10^8		35
BC 101 (*tms-12*)	0	34	3.0×10^7	5×10^{-4}	
	4	34	6.0×10^8		35
	4	48	3.7×10^5		35
BC 102 (*tms-26*)	0	34	2.5×10^7	0.44	
	4	34	8.2×10^8		35
	4	48	3.6×10^8		40

[a] Cells grown in enriched salts medium. Determinations made on culture used for growth studies in Fig. 1.

[b] Mass-doubling time estimated from the straight-line portion of the growth curves in Fig 1.

times are approximations taken from the growth curves. Generation times determined from viable-cell doubling are unreliable with these strains because of chain formation and loss of viability at 48 C. The *wt* cells had about 65% as many viable cells at 48 C as at 34 C. The mass-doubling times at both temperatures were the same. Mutant *tms-12* showed a considerable loss in viability at 48 C, even though its rate of growth and the final level of cell mass attained were unaffected. Mutant *tms-26* showed a different effect. The number of viable cells after 4 hr of incubation at 48 C was reduced to about 40% of that at 34 C. The mass-doubling time at 48 C was significantly longer than that at 34 C.

Macromolecular Synthesis

Synthesis of DNA was measured by the incorporation of ^{3}H-thymidine at the two temperatures. The results (Fig. 2) show that DNA synthesis was stimulated at the elevated temperature in all three strains. Thus, DNA synthesis was not inhibited in these mutants during the time measured.

The incorporation of ^{3}H-uridine was used to measure RNA synthesis (Fig. 3). An unexplained delay in the incorporation or uptake of ^{3}H-uridine was evident soon after shifting the cells to 48 C. Total RNA, measured chemically in *wt*, showed that RNA was synthesized at 48 C at the same rate as it was synthesized at 34 C, without any delay. It is possible that the sudden shift from 34 to 48 C produced some thermal injury to the ribosomes, as has been noted in *Staphylococcus* (34). The breakdown of some ribosomes could add to the RNA precursor pool, delaying the uptake and incorporation of ^{3}H-uridine without significantly affecting total RNA synthesis. The tem-

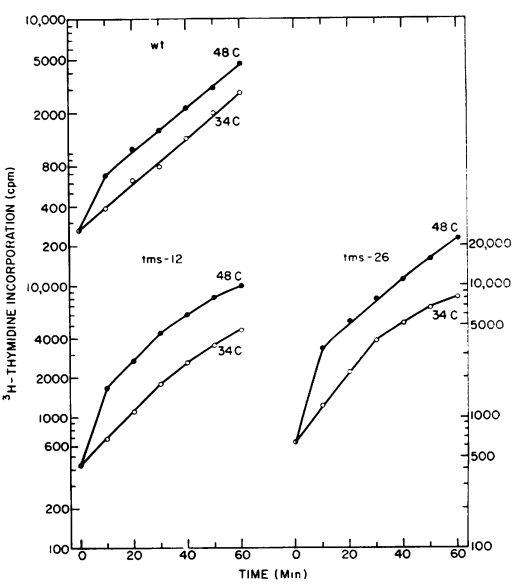

FIG. 2. *DNA synthesis in BC 100 (wt), BC 101 (tms-12), and BC 102 (tms-26) at 34 and 48 C. Cells grown to log phase at 34 C in enriched salts medium and prelabeled for 30 min before start of experiment.*

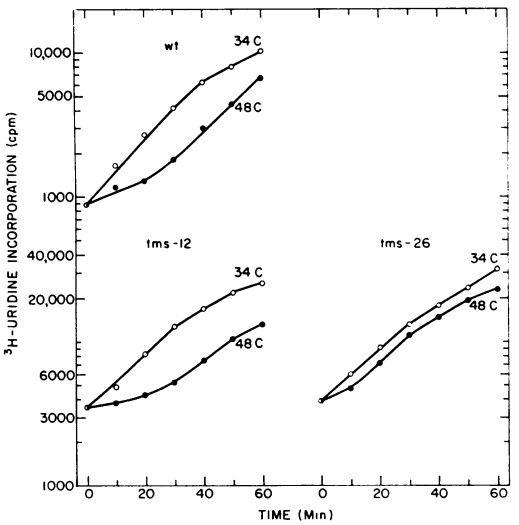

FIG. 3. *RNA synthesis in BC 100 (wt), BC 101 (tms-12), and BC 102 (tms-26) at 34 and 48 C. Cells grown to log-phase at 34 C in enriched salts medium and prelabeled for 30 min before start of experiment.*

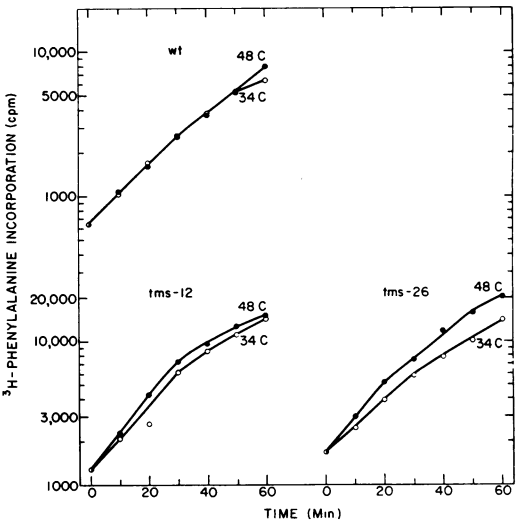

FIG. 4. *Protein synthesis in BC 100 (wt), BC 101 (tms-12), and BC 102 (tms-26) at 34 and 48 C. Cells grown to log phase at 34 C in enriched salts medium and prelabeled for 30 min before start of experiment.*

perature shift affected RNA synthesis in neither of the mutants any more than it did in *wt*. In fact, mutant *tms-26* appeared less affected in its RNA synthesis by the shift to 48 C than did *wt*.

Protein synthesis was measured by ^{3}H-phenylalanine incorporation (Fig. 4). Kinetics of protein synthesis were about the same at both temperatures for all three strains.

Morphology

Changes in morphology at the elevated temperature provide the pertinent observation as to the kind of functions affected in these mutants. All strains showed essentially the same morphology when grown at 34 C. However, after a 2-hr incubation at 48 C, *tms-12* and *tms-26* exhibited morphological differences, as shown by phase-contrast microscopy (Fig. 5), whereas the morphology of *wt* was unaltered. Mutant *tms-12* formed long cells without septa. The *tms-26* mutant cells showed distinct swellings characteristically at the terminal ends. After the 2-hr incubation, 60 to 80% of the cell population showed definite morphological alterations. Continued incubation at 48 C enhanced these morphological changes, as shown for *tms-26* in Fig. 5e.

Table 3 gives the ratio of incorporation of the three macromolecular precursors at 48 C compared to 34 C after 2 hr of incubation. The largest difference existed for DNA synthesis in the mutant *tms-12*. However, the magnitude of this difference, about a 25% lowering in isotope incorporated compared to *wt*, was considerably smaller than the extent of morphological change that had occurred in the cell population at this time. Furthermore, the filaments formed by this mutant after 4 hr of incubation at 48 C contained discrete nuclear bodies regularly distributed throughout the filament (Fig. 6). This indicates that DNA synthesis continued in *tms-12* during the time the filament was being formed.

Mapping

Replicative mapping shows the order of these *tms* mutant loci relative to several other known loci on the genetic map of *B. subtilis* (Table 4). This was carried out by the density transfer method (30, 45, 47). Results show that the locus for *tms-12* lies between *leu-8* and *metB5*. The locus for *tms-26* would appear to lie before *purA16* in replication order, but the measure of transforming activity in the hybrid fraction is inflated by the background of temperature-resistant revertants in this strain.

Linkage analysis by transduction (Table 4) reveals that the *tms-12* locus is not linked to any of the markers in the known linkage groups between *leu-8* and *metB5*. It has been shown that *leu-8* and *nia-38* are linked by transduction with PBS1 (10). The locus *argA* is before *leu-8*, and *phe-12* lies between *leu-8* and *nia-38*. Also, *lys-21* is linked to *metB5* by transduction, and the locus for *tyr-1* resides between them. The *tms-12* locus was not found to be linked to any of these loci. Within this region of the genetic map, there exists

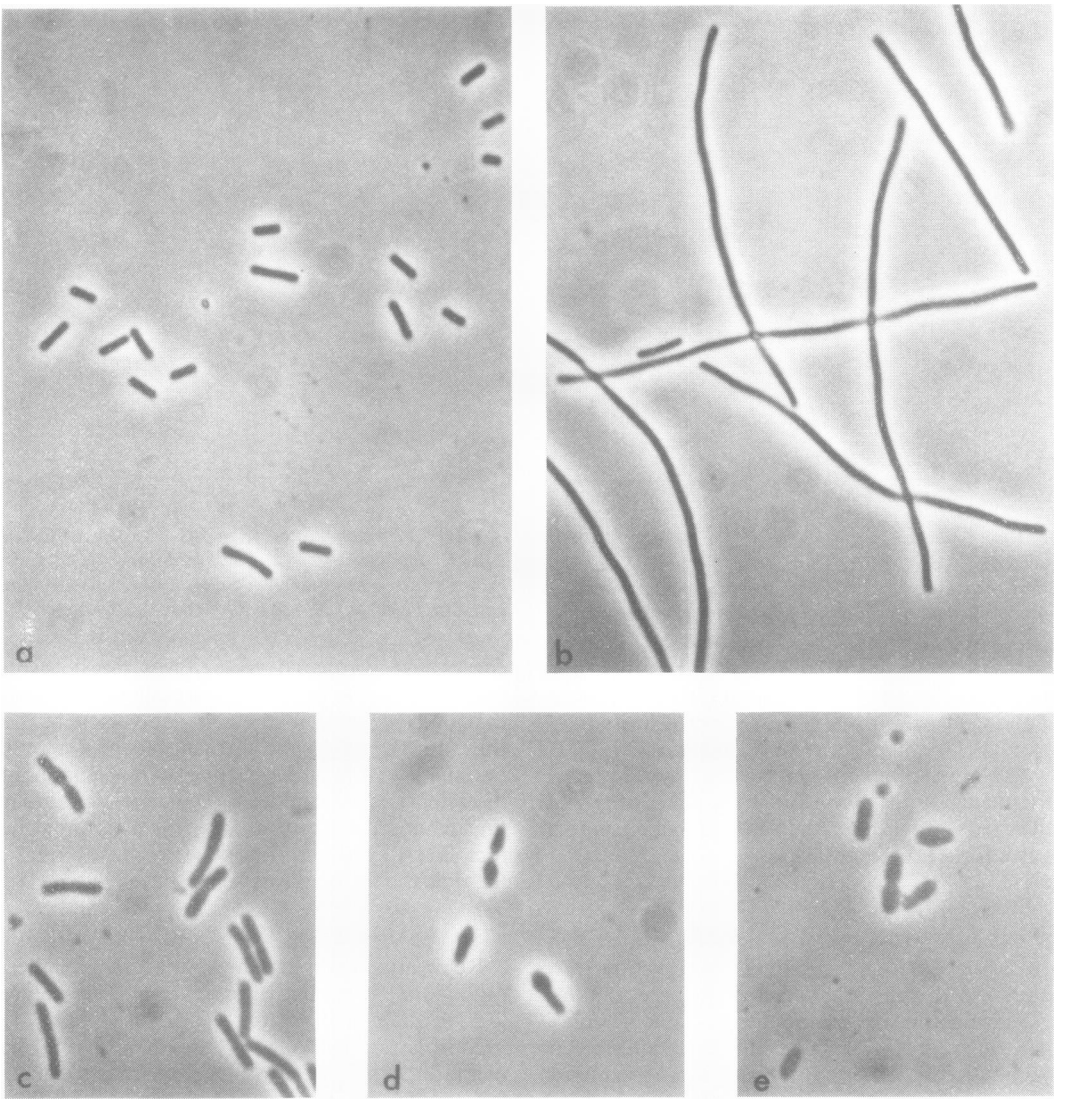

FIG. 5. *Morphology of (a) BC 100 (wt), (b) BC 101 (tms-12), (c and d) BC 102 (tms-26) after 2 hr, and (e) BC 102 after 4 hr of incubation at 48 C. Samples taken from cultures used for growth studies in Fig. 1.*

a gap between *nia-38* and *lys-21* in which linkage is not continuous. It is possible that the *tms-12* locus lies within this region on the map. If it does and if this region is structurally continuous, then the size of this linkage gap must be equivalent to at least twice the size of the largest piece of host DNA that can be carried by the PBS1 phage, which would be about 4×10^8 daltons (10, 42).

The *tms-26* locus is situated between *purA16* and *ery-1* (Table 4). Linkage analysis places it near *cysA14*. The probable order is *purA16*, *tms-26*, and *cysA14*.

Figure 7 is a representation of the genetic map of *B. subtilis*, which shows the positions of *tms-26*, near the origin, and *tms-12*, toward the terminal region of the map.

Genetic Homology

Competent cells of *wt*, *tms-12*, and *tms-26* were examined for interspecific transformation frequencies by use of heterologous *B. niger* DNA

TABLE 3. *Comparison of macromolecular synthesis at 34 and 48 C in BC 100, BC 101, and BC 102*

Macromolecule	^{3}H precursor	Ratio of ^{3}H counts/min, 2 hr (48 C/34 C)[a]		
		BC 100 (*wt*)	BC 101 (*tms-12*)	BC 102 (*tms-26*)
DNA	^{3}H-thymidine	2.40	1.80	1.96
RNA	^{3}H-uridine	0.87	0.90	0.87
Protein	^{3}H-phenylalanine	1.20	0.98	1.20

[a] Samples of 0.1 ml used throughout.

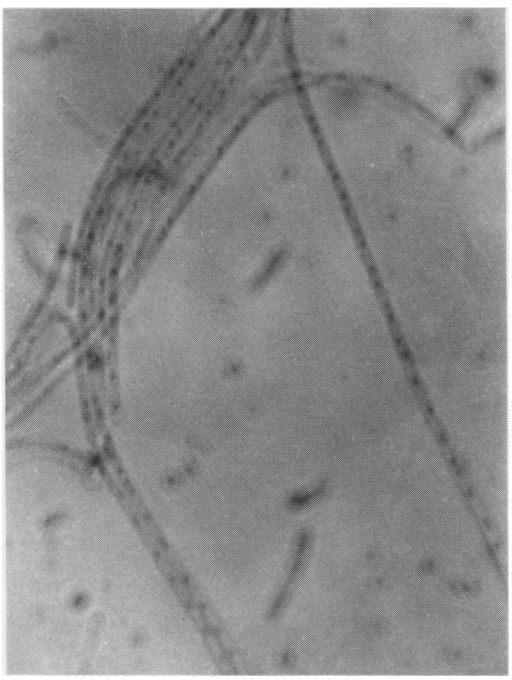

FIG. 6. *Distribution of nuclear-bodies in filaments formed by BC 101 (tms-12) after 4 hr of growth at 48 C. Conditions for growth were the same as given for Fig. 1.*

and *B. licheniformis* DNA and by comparison of these frequencies to those obtained with homologous *B. subtilis* DNA. Dubnau et al. (12) have shown that the *B. subtilis pur B6* locus shows limited homology with *B. niger* DNA and no detectable homology with *B. licheniformis* DNA. The *metB5* locus showed no detectable homology with either DNA, and drug-resistance markers showed appreciable homology with both types of DNA. The relative transformation frequencies for *purA16* and *metB5* were in accord with the reported results (Table 5). Furthermore, both *tms-12* and *tms-26* were genetically conserved. The *tms-26* locus was genetically conserved to a much greater extent than the *tms-12* locus.

DISCUSSION

The observations reported here for two mutants should serve as indicators of the functions most probably affected by the temperature-sensitive alterations. The onset and magnitude of the morphological changes are difficult to assess at times earlier than 2 hr at 48 C because of the nature of the change. It is reasonable to assume that events leading to these morphological alterations have begun well before visual confirmation can be made. In fact, they might begin soon after transfer from 34 to 48 C. Clearly, these morphological changes are the first measured traits that are characteristic of the mutants. When expressed, the *tms-12* mutation presumably leads to a blocking in the cell division process. The *tms-26* mutation affects the cells' rigidity when expressed and might act on the synthesis, assembly, or maintenance of cell wall components. However, the locus for *tms-26* is situated in a region known to contain genetic determinants for sporulation (40, 41). It was noted that the cells of this strain, when incubated at 48 C, characteristically swell at the terminal end. Under phase-contrast microscopy, the swollen end shows a phase difference, suggesting structural changes in this region. At this stage, the swollen cells strikingly resemble cells in a prespore phase of development. Recently, structures in the regions of swelling have been detected by electron microscopy (J. Mangan, *personal communication*). It is possible that the temperature-sensitive lesion in this mutant may primarily affect some aspect of the sporulation process and secondarily affect the cell wall. It is most interesting that both of the functions identified by *tms-12* and *tms-26* are genetically conserved.

The genetic map locations of *tms-12* and *tms-26* show that more than one particular region has been conserved. The *tms-26* locus is situated near or within a region of strong genetic conservation (12). This region contains the drug-resistance markers, which probably affect ribosomal components, as well as a majority of the ribosomal RNA cistrons and the 4*S* transfer RNA cistrons (11, 27, 28). The other locus *tms-12* is positioned distally on the genetic map in a region that previously had not been known to contain conserved genetic functions. However, it recently has been demonstrated that this region also contains some of the cistrons for ribosomal RNA and 4*S* RNA (33). It is not known whether these RNA cistrons and that for *tms-12* are closely associated.

TABLE 4. *Genetic analysis for mapping tms-12 and tms-26*

Replicative order		Transduction by PBS1 grown on BC 101 (*tms-12*)				Transduction by PBS1 grown on BC31					
									Unselected phenotype		
Selective phenotype	Per cent transforming activity in hybrid fraction (recipient strains, BC 101 and 102; donor strain, BC 30)	Recipient strain	Selected phenotype	No. of transductants tested	No. Tms	Recipient strain	Selected phenotype	No. of transductants tested	Pur+	Ery+	Tmr-26
Pur+	74	BC 26	Arg+	150	0	BC 102	Pur+	584	—	4%	36%
Ery^r	72	BC 26	Phe+	274	0	(*tms-26*)	Pur+ Ery^r	24	—	—	58%
Leu+	38	BC 50	Nia+	370	0		Tmr-26	133	22%	24%	—
Met+	4	BC 51	Lys+	267	0						
Tms-12[a]	19	BC 27	Tyr+	132	0	Transduction by PBS1 grown on BC 102 (*tms-26*)					
Tmr-26	79								Pur−	Tms-26	
						BC 28	Cys+	148	25%	92%	

[a] Tmr-12 represents the temperature-resistant phenotype in BC 101.

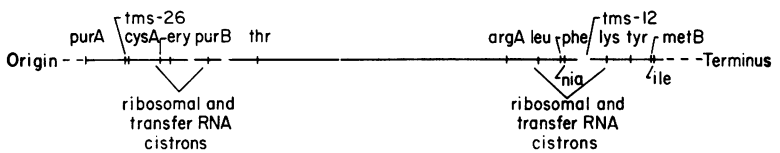

FIG. 7. *Genetic map for B. subtilis. Breaks in the line indicate regions where linkage is not continuous.*

TABLE 5. *Genetic homology*

Donor DNA (2 μg/ml)	Per cent GC	Selected phenotype[a]			
		Pur+	Met+	Tmr-12	Tmr-26
B. subtilis BC 31	43	100%[b] (9.7 × 10^5)[c]	100% (6.6 × 10^5)	100% (2.5 × 10^5)	100% (7.2 × 10^5)
B. niger	43	0.05%	<0.02%	0.64%	48%
B. licheniformis	45–46	<0.01%	<0.02%	0.51%	~300%

[a] Recipient strains: BC 100, BC 101, and BC 102. Gene loci transformed to produce selected phenotype are: *purA16*, *metB5*, *tms-12*, and *tms-26*.

[b] Results for the same genetic locus in different recipients and from separate experiments averaged in determining these values. Transformation frequency with homologous DNA arbitrarily taken as 100%. Heterologous transformation frequencies compared to homologous value for same marker and expressed as percentage of that value.

[c] Number of transformants per milliliter.

It is interesting to speculate on the meaning of gene conservation. Measurable transforming activity for a given function, between two different organisms, indicates: (i) that both organisms possess that function and (ii) that the nucleotide sequences determining that function are not sufficiently different to block pairing and recombination. Specific gene conservation implies that some genes were changed significantly in nucleotide sequence while others were not appreciably changed. This further implies that some selective force has acted (and probably is acting) to retain nucleotide sequence in some functions but not in others. One can further speculate about the fac-

tors that brought about this condition. Possibly, some genetic functions are vital to the cell whereas others are less significant. These vital functions may be conserved because they have fewer alternatives, with regard to change, without losing operational efficiency. This could be due to their complexity. Random changes in such functions would tend to reduce efficiency. Another consideration is that a function may be genetically conserved because its appearance on the evolutionary time scale has not allowed for sufficient change to occur. Definitive answers to these speculations are difficult and may be impossible to obtain. However, future work will show whether conserved functions are localized in particular regions of the genetic map or are distributed more randomly on it. The use of conditional mutants to identify some of those genetically conserved functions is clearly demonstrated.

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