JOURNAL OF BACTERIOLOGY, Sept. 1971, p. 636-645

Vol. 107, No. 3
Printed in U.S.A.

Genetic Mapping of Genes Concerned with Glutamyl Polypeptide Production by *Bacillus licheniformis* and a Study of Their Relationship to the Development of Competence for Transformation[1]

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Received for publication 1 June 1971

By transduction with SP-15, we have mapped some of the genetic sites involved in glutamyl polypeptide (Pep) production by *Bacillus licheniformis* 9945A. Eleven peptide mutations were assigned to group 1 which lies between the *gly-42* and *his-6* markers. Three other mutations were assigned to group 2 which lies between the *ade-2* and *str-1* sites. A series of 28 additional Pep$^-$ mutants did not belong to either group 1 or 2; the chromosomal location of those peptide mutations is unknown. All three mutants of group 2 were highly transformable, but only two of group 1 transformed well under the conditions employed. Knowing the chromosomal markers that were linked to peptide mutations made it possible to investigate the effect peptide mutations might have on the development of competence for transformation. Nontransformable organisms were rendered transformable upon the introduction, by transduction, of Pep$^-$ mutations from transformable strains. These results supported the conclusion that the ability of cells to develop competence for transformation when grown under appropriate conditions was related to the physiological effects of particular peptide mutations.

Bacillus licheniformis strain 9945A produces large amounts of glutamyl polypeptide (peptide) when grown under appropriate conditions (6), and on minimal agar plates it produces extremely mucoid colonies (Pep$^+$) as a result of the accumulation of peptide. Gwinn and Thorne (4) and Leonard et al. (8) independently developed transformation systems for strain 9945A, and in both systems the highly transformable auxotrophs were mutants that were deficient in peptide production (Pep$^-$). Thus, it appeared that transformability was in some way related to the inability of cells to produce peptide, although not all Pep$^-$ mutants were transformable by the procedures used. Thorne and Stull (13) compared the two transformation systems invólving the independently isolated transformable Pep$^-$ mutants. Their study demonstrated that each system required a specific medium for primary growth of the cells but that the same transformation medium could be used for both.

The availability of transformation systems as well as two transducing phages (11) for strain 9945A rendered the organism amenable to genetic investigation. Tyeryar et al. (14) constructed a chromosomal map which consisted of locations of genes for nine amino acids, a gene involved in purine biosynthesis, and one involved in peptide production. The studies to be presented here concerned efforts to locate other genetic sites involved in peptide production and to ascertain whether there is a correlation between the transformability of a particular Pep$^-$ mutant and the chromosomal location of its peptide mutation.

MATERIALS AND METHODS

Organisms. The various mutants of *B. licheniformis* used are listed in Table 1. They were all derived from the ATCC 9945A strain. Those mutants which have not been described previously were assigned tentative

[1] This paper was taken in part from a dissertation submitted by R. W. McCuen in partial fulfillment of the requirements for the Ph.D. degree at the University of Massachusetts.

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TABLE 1. *Description of Bacillus licheniformis cultures*[a]

Designation	Characteristics	Previous designation, origin, or reference
9945A	Wild-type, prototrophic, Pep$^+$	ATCC; Gwinn and Thorne (4)
FD01	*str-1*	Tyeryar et al. (14)
FD012	*lys-4*	9945A M2 of Taylor and Thorne (11)
FD030	*gly-3 pep-1*	9945A M28 of Gwinn and Thorne (4)
FD039	*pep-1*	9945A → FD030; M28-RT of Thorne and Stull (13)
FD042	*pep-3*	Acriflavine treatment of 9945A; Thorne
FD043	*pep-4*	Acriflavine treatment of 9945A; Thorne
FD0176	*gly-42 pep-1*	M28-RT-M63 of Thorne and Stull (13)
FD0250	*his-6 pep-1*	M28-RT-M20-RT-T4 of Thorne and Stull (13)
FD0259	*ser/gly-1 his-6 pep-1*	M28-RT-M20-D1 of Thorne and Stull (13)
FD0264	*pur-1 pep-6*	M17-R1 of Leonard and Mattheis (7)
FD0265	*arg-13 pep-7*	M18-R1 of Leonard and Mattheis (7)
UM1	*gly-42*	9945A → FD0176
UM2	*his-6*	9945A → FD0250
UM3	*pep-7*	9945A → FD0265
UM5	*ade-2 pep-7*	Ultraviolet irradiation of UM3
UM6	*ade-2*	9945A → UM5
UM7	*his-6 pep-7*	FD0259 → FD0265
UM10	*his-6 arg-13 pep-7*	FD0265 → UM7
UM12	*ade-2 his-6 pep-7*	UM5 → UM10
UM13	*ade-2 his-6 pep-8*	UM6 → UM19
UM15	*arg-13*	9945A → FD0265
UM16	*arg-13 pep-9*	Spontaneous mutation from UM15
UM18	*ser/gly-1 his-6*	9945A → FD0259
UM19	*ser/gly-1 his-6 pep-8*	Spontaneous mutation from UM18
UM22	*gly-42 his-6 pep-8*	UM1 → UM13
UM23	*ser/gly-1 his-6 pep-13*	Spontaneous mutation from UM18
UM24	*ser/gly-1 his-6 pep-14*	Spontaneous mutation from UM18
UM25	*ser/gly-1 his-6 pep-15*	Spontaneous mutation from UM18
UM26	*ser/gly-1 his-6 pep-16*	Spontaneous mutation from UM18
UM27	*ser/gly-1 his-6 pep-17*	Spontaneous mutation from UM18
UM28	*ser/gly-1 his-6 pep-18*	Spontaneous mutation from UM18
UM30	*ser/gly-1 his-6 pep-20*	Spontaneous mutation from UM18
UM39	*arg-13 pep-29*	Spontaneous mutation from UM15
UM44	*arg-13 pep-34*	Ultraviolet irradiation of UM15
UM48	*arg-13 pep-38*	Ultraviolet irradiation of UM15
UM50	*lys-4 pep-40*	Spontaneous mutation from FDO12
UM62	*ade-2 pep-1 pep-7*	FD039 $\xrightarrow{td}$ UM12
UM68	*ade-2 his-6*	9945A → UM12
UM69	*pep-13*	9945A → UM23
UM75	*str-1 pep-7*	FD01 $\xrightarrow{td}$ UM3
UM76	*ade-2 pep-4 pep-7*	FD043 $\xrightarrow{td}$ UM12
UM84	*his-6 pep-7*	UM3 $\xrightarrow{td}$ UM68
UM86	*ade-2 pep-1*	FD039 $\xrightarrow{td}$ UM68
UM89	*lys-4 str-1 pep-7*	UM75 $\xrightarrow{td}$ FD012
UM90	*lys-4 str-1*	UM75 $\xrightarrow{td}$ FD012
UM107	*ade-2 pep-13*	UM69 $\xrightarrow{td}$ UM68

[a] The following abbreviations are used: ade, adenine; arg, arginine; gly, glycine; his, histidine; lys, lysine; pur, purine; ser, serine; pep, glutamyl polypeptide; str, streptomycin; r, resistance; s, susceptibility. All organisms with UM designations were derived during the present investigation at the University of Massachusetts. An arrow indicates that the mutant was constructed by congression; the head of the arrow points to the recipient. The letters td situated on the arrow indicate the construction was by SP-15 mediated transduction; otherwise transformation was employed. Unless indicated otherwise the cultures were Pep$^+$

genotypic designations in accordance with the recommendations of Demerec et al. (2). Bacteriophage SP-15 (11) was used for all transductions.

Media. The media used in transformation experiments, NBSG-X, BLSG, and TM, were prepared as described by Thorne and Stull (13). MSNG broth, used for growing recipient cells for transduction, was the nutrient broth-glycerol-salts medium described by Taylor and Thorne (11). PA medium (11) was used for phage propagation and assays. For viable cell counts, cells were diluted in TM medium or 1% peptone (Difco) and plated on NBY agar (13). For scoring transformants and transductants M1 medium (13), M1 supplemented with 1% (v/v) of NBY broth (termed M1E), and medium E (6) were used. Potato extract medium (12) was used for growing spores.

Isolation of mutants. Auxotrophic mutants were isolated following ultraviolet (UV) irradiation of spores as described by Iyer (5). Multiply marked mutants were obtained by UV irradiation or were constructed by congression. Transformation was employed when the marker transferred from the donor was not linked to the selected marker in the recipient, and transduction was frequently used when the construction involved linked markers.

Pep^- mutants were isolated from untreated or UV-irradiated spores by spreading suitable dilutions on medium E agar plates supplemented with Difco Casamino Acids (2 g/liter) and Difco yeast extract (1 g/liter) as described by Leonard and Mattheis (7). After 6 to 7 days at 37 C, dry, rough areas at the edges of Pep^+ colonies were streaked on M1 agar to obtain isolated colonies.

Deoxyribonucleic acid (DNA) preparations. DNA was prepared by the method of Marmur (9) employing the modifications of Gwinn and Thorne (4). DNA concentrations were determined by the method of Burton (1).

Production of spores. Spores were usually grown on potato agar slants or in potato extract broth. They were harvested in distilled water, heated at 65 C for 30 min, and stored at 4 C.

Bacteriophage propagation and assay. SP-15 was propagated in soft agar overlays. Spores (10^8 in 0.1 ml) of the appropriate organism and phage (10^8 plaque-forming units in 0.1 ml) were mixed in tubes containing 3 ml of PA broth, and 3 ml of soft PA agar (0.7% agar) at 46 C was added. Three milliliters of the mixture was plated on freshly poured PA agar plates (1.5% agar), and the plates were incubated 16 hr at 37 C. The phage was harvested by macerating the soft agar layer in 5 ml of PA broth and centrifuging at low speed to sediment the agar and cells. The supernatant fluid was treated with deoxyribonuclease (1× crystallized from Worthington Biochemical Corp.; 50 μg/ml at 37 C for 30 min) and filtered through an HA membrane filter (Millipore Corp., Bedford, Mass.). Phage suspensions were tested for sterility and stored at 4 C in PA broth supplemented with 1 μg of $ZnSO_4 \cdot 7H_2O$ and 40 μg of $FeCl_3 \cdot 6H_2O$ per ml (15). SP-15 lysates prepared in this manner retained good transducing activity for several months.

The phage was assayed by the procedure described by J. Mele (Master's Thesis, Univ. of Massachusetts, Amherst, 1968).

Transduction. Recipient cells for transduction were grown for 5 hr in 250-ml Erlenmeyer flasks containing 50 ml of MSNG medium and incubated on a rotary shaker (250 rev/min) at 37 C. Flasks were inoculated with 5 ml of an overnight culture grown in the same medium from a spore inoculum. A sample of the 5-hr culture was centrifuged at low speed, and the cell pellet was resuspended in one-half its original volume of PA broth (2×10^9 to 5×10^9 cells/ml). Samples (1 ml) of resuspended cells were placed into tubes (18 by 150 mm) containing phage (multiplicity of infection was about 1), and the final volume of the mixture was brought to 2 ml with PA broth. Control mixtures of the same composition, but lacking phage, were always included in each experiment. The tubes were incubated on a reciprocating shaker (144 strokes/min) in a 37 C water bath for 30 to 45 min. Samples were plated on M1E agar supplemented with the appropriate nutritional requirements (50 μg of amino acids per ml and 30 μg of purines per ml), and transductants were scored after 48 hr at 37 C. Recombinant genotypes were tentatively confirmed by testing the transductants for growth on M1 agar with and without the appropriate supplements.

Transductants for streptomycin resistance were selected by spreading suitable amounts of the transduction mixture on NBY agar plates. These were incubated at 37 C for 3 hr to allow for expression of the streptomycin resistance marker, and the plates were then sprayed lightly with a streptomycin-sulfate solution (200 mg/ml). Streptomycin-resistant transductants were scored after 48 hr.

Although several methods were attempted, we were not able to select efficiently those cells transduced from Pep^+ to Pep^- or from Pep^- to Pep^+; therefore single transductants for a peptide marker could not be scored. Cells simultaneously transduced for an auxotrophic marker and a peptide locus were easily scored on the basis of their distinctive colonial morphology.

Transformation. Two transformation procedures were used: the M28 procedure, so called because it was originally developed (4) for transformation of mutant M28 (the original *pep-1* mutant, now designated FD030), and the M18 procedure, so called because it was originally and independently developed (7, 8) for mutant M18 (the original *pep-7* mutant, now designated FD0265). The previously published accounts of the two procedures include studies of various factors affecting the transformation systems, and thus, to avoid confusion, the details of the two procedures as used in the present investigation are given here.

For transformation in the M28 system, 10^7 spores of the desired mutant were inoculated into a 250-ml Erlenmeyer flask containing 25 ml of NBSG-X medium. The flask was incubated at 37 C on a rotary shaker (250 rev/min) for a period of time optimal for a particular mutant under study. One-tenth milliliter of cells was added to tubes (18 by 150 mm) containing 0.8 ml of TM broth and 0.1 ml of DNA (in 2 M NaCl), and these tubes were incubated for 3 hr in a slanted position on a reciprocating shaker (144 strokes/min) in a 37 C water bath. Similar tubes without DNA were run as controls in all experiments. Following the 3-hr incubation period, 50 μg of deoxyribonuclease in 0.05 ml of water were added to each tube, and incubation was

continued for 15 min. Samples were diluted in TM broth and plated on the appropriate medium.

For transformation in the M18 system, 10^7 spores of the mutant to be transformed were inoculated into a 500-ml Erlenmeyer flask containing 50 ml of BLSG medium, and the flask was incubated on the shaker at 37 C for 19 hr or another period of time optimal for a particular mutant. For transformation, the culture was diluted 1:20 in TM broth, and 0.9 ml was transferred to tubes (18 by 150 mm). The tubes were incubated for 2 hr on the shaker described above. DNA (0.1 ml in 2 M NaCl) was then added and incubation was continued for an additional 2 hr. The samples were treated with deoxyribonuclease as described above, diluted in TM broth, and plated on the appropriate medium.

In both systems transformants were scored after 48 hr at 37 C. For selecting cells transformed for auxotrophic markers, we used M1E agar supplemented as appropriate (50 μg of amino acids and 30 μg of purines per ml). Medium E agar supplemented with 100 to 200 μg of the auxotrophic requirements per ml was used for selecting cells transformed to Pep^+ in the M28 procedure. Cells transformed to Pep^+ in the M18 procedure were selected efficiently on M1 agar supplemented when necessary with 50 μg of amino acids and 30 μg of purines per ml.

Cells transformed to streptomycin resistance were selected in the same manner as streptomycin-resistant transductants, except that 6 hr were allowed for expression time before the NBY agar plates were sprayed with streptomycin. Spontaneously induced prototrophic revertants or streptomycin-resistant mutants were rarely observed in either transformation procedure. Low numbers of Pep^+ revertants were occasionally observed for some of the group 1 Pep^- mutants. Pep^+ revertants were not observed for those Pep^- mutants of group 2.

RESULTS AND DISCUSSION

Tests for linkage of pep-1 and pep-7 mutations to other markers. The M28 series of transformable auxotrophs studied by Thorne and Stull (13) were derived from a Pep^- mutant (*pep-1*) originally isolated by Gwinn and Thorne (4). The M18 series of transformable auxotrophs (13) were derived from a Pep^- mutant (*pep-7*) originally isolated by Leonard et al. (8). Tyeryar et al. (14) have shown with phage SP-15 that *pep-1* was cotransduced with *gly-2* and *his-3*. The *gly-2* and *his-3* mutants were not available in our laboratory; however, we did possess *gly-42 pep-1* and *his-6 pep-1* mutants from which we constructed an organism containing both the *gly-42* and *his-6* mutations. The data presented in Table 2 show that pep-1 was cotransduced with *gly-42* at a frequency of 30% and with *his-6* at a frequency of 61%. *Gly-42* was cotransduced with *his-6* at a frequency of 8%. These frequencies of cotransduction suggest that *pep-1* lies between the *gly-42* and *his-6* mutations and probably closer to the histidine than to the glycine marker. The cotransduction frequencies of *pep-1* with *gly-42* and *his-6* were similar to those reported by Tyeryar et al. (14) for cotransduction of *pep-1* with *gly-2* and *his-3*. Therefore, it was concluded that the auxotrophic markers occupy similar map positions.

DNA extracted from FD0265 cells (*arg-13 pep-7*) was able to transform competent cells of FD0250 (*his-6 pep-1*) to Pep^+. Thus, *pep-1* and *pep-7* were not identical. Tests for cotransduction of *pep-7* with *gly-42* or *his-6* were negative, indicating that the *pep-7* mutation was not located in the same region of the chromosome as the *pep-1* mutation. Therefore, attempts were made to find auxotrophic mutations that were linked to *pep-7*. Spores of UM3 (prototrophic *pep-7*) were UV-irradiated and auxotrophic mutants were isolated. These mutants were then transduced to prototrophy by SP-15 grown on 9945A (prototrophic Pep^+). By this procedure *pep-7* was found to be linked to *ade-2*. The information given in Table 3 shows that *ade-2* and *pep-7* were cotransduced at a frequency of 28 to 30%. Results of other experiments showed that *ade-2* was not cotransduced with *pep-1*, *gly-42*, or *his-6*.

Neither *ade-2* nor *pep-7* was among the markers placed on the chromosomal map of *B. licheniformis* by Tyeryar et al. (14). Comparison of their *B. licheniformis* map with the *B. subtilis* map (3) led to the suggestion that the *pur-1* marker positioned at the origin on the *B. licheniformis* map might be analogous to the *purB* locus of *B. subtilis*. *PurB* is located in the origin-proximal region of the *B. subtilis* chromosome in the second gene-linkage group. Another locus concerned with purine biosynthesis, *purA*, is positioned near the origin of the *B. subtilis* chromosome in the first gene-linkage group. The *pur-1* mutant of *B. licheniformis* and *purB* mutant of *B. subtilis* were able to use either adenine or guanine for growth, whereas a mutant of *B. subtilis* carrying a mutation in the *purA* locus could use only adenine. The *ade-2* mutant of *B. licheniformis* also had a strict requirement for adenine. Thus, it seemed likely that if the chromosome maps of the two organisms were similar, *pur-1* was not located at the origin of the *B. licheniformis* chromosome, and that *ade-2* might occupy this position.

Dubnau et al. (3) demonstrated cotransduction of the *purA* and streptomycin resistance markers in *B. subtilis*. By using *B. licheniformis* Rogolsky (10) was unable to show cotransduction of the streptomycin resistance marker (*str-1*) with *pur-1*. This was consistent with the hypothesis that *ade-2* and *pur-1* of *B. licheniformis* were located in map positions analogous to those of *purA* and *purB*, respectively, of *B. subtilis*. If this were the case, then *ade-2* should be cotransducible with

TABLE 2. *Cotransduction of pep-1 and gly-42 or his-6 by SP-15 grown on 9945A (prototrophic Pep⁺)*[a]

Recipient	Transductants/0.1 ml			Cotransduction (%)
	Gly⁺	His⁺	Doubles	
FD0176 (*gly-42 pep-1*)	120		36 (Gly⁺ Pep⁺)	30
FD0250 (*his-6 pep-1*)		82	50 (His⁺ Pep⁺)	61
UM22 (*gly-42 his-6 pep-8*)	131	214	13 (Gly⁺ His⁺)	10 (based on Gly⁺) 6 (based on His⁺)

[a] All values are based on the average of at least three experiments. See footnote to Table 1 for abbreviations used.

TABLE 3. *Cotransduction of ade-2, pep-7, and str-1*[a]

Donor	Recipient	Transductants				Cotransduction (%)[b]
		No. examined		No. of Str-r[c] among		
				Ade⁺ Pep⁻	Ade⁺ Pep⁺	
		Ade⁺ Pep⁻	*Ade⁺ Pep⁺*			
9945A (Pep⁺ prototrophic)	UM5 (*ade-2 pep-7*)	110	33			30 (*ade-2* and *pep-7*)
FD01 (*str-1* Pep⁺ prototrophic)	UM5	241	94	2	25	28 (*ade-2* and *pep-7*) 8 (*ade-2* and *str-1*)
		Str-r Pep⁻	*Str-r Pep⁺*			
FD01	UM3 (*pep-7* Str-s)	45	33			42 (*str-1 pep-7*)

[a] See footnote to Table 1 for abbreviations used.

[b] All values are based on the average of at least three experiments.

[c] These were determined by picking all the Ade⁺ transductants from M1E to M1 with and without streptomycin.

str-1. The results of the second transduction experiment presented in Table 3 show that this was true. When *ade-2 pep-7* cells were transduced by SP-15 grown on FD01 (prototrophic *str-1* Pep⁺), 8% of the Ade⁺ transductants were also transduced to streptomycin resistance. Among 94 Ade⁺Pep⁺ transductants, 25 were also Str-r; and among 241 Ade⁺Pep⁻ transductants, only 2 were Str-r. These data are consistent with the hypothesis that *pep-7* lies between the *ade-2* and *str-1* sites. Further evidence that this is the case is supplied by the data from the third experiment in Table 3. Cells of UM3 (Str-s *pep-7*) were transduced by SP-15 grown on FD01 (*str-1* Pep⁺) and Str-r transductants were selected. Among the Str-r recombinants tested, 42% were also transduced to Pep⁺. Thus, the cotransduction frequencies of 42% for *str-1* and *pep-7*, 30% for *ade-2* and *pep-7*, and 8% for *ade-2* and *str-1* can be taken as evidence that *pep-7* probably lies between the *ade-2* and *str-1* markers.

Classification of some Pep⁻ mutants. Based on the data presented thus far, at least one cistron involved in glutamyl polypeptide production lies between the *gly-42* and *his-6* sites, and at least one other lies between the *ade-2* and *str-1* markers. Since it is not known how many peptide cistrons lie in each of the regions, all of the peptide mutations that lie between *gly-42* and *his-6* will be referred to as group 1 mutations, and all of those that lie between *ade-2* and *str-1* will be designated as group 2 peptide mutations. Further classification of the mutations into cistrons cannot be done until additional information is available.

A number of Pep⁻ mutants were tested for cotransduction of their peptide mutation with specific markers. Usually the Pep⁻ mutant was used as a donor, and a set of three standard tester strains were used as recipients. UM1 (*gly-42* Pep⁺), UM2 (*his-6* Pep⁺), and UM6 (*ade-2* Pep⁺) were transduced to prototrophy and the transductant colonies were examined for their peptide phenotype. If some of the transductants from UM1 and UM2 were also Pep⁻, this indicated that the peptide mutation in the donor belonged to group 1. If some of the transductants from UM6 were Pep⁻, this suggested that the peptide mutation in the donor might belong to group 2.

Some of the Pep⁻ mutants initially possessed the *his-6* mutation. These were screened for

group 1 mutations by using them as recipients in transductions in which the donor was prototrophic Pep$^+$. If some of the His$^+$ transductants were also Pep$^+$, the mutation was tentatively assigned to group 1. SP-15 was then grown on the mutant containing the suspected group 1 mutation and used to transduce UM1 (*gly-42* Pep$^+$). The recovery of Gly$^+$ Pep$^-$ transductants confirmed that the peptide mutation belonged to group 1.

The results of the transduction experiments that were conducted to classify the mutations are presented in Table 4. Of 42 Pep$^-$ mutants that we attempted to map, only 14 could be placed into groups 1 and 2. In addition to *pep-1*, no. 3, 4, 13–18, 38, and 40 were placed in group 1, and in addition to *pep-7*, no. 6 and 9 were tentatively placed in group 2. Based on an average of 150 to 200 transductants examined, 28 other Pep$^-$ mutants could not be placed into either group 1 or 2; the locations of these on the *B. licheniformis* chromosome are unknown.

Examination of the results presented in Tables 2 and 4 reveals some differences in the respective values for cotransduction of *pep-1* with *gly-42* and *his-6*. The values in Table 2 were obtained when the recipient contained the auxotrophic marker and the peptide mutation to be tested for linkage. Thus, on the scoring plates the background cells were Pep$^-$. However, the values in Table 4 for cotransduction of *pep-1* were determined when the donor contained the peptide mutation; in this case the background cells were Pep$^+$. This suggested that discrepancies among cotransduction frequencies might be related to the peptide phenotype of the recipient or background cells. This was examined further and results were presented elsewhere (R. W. McCuen, Ph.D. thesis, Univ. of Massachusetts, Amherst, 1971) showed that when recipient cells were Pep$^+$ the frequencies of cotransduction of peptide mutations with linked markers were 10 to 24% lower than those obtained when the recipient cells were Pep$^-$. Since the cotransduction values for the group 1 peptide mutations in Table 4 were not all determined in the same manner, the mutations cannot be placed in a precise order.

The *pep-6* and *pep-9* mutations exhibited linkage to *ade-2* (Table 4) and were tentatively classed as group 2 mutations. To confirm this, the following results were considered. In transduction experiments with *ade-2*, *pep-6*, and *str-1* mutants, *pep-6* was cotransduced with *ade-2* at a frequency of 22% and with *str-1* at a frequency of 51%. This suggested that *pep-6* also lies between *ade-2* and *str-1*. Transformation of UM16 cells (*arg-13 pep-9*) yielded Pep$^+$ transformants when DNA from FD0264 (*pur-1 pep-6*) or wild-type 9945A was used but not when the DNA was from UM3 (*pep-7*). Similarly, Pep$^+$ transformants were recovered when cells of FD0264 (*pur-1 pep-6*) were transformed with DNA from UM16 (*arg-13 pep-9*) or wild-type but not with DNA from UM3 (*pep-7*). Thus, mutants bearing *pep-9* and *pep-6* would cross with each other to yield Pep$^+$ but neither would cross with *pep-7* to give Pep$^+$. These results concerning the three peptide mutations can best be explained if all three lie between *ade-2* and *str-1* and if *pep-7* is a deletion overlapping *pep-6* and *pep-9*.

Examination of the two transformation systems. A study of M18 and M28 transformation procedures was undertaken to gain insight into the requirement of certain Pep$^-$ mutants for a specific transformation procedure. The two procedures were similar except for the specificity of the growth medium for each series of transformable mutants. *Pep-1* mutants, previously designated as the M28 series of mutants, were grown in NBSG-X medium, and *pep-7* mutants, previously designated as the M18 series, were grown in BLSG medium. The two media are similar, the main differences being that NBSG-X has a much higher phosphate content, a higher initial *p*H, and a lower concentration of glycerol than BLSG. Thorne and Stull (13) have shown that cell populations in each growth medium reached a potentially competent state after the culture entered the stationary phase and that they attained maximal competence only after being transferred from the growth medium to transformation medium. Those investigators also demonstrated that the length of incubation time in the growth medium was a critical factor in producing potentially competent cell populations.

Table 5 shows data from the transformation of *pep-1* cells by the M28 procedure and *pep-7* cells by the M18 procedure. These data confirmed the previously published results (13). The optimal time for growing potentially competent *pep-1* cells in NBSG-X medium was found to be about 20 hr, and for *pep-7* cells in BLSG medium it was about 19 hr. Earlier studies (13) had demonstrated that *pep-7* mutants were poorly transformable when grown in NBSG-X medium, and, in like manner, *pep-1* cells transformed poorly when grown in BLSG medium. The data from similar experiments conducted during the present investigation confirmed and extended those results. FD0250 cells (*his-6 pep-1*) grown in BLSG medium gave a maximum transformation frequency of only 0.15% when tested at 4-hr intervals between 16 and 42 hr. When FD0265 cells (*arg-13 pep-7*) were transformed by the M28 procedure, the transformation frequency after 16 to 20 hr of growth in NBSG-X was between

TABLE 4. *Classification of Pep⁻ mutants of Bacillus licheniformis by SP-15-mediated transduction*[a]

Peptide mutation tested	Donor	Recipient	Transductants/0.1 ml		Cotransduction (%)	Group assignment
			marker⁺ Pep⁺	marker⁺ Pep⁻		
pep-1	FD039 (prototrophic *pep-1*)	UM1 (*gly-42*)	326	78	19	
		UM2 (*his-6*)	101	115	53	1
		UM6 (*ade-2*)	150	0	0	
pep-3	FD042 (prototrophic *pep-3*)	UM1 (*gly-42*)	217	44	17	
		UM2 (*his-6*)	30	229	89	1
		UM6 (*ade-2*)	242	0	0	
pep-4	FD043 (prototrophic *pep-4*)	UM1 (*gly-42*)	48	13	20	
		UM2 (*his-6*)	12	81	87	1
		UM6 (*ade-2*)	74	0	0	
pep-6	FD0264 (*pur-1 pep-6*)	UM1 (*gly-42*)	114	0	0	
		UM2 (*his-6*)	90	0	0	2
		UM6 (*ade-2*)	105	30	22	
pep-7	9945A (prototrophic Pep⁺)	UM5 (*ade-2 pep-7*)	53	123	30	2
pep-9	UM16 (*arg-13 pep-9*)	UM1 (*gly-42*)	366	0	0	
		UM2 (*his-6*)	374	0	0	2
		UM6 (*ade-2*)	138	65	32	
pep-13	UM23 (*ser/gly-1 his-6 pep-13*)	UM1 (*gly-42*)	180	42	19	
	9945A (prototrophic Pep⁺)	UM23 (*ser/gly-1 his-6 pep-13*)	108 (His⁺)	112 (His⁺)	49	1
pep-14	UM24 (*ser/gly-1 his-6 pep-14*)	UM1 (*gly-42*)	192	28	13	
	9945A (prototrophic Pep⁺)	UM24 (*ser/gly-1 his-6 pep-14*)	140 (His⁺)	132 (His⁺)	52	1
pep-15	UM25 (*ser/gly-1 his-6 pep-15*)	UM1 (*gly-42*)	245	30	11	
	9945A (prototrophic Pep⁺)	UM25 (*ser/gly-1 his-6 pep-15*)	125 (His⁺)	160 (His⁺)	45	1
pep-16	UM26 (*ser/gly-1 his-6 pep-16*)	UM1 (*gly-42*)	187	30	14	
	9945A (prototrophic Pep⁺)	UM26 (*ser/gly-1 his-6 pep-16*)	110 (His⁺)	112 (His⁺)	50	1
pep-17	UM27 (*ser/gly-1 his-6 pep-17*)	UM1 (*gly-42*)	163	27	14	
	9945A (prototrophic Pep⁺)	UM27 (*ser/gly-1 his-6 pep-17*)	157 (His⁺)	153 (His⁺)	51	1
pep-18	UM28 (*ser/gly-1 his-6 pep-18*)	UM1 (*gly-42*)	143	30	17	
	9945A (prototrophic Pep⁺)	UM28 (*ser/gly-1 his-6 pep-18*)	77 (His⁺)	127 (His⁺)	38	1
pep-38	UM48 (*arg-13 pep-38*)	UM1 (*gly-42*)	190	50	20	
		UM2 (*his-6*)	60	80	57	1
		UM6 (*ade-2*)	235	0	0	
pep-40	UM50 (*lys-4 pep-40*)	UM1 (*gly-42*)	250	20	7	
		UM2 (*his-6*)	160	80	31	1
		UM6 (*ade-2*)	300	0	0	

[a] All values are based on the average of at least three experiments. A marker⁺ transductant refers to the auxotrophic marker in the recipient. See footnote to Table 1 for abbreviations used.

TABLE 5. *The optimal times for growing potentially competent cultures for the two transformation procedures*[a]

Mutant tested	Procedure used	Age of culture (hr)	Prototrophic transformants/ml	Recipient cells/ml	Transformation (%)
FD0250 (*his-6 pep-1*)	M28	16	6.1×10^5	2.4×10^8	0.3
		20	9.1×10^6	2.3×10^8	4.0
		22	7.1×10^6	2.0×10^8	3.5
		24	4.8×10^6	1.6×10^8	3.0
		26	4.2×10^6	1.5×10^8	2.8
		28	1.5×10^6	1.3×10^8	1.1
FD0265 (*arg-13 pep-7*)	M18	15	1.4×10^6	8.1×10^7	1.7
		17	3.3×10^6	8.2×10^7	4.1
		19	4.0×10^6	8.1×10^7	4.9
		21	1.2×10^6	7.6×10^7	1.6
		23	6.4×10^5	7.5×10^7	0.9
		26	1.7×10^5	7.5×10^7	0.2

[a] Saturating concentrations of deoxyribonucleic acid from 9945A (prototrophic pep$^+$) were employed. See footnote to Table 1 for abbreviations used.

0.001 and 0.2%. However, if the incubation time of the cells in NBSG-X was increased to 28 hr, they were transformed at a frequency of 3% (Table 6).

From these comparisons of the two transformation procedures, we concluded that *pep-1* mutants were highly transformable by only the M28 procedure but that *pep-7* mutants were highly transformable by both procedures if the proper time of incubation in the growth medium was employed. Because of these results, we have adopted the M28 procedure as the standard method for transforming Pep$^-$ *B. licheniformis* mutants. The time of incubation in the primary growth medium is adjusted to suit the requirements of the mutant under study.

Transformation of Pep$^+$ and Pep$^-$ mutants. When studying the transformation characteristics of *B. licheniformis* mutants, previous investigators observed that Pep$^+$ auxotrophs were poorly transformable even though they were derived from highly transformable Pep$^-$ auxotrophs (4, 7, 8). The poor transformation observed with Pep$^+$ cells was not due to the auxotrophic marker carried by the mutant. These observations led to the hypothesis that transformability is related to the inability of an organism to produce peptide.

When all the mutants that contained a mapped peptide mutation were tested for transformation under standard conditions, only five transformed well; two of these were in group 1 (*pep-1* and *pep-38*) and the other three were those of group 2 (*pep-6*, *pep-7*, and *pep-9*). The *pep-1* and *pep-7* mutants correspond to the original M28 and M18 Pep$^-$ mutants, respectively. The *pep-6* mutant, which was originally designated M17R-1, was shown to be transformable along with *pep-7* mutants (M18 mutants) by Leonard and Mattheis (7).

TABLE 6. *Optimal time for transformation of FD0265 cells (arg-13 pep-7) by the M28 procedure*[a]

Age of culture (hr)	Arg$^+$ transformants/ml	Recipient cells/ml	Transformation (%)
16	1.1×10^3	1.2×10^8	0.0009
20	2.6×10^5	1.6×10^8	0.16
24	1.1×10^5	1.7×10^8	0.07
26	1.4×10^6	1.4×10^8	1.0
28	5.2×10^6	1.7×10^8	3.0
32	4.0×10^6	1.4×10^8	2.9
34	1.7×10^6	6.8×10^7	2.5

[a] Approximately 20 μg of deoxyribonucleic acid from 9945A was used. See footnote to Table 1 for abbreviations used.

The other nine mutants carrying peptide mutations that mapped in group 1 gave transformation frequencies ranging from 0 to 0.003%. Similarly, mutants containing the unmapped peptide mutations, *pep-8*, *pep-20*, *pep-29*, and *pep-34* did not transform well, if at all. The remaining 24 unmapped Pep$^-$ mutants were not tested for transformation.

Transduction of the ability to be transformed. From the preceding results it appeared that the ability of *B. licheniformis* cells to be transformed under the specific conditions employed was related to the presence of particular Pep$^-$ mutations. Thus, it became of interest to test whether nontransformable auxotrophic mutants would become transformable upon the acquisition of one of the Pep$^-$ mutations associated with transformability. The linkage sites for the Pep$^-$ mutations were known, and thus appropriate test strains could be constructed.

Nontransformable UM68 (*ade-2 his-6* Pep$^+$) was constructed from UM12 (*ade-2 his-6 pep-7*) by transformation. SP-15 grown on either FD039 (prototrophic *pep-1*) or UM3 (prototrophic *pep-7*) was used to transduce UM68. His$^+$ *pep-1* and Ade$^+$ *pep-7* transductants were isolated and tested for transformability. As controls, the following organisms were also included in the tests for transformation: UM68, His$^+$ Ade$^-$ Pep$^+$ and His$^-$ Ade$^+$ Pep$^+$ transductants from the transduction described above, and some Pep$^-$ recombinants isolated from the transduction of UM68 with SP-15 grown on UM69 (prototrophic *pep-13*). The *pep-13* mutation was cotransducible with *his-6* (table 4), and cells carrying this peptide mutation were poorly transformable. The genotype of the peptide mutation contained in each of the Pep$^-$ transductants was confirmed by transformation tests employing DNA species known to contain the peptide mutations being studied. The data presented in Table 7 reveal that only those recombinants inheriting *pep-1* or *pep-7* transformed at high frequencies. The control organisms yielded frequencies ranging from 0 to 0.002%. These results were consistent with the hypothesis that high transformability was related to specific peptide mutations.

The experiments discussed above were done with a nontransformable Pep$^+$ organism (UM68) derived from a Pep$^-$ mutant (UM12) that was highly transformable. Thus, the question arose as to whether the past genetic history of UM68 could have influenced the results. Would similar results be obtained if the recipient used in constructing the test organism were more directly derived from the nontransformable wild-type 9945A? Experiments to answer this were based on the fact that *pep-7* was cotransducible with *str-1*. SP-15 was grown on UM75 (prototrophic *pep-7 str-1*) and used to transduce nontransformable organisms, e.g., FD012 (*lys-4* Pep$^+$). Among transductants selected for resistance to streptomycin, those that inherited the *pep-7* mutation also acquired the ability to be transformed.

Transformation characteristics of organisms containing two Pep$^-$ mutations. UM62 (*ade-2 pep-1 pep-7*) and UM76 (*ade-2 pep-4 pep-7*) were constructed by transduction of UM12 (*ade-2 his-6 pep-7*) with phage grown on either FD039 (prototrophic *pep-1*) or FD043 (prototrophic *pep-4*). Each of the peptide mutations, *pep-1* and *pep-4*, was cotransducible with *his-6*, and therefore, selection of His$^+$ recombinants yielded some double Pep$^-$ mutants. The *pep-1* and *pep-7* mutations were chosen as representatives of those associated with transformability, and *pep-4* was chosen as representative of Pep$^-$ mutations not associated with transformability. Genotypes of the constructed double Pep$^-$ mutants were confirmed by the fact that DNA bearing either of the peptide mutations in question did not transform the double mutant to Pep$^+$, whereas

TABLE 7. *Transduction of the ability to be transformed*

SP-15 transduction		Description of recombinant or control strain tested for transformation	Transformation tests[a]		
Donor	Recipient		Transformants/ml	Recipient cells/ml	Frequency (%)
FD039 (prototrophic *pep-1*)	UM68 (*ade-2 his-6* Pep$^+$)	UM86 (*ade-2 pep-1*)	1.8×10^7 (Ade$^+$)	2.3×10^8	7.5
FD039 (prototrophic *pep-1*)	UM68 (*ade-2 his-6* Pep$^+$)	—(*ade-2* Pep$^+$)	0 (Ade$^+$)	2.2×10^8	0
UM3 (prototrophic *pep-7*)	UM68 (*ade-2 his-6* Pep$^+$)	UM84 (*his-6 pep-7*)	1.0×10^7 (His$^+$)	1.7×10^8	6.0
UM3 (prototrophic *pep-7*)	UM68 (*ade-2 his-6* Pep$^+$)	—(*his-6* Pep$^+$)	1.4×10^2 (His$^+$)	2.4×10^8	0.00006
UM69 (prototrophic *pep-13*)	UM68 (*ade-2 his-6* Pep$^+$)	UM107 (*ade-2 pep-13*)	6.2×10^3 (Ade$^+$)	3.2×10^8	0.002
		UM68 (*ade-2 his-6* Pep$^+$)	0 (Ade$^+$ His$^-$)	2.1×10^8	0

[a] Inoculum for each experiment consisted of one large representative colony from an NBY agar plate incubated at 37 C for 48 hr. Each colony was tested according to the M28 procedure with incubation times in NBSG-X as follows: UM86 and UM68, 22 hr; UM107, 24 hr; UM84, *ade-2* Pep$^+$, and *his-6* Pep$^+$, 28 hr. Saturating concentrations of deoxyribonucleic acid from 9945A (prototrophic Pep$^+$) were used. See footnote to Table 1 for abbreviations used.

saturating concentrations of a mixture of two DNA species, each carrying one of the respective mutations, did produce Pep$^+$ transformants.

Each of the double Pep$^-$ mutants was tested to determine its tranformation characteristics. The double mutant, UM62, which contained the *pep-1* and *pep-7* mutations transformed well and at frequencies (3 to 5%) similar to those obtained with single *pep-1* or *pep-7* mutants from which the double mutant was derived. The other double mutant, UM76, also transformed well (frequencies of 3 to 6%) even though one of the peptide mutations, *pep-4*, was derived from a nontransformable parent. These results indicate that introducing a peptide mutation associated with nontransformability into a transformable Pep$^-$ recipient did not alter the ability of the recipient to become competent for transformation.

The mechanism of glutamyl polypeptide synthesis by *B. licheniformis* has not been elucidated, and the nature of the biochemical lesions imposed by the various peptide mutations is, therefore, unknown. It seems quite clear that some peptide mutations confer upon the cell the ability to develop competence under certain specific conditions. The peculiar colonial morphology of transformable Pep$^-$ mutants (4, 7) suggests that the cells may have an altered cell wall. In this connection it is perhaps pertinent to point out some peculiarities of the constructed mutant, UM62, that contained two peptide mutations associated with transformability. This mutant grew very slowly and it did not reach maximal potential competence before 42 hr. Under the electron microscope, thin sections of the double mutant revealed cells that were very heterogeneous in size, and there were frequent long, nonseptate, filamentous organisms. This was in contrast to cells containing a single peptide mutation or to cells of the constructed double mutant, UM76, which contained one peptide mutation associated with transformability and one that was associated with nontransformability; under the microscope these were morphologically homogeneous and did not appear different from wild-type Pep$^+$ cells.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-07654 from the National Institute of Allergy and Infectious Diseases.

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