

TRANSFORMATION TO PROTOTROPHY AND POLYGLUTAMIC ACID SYNTHESIS IN *BACILLUS LICHENIFORMIS*

C. GOMEZ LEONARD, D. K. MATTHEIS, M. J. MATTHEIS, AND R. D. HOUSEWRIGHT

U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland

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ABSTRACT

LEONARD, C. GOMEZ (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), D. K. MATTHEIS, M. J. MATTHEIS, AND R. D. HOUSEWRIGHT. Transformation to prototrophy and polyglutamic acid synthesis in *Bacillus licheniformis*. *J. Bacteriol.* **88**:220-225. 1964.—Growth conditions necessary for transformation to prototrophy of 14 auxotrophs of *Bacillus licheniformis* were studied. The unexpected finding of different growth requirements by each auxotroph for the development of transformable cells is discussed. Under optimal growth conditions for a serine-deficient mutant transformation frequencies of 0.1% were obtained. A medium for transformation of competent cells in liquid suspensions is described. In addition, this report presents evidence for the transformation of three nonencapsulated mutants of *B. licheniformis* for the ability to synthesize polyglutamic acid (capsular material).

Bacillus licheniformis strain 9945A (previously designated by the American Type Culture Collection as *B. subtilis* strain 9945A) synthesizes large amounts of polyglutamic acid (capsular material) during growth in a chemically defined medium (Leonard, Housewright, and Thorne, 1958). We have been studying the mechanism of polyglutamic acid synthesis by *B. licheniformis* (Leonard et al., 1958; Thorne and Leonard, 1958; Leonard and Housewright, 1963). Genetic and biochemical studies of factors affecting polyglutamic acid synthesis by encapsulated and nonencapsulated mutants of this organism will be of great help in further elucidating the mechanism of polyglutamic acid synthesis.

Transformation of *B. licheniformis* auxotrophs had not been reported until Gwinn and Thorne (1964) were able to transform to prototrophy 3 of a series of 28 auxotrophs of *B. licheniformis*.

This report presents studies on the growth conditions necessary for transformation to prototrophy of each of the 14 mutants we tested. The finding of different growth requirements by

each auxotroph for the development of transformable cells is discussed. This report also presents evidence for the transformation of three nonencapsulated mutants of *B. licheniformis* for the ability to synthesize polyglutamic acid (capsular material). A medium for transformation of *B. licheniformis* cells in liquid suspensions is described.

MATERIALS AND METHODS

Organisms. *B. licheniformis* 9945A was used throughout as the donor of wild-type deoxyribonucleic acid (DNA) and for obtaining auxotrophic mutants after irradiation of spores with ultraviolet light (Iyer, 1960). This strain produces large quantities of capsular material (L- and D-polyglutamic acid) when grown on minimal agar plates (Gwinn and Thorne, 1964) or on medium E (Leonard et al., 1958). Nonencapsulated mutants arising spontaneously were isolated from the auxotrophic mutants and from the wild-type parent. The nonencapsulated mutants (capsule⁻) did not produce capsular material in any media tested, including supplemented minimal or medium E agar plates. (Under similar conditions encapsulated strains produced large amounts of capsular material.)

Stock cultures. Spores of wild-type and mutant strains were prepared in potato broth (Thorne, 1962) and kept at 4 C. Cells were grown from spore inoculum (10⁶ spores per ml) in 50 ml of the desired medium in 500-ml flasks. The flasks were shaken at 37 C on a reciprocating shaker set at 100 excursions per min (5-cm stroke) for the desired length of time.

Media and cultural conditions. The minimal medium consisted of (NH₄)₂SO₄, 2 g; K₂HPO₄, 14 g; KH₂PO₄, 6 g; Na citrate·2H₂O, 1 g; MgSO₄·7H₂O, 0.2 g; FeCl₃·6H₂O, 0.04 g; MnSO₄·H₂O, 0.002 g; glucose, 0.5 g (added aseptically); and triple-distilled water to 1 liter (pH 7.0). Nutrient medium (NBSG) was prepared by adding 8 g of nutrient broth (Difco) and

5 g of glycerol (added aseptically) per liter of minimal medium with glucose omitted.

Transformants were scored on medium E or on minimal agar plates. Minimal agar plates consisted of the same minimal medium described above, except that 1 g of L-glutamic acid and 15 g of agar were added aseptically per liter of medium.

DNA. DNA was isolated by Marmur's (1961) procedure from lysozyme lysate of 10-hr-old cells grown in NBSG. The treatment with ribonuclease and the precipitation of DNA with isopropyl alcohol were omitted. The DNA was determined by the method of Burton (1956) and was stored in 2 M NaCl at 4 C.

Transformation methods. The mutants were tested for transforming ability at 37 C, by either the agar plate technique or by the tube transformation technique in liquid suspensions. Plate transformation consisted of spreading together 0.1 ml of cells (grown in NBSG medium for any desired length of time) and 0.1 ml of DNA (40 μ g of DNA per plate) on minimal or medium E agar plates. When the cells were diluted before plating, the diluent used was minimal medium with 10^{-3} M CaCl_2 added aseptically and glucose omitted. Control plates included one with cells alone and one with cells, DNA, and deoxyribonuclease (40 μ g of 1 \times crystallized product of Worthington Biochemical Corp., Freehold, N.J.). All the mutants included in these studies were very stable, and revertants rarely, if ever, occurred. The plates were incubated for 2 days. The tube transformation method in liquid suspensions was done in cotton-plugged test tubes (150 by 15 mm) in a final volume of 1 ml. Transformation medium (BL) consisted of minimal medium with 0.5% glucose, 0.01% acid-hydrolyzed casein (vitamin-free), 5 μ g of L-tryptophan per ml, 5×10^{-3} M MgSO_4 , 10^{-3} M CaCl_2 , 9×10^{-4} M MnSO_4 , and 0.2 M NaCl (pH 7.0). As a rule, 5 μ g of the nutritional requirement of the mutant being tested were added per ml of BL transformation medium. The cells were grown in NBSG for the desired length of time, washed twice, and diluted with BL transformation medium to the desired cell concentration. As a rule, 0.1 ml of cells (3 to 6×10^8 cells), 0.1 ml of DNA in 2 M NaCl (40 μ g), and 0.8 ml of BL transformation medium were placed in a test tube. The tubes were slanted on a rack and shaken on a reciprocating shaker for

3 to 4 hr at 37 C. The samples were exposed to 40 μ g of deoxyribonuclease per ml for 15 min, diluted in minimal medium with 10^{-3} M CaCl_2 added aseptically and glucose omitted, and plated on minimal medium or medium E plates.

RESULTS

Growth studies on the appearance of transformable cultures. We were unable to transform auxotrophs of *B. licheniformis* when we used the routine procedures described for *B. subtilis* strain 168 (Anagnostopoulos and Spizizen, 1961), for *Haemophilus influenzae* (Stuy, 1962; Goodgal and Herriott, 1961), and for *Diplococcus pneumoniae* (Lacks, 1962), although many modifications of these methods were tested. Penassay Broth, Tryptose Broth, and Brain Heart Infusion (Difco) were also tested for growth of transformable cells of the serine auxotroph, and no significant number of transformable cells was obtained. Efforts to develop a chemically defined medium for growing competent cells of various auxotrophs with either a spore or a cell inoculum were unsuccessful.

We then tested cell cultures of 14 mutants grown in NBSG, as described in Materials and Methods. The cells were tested directly by the plate transformation method at 2-hr intervals from 0 to 54 hr of growth. Transformation was obtained with only 4 of these 14 mutants at different periods of incubation. When varying concentrations of glycerol were tested, all 14 mutants were transformed to prototrophy (Table 1). The concentration of glycerol required, and the time of incubation in NBSG required for development of competence, may vary for each auxotroph. In all cases, addition of deoxyribonuclease to the plates resulted in complete loss of colonies.

Addition of 10^{-3} M CaCl_2 to NBSG medium resulted in a several-fold increase in the number of transformants of most auxotrophs tested. In all subsequent experiments, 10^{-3} M CaCl_2 was added aseptically to NBSG.

We then tested the effect of several other components of NBSG on the transformability of cells of a serine auxotroph, M5V-3 (Table 2). The cells were diluted to the desired cell concentration and tested by plate transformation. Although not shown in this table, the concentration of nutrient broth was also varied. When 0.4 and 0.6% nutrient broth were used, no significant trans-

TABLE 1. *Mutants of Bacillus licheniformis 9945A transformed to prototrophy**

Mutant	Requirement	Glycerol concn	Optimal time of incubation for competence	Plate transformants per 3×10^8 recipient cells†
		%	hr	
M1	Thiamine	0.30	14	34
M1R-1	Thiamine	0.30	13	91
M2R-1	Lysine	0.40	20	11
M4R-1	Arginine	0.40	18	174
M5	Serine	0.50	17	49
M5V-3	Serine	0.50	17	58
M8	Methionine	0.50	27	18
M8R-1	Methionine	0.35	30	52
M11	Methionine	0.50	22	60
M12	Unidentified	0.75	23	106
M14	Adenine	0.50	18	57
M17	Adenine	1.00	25	17
M18	Arginine	1.00	26	220
M19	Arginine	0.50	18	25

* Each of the mutants was tested for transformation on minimal agar plates with DNA isolated from wild-type *B. licheniformis*. Cultures of recipient cells were grown for the number of hours indicated in NBSG medium under the conditions described under Materials and Methods.

† Control plates without DNA or with DNA and deoxyribonuclease gave no colonies.

formation of various age cells was obtained with this serine auxotroph. This auxotroph transformed at a frequency of 0.01% by plate transformation (and 0.1 to 0.3% by tube transformation) when grown for 36 to 42 hr in NBSG with 0.8% nutrient broth, 1.5% glycerol, 0.3% Na citrate, and 0.2% $(\text{NH}_4)_2\text{SO}_4$. These concentrations of glycerol, Na citrate, and $(\text{NH}_4)_2\text{SO}_4$ were found to be optimal; varying the concentration of any of these resulted in a decrease in transformation frequency. The concentrations of glycerol used also affected the age and the length of time the cultures remained transformable. When glucose was substituted for glycerol in this medium, cells could not be transformed.

Similar studies were done with a nonencapsulated, thiamine-requiring mutant (M1R-1). These cells transformed at a frequency of 0.0001% when grown in specifically modified NBSG with 0.6 to 0.8% nutrient broth, 0.3% glycerol, 0.2% Na citrate, and 0.2% $(\text{NH}_4)_2\text{SO}_4$ by the plate or tube transformation method. Cell cultures

became competent after 14 hr of incubation and remained competent for about 1 hr. It was found later that cells of this thiamine-deficient auxotroph transformed at frequencies of 0.003% when grown in NBSG (as described above) with added 3×10^{-4} M MnSO_4 and 0.15 M NaCl. The transformation frequencies of M1R-1 were improved about 1,000-fold from those obtained with unmodified NBSG, but were still low compared to the serine auxotroph. It is significant to point out that a complex growth medium (NBSG) specifically modified for each mutant was the only successful means found that gave a significant number of transformable cells of the 14 auxotrophs studied.

Efforts to transfer the cells after various times of incubation from NBSG to other complex media or to chemically defined media for further development of competence failed.

In all cases, the transformable cultures were in the late stationary phase of growth, because all the mutants attained maximal growth in modified NBSG after 8 to 10 hr of incubation. Total growth in NBSG or modified NBSG was about

TABLE 2. *Effect of medium components on the competence of M5V-3 cells (serine⁻)**

Concn of medium components			Optimal time of incubation for competence	Plate transformants per 6×10^9 recipient cells†
Glycerol	Na citrate	$(\text{NH}_4)_2\text{SO}_4$		
%	%	%	hr	
0.2	0.1	0.2	—	0
0.5	0.1	0.2	15	2.2×10^1
1.0	0.1	0.2	32	3.8×10^2
1.25	0.1	0.2	32-34	6.9×10^2
1.5	0.1	0.2	32-42	3.4×10^3
2.0	0.1	0.2	38-48	1.4×10^3
1.5	0.2	0.2	36-42	4.5×10^3
1.5	0.3	0.2	36-42	6.7×10^3
1.5	0.5	0.2	36-42	1.2×10^4
1.5	0.3	0	36-42	3.0×10^2
1.5	0.3	0.1	36-47	1.3×10^3
1.5	0.3	0.3	36-40	6.2×10^3
1.5	0.3	0.5	32-39	1.0×10^4

* Other medium components at regular concentration in NBSG with 10^{-3} M CaCl_2 were added aseptically. The recipient cells were diluted with minimal medium (10^{-3} M CaCl_2 added aseptically), and varying cell concentrations were tested by plate transformation.

† Control plates without DNA or with DNA and deoxyribonuclease gave no colonies.

3 to 6×10^9 cells per ml, and was maintained for over 48 hr. All of the mutants gave less than 1% sporulation in this medium.

Transformation in liquid suspensions. Several media were tested for optimal tube transformation. Poor results were obtained with the transformation medium described by Anagnostopoulos and Spizizen (1961). A modification of this transformation medium, designated here as BL transformation medium, supported transformation of the auxotrophs tested. Competent cells of serine-requiring and thiamine-requiring auxotrophs required NaCl and two divalent cations for optimal transformation in BL medium. Table 3 shows some of these results with a serine-deficient auxotroph. Transformation frequencies of 0.11% were obtained with this auxotroph when 0.1 to 0.4 M NaCl was added. Occasionally, transformation frequencies of 0.3% were obtained. A decrease or increase from these NaCl concentrations resulted in poor transformation. The two-cations requirement was satisfied by a combination of Mn^{++} and Mg^{++} or Mn^{++} and Ca^{++} , at the concentrations given in Table 3. A combination of Mg^{++} and Ca^{++} , without Mn^{++} , was not as satisfactory. A combination of all three cations gave more consistent results with the mutants tested, and therefore all three cations in addition to 0.2 M NaCl were used routinely in BL transformation medium. Similar requirements were found for tube transformation of the thiamine-deficient auxotroph (M1R-1). This auxotroph transformed at frequencies of 0.003% by tube transformation.

Tube transformation was relatively low during the first hour of incubation in BL transformation medium, but transformation was optimal after 3 to 4 hr of incubation. No significant growth of the cells occurred in this medium during the 4-hr incubation period.

The optimal incubation time, and metal ion concentration in BL transformation medium, may vary with each mutant. Optimal conditions for growth and tube transformation of the other auxotrophs shown in Table 1 are under study.

Transformation of the ability to synthesize polyglutamic acid (capsular material). Because no specific method is available for selecting encapsulated transformants from the nonencapsulated recipients, only double transformants to prototrophy and encapsulation can be scored. Therefore, high frequencies of transformation

TABLE 3. *Effect of metal cations on transformation of M5V-3 (serine⁻) in liquid suspensions**

Metal cations studied in BL medium				Transformants per 6×10^8 recipient cells†
NaCl (2×10^{-1} M)	MnSO ₄ (9×10^{-4} M)	CaCl ₂ (10^{-3} M)	MgSO ₄ (5×10^{-3} M)	
+	+	+	+	5.8×10^5
+	+	+	-	6.7×10^5
+	+	-	+	5.2×10^5
+	+	-	-	2.7×10^5
+	-	+	+	1.7×10^5
+	-	+	-	2.5×10^4
+	-	-	+	1.1×10^5
+	-	-	-	5.0×10^1
-	+	+	+	2.5×10^4

* M5V-3 (serine⁻) cells were grown for 39 hr in modified NBSG. The cells were washed twice and tested by tube transformation as described in the text. The tubes were shaken for 4 hr, and the transformants were scored on minimal agar plates, with 50 μ g of deoxyribonuclease added.

† Control samples without DNA or with DNA and deoxyribonuclease gave no colonies.

with nonencapsulated auxotrophs are required. As the transformation frequency to prototrophy was increased, the number of double transformants to prototrophy and capsule⁺ was also increased. A double mutant thiamine⁻, capsule⁻ (M1R-1) was used in most of these studies. Double transformants, thiamine⁺, capsule⁺, are easily distinguished from single transformants, thiamine⁺, capsule⁻, when plated on minimal agar plates (Fig. 1).

Table 4 shows transformation of this mutant by use of an excess of DNA isolated from several sources. When wild-type 9945A DNA or DNA extracted from a double transformant (thiamine⁺, capsule⁺) was used, about 5 to 20% of the total transformants to prototrophy were also encapsulated (i.e., were able to synthesize polyglutamic acid). When DNA extracted from a nonencapsulated transformant to prototrophy (thiamine⁺, capsule⁻) was used, all the prototrophs were capsule⁻. DNA extracted from some other capsule⁻ mutants of *B. licheniformis* transformed this mutant to capsule⁺, whereas others did not. This indicated that some of our nonencapsulated mutants mutated at different points in the chromosome. Homologous DNA gave no transformation. Also, addition of

deoxyribonuclease to the samples resulted in total loss of transformants.

The ability to synthesize capsular material was also transformed to an arginine⁻, capsule⁻ mutant and to a methionine⁻, capsule⁻ mutant. Other nonencapsulated mutants are under investigation.

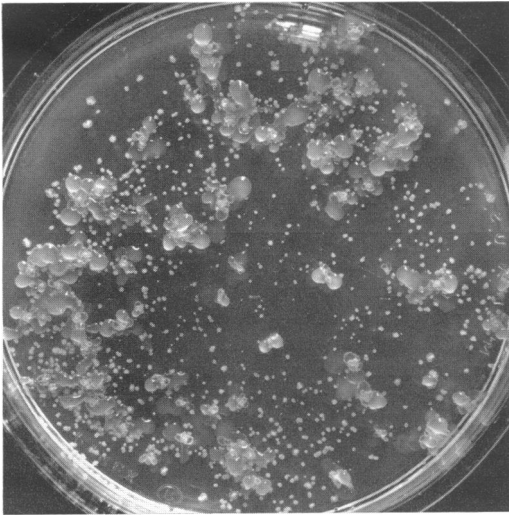


FIG. 1. Encapsulated and nonencapsulated transformants of *Bacillus licheniformis* M1R-1 (thiamine⁻, capsule⁻) on minimal agar plates.

TABLE 4. Transformation of the ability to synthesize polyglutamic acid (capsular material) to M1R-1 (thiamine⁻, capsule⁻)*

DNA source	Single transformants to thiamine ⁺	Double transformants to thiamine ⁺ , capsule ⁺
Wild-type 9945A.....	502	18
M1R-1 (thiamine ⁺ , capsule ⁺).....	480	23
M1R-1 (thiamine ⁺ , capsule ⁻).....	463	0
M1R-1 (thiamine ⁻ , capsule ⁻).....	0	0
9945A-1 (capsule ⁻).....	280	0
9945A-4 (capsule ⁻).....	310	14

* M1R-1 (thiamine⁻, capsule⁻) cells were grown in NBSG medium with 0.3% glycerol, 0.2% Na citrate, and 10⁻³ M CaCl₂ for 14.5 hr; 3 × 10⁸ cells were tested for transformation on minimal agar plates with 50 μg of the desired DNA per plate. Control plates without DNA or with DNA and deoxyribonuclease gave no colonies.

DISCUSSION

We were unable to transform auxotrophs of *B. licheniformis* when we used routine procedures described for *B. subtilis* 168 (Anagnostopoulos and Spizizen, 1961), for *H. influenzae* (Stuy, 1962; Goodgal and Herriott, 1961), and for *D. pneumoniae* (Lacks, 1962), although many modifications of these methods were tested. It was an unexpected finding that the use of a complex medium (NBSG) specifically modified for each auxotroph tested was the only successful means found that gave significant numbers of transformable cells of all the 14 auxotrophs studied.

These special growth media may be necessary for getting each mutant to a specific physiological state of growth. The requirement of a specifically modified medium for optimal transformation of each auxotroph studied may account for the different transformation frequencies found among different auxotrophs of a given strain of *B. subtilis* and other organisms (as well as those of different isolates of the same auxotroph), when the different auxotrophs of a given organism are tested under the same set of conditions.

A medium for transformation of *B. licheniformis* cells in liquid suspensions is described. Competent cells of several auxotrophs of *B. licheniformis* required NaCl, Mn⁺⁺, and either Ca⁺⁺ or Mg⁺⁺ for optimal transformation. The metal requirements for optimal transformation may vary with each auxotroph of *B. licheniformis*. The possible roles of these metals on DNA uptake and integration in *B. licheniformis* are not known. No attempts were made in these studies to determine their possible roles. The importance of metals and ionic strength in transformation of *B. subtilis* (Young and Spizizen, 1963) and in transformation of *H. influenzae* (Barnhart and Herriott, 1963).

This report also presents evidence for the transformation of the ability to synthesize capsular material, namely, polyglutamic acid in three nonencapsulated mutants of *B. licheniformis*. Because no specific method was available for selecting encapsulated transformants from the nonencapsulated recipients, only double transformants to prototrophy and encapsulation were scored. When an excess of DNA was used, about 20% of the transformants were also cotrans-

formed for the ability to synthesize polyglutamic acid (capsular material). Under similar conditions of growth, the polyglutamic acid synthesized by the transformants to encapsulation was similar both qualitatively and quantitatively to that synthesized by wild-type *B. licheniformis*.

We have several different nonencapsulated mutants, and we plan to do genetic and biochemical studies with these mutants. These studies, in addition to our work on polyglutamic acid synthesis by cell-free extracts of *B. licheniformis*, may help to elucidate the mechanism of polyglutamic acid synthesis (Leonard and Housewright, 1963).

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