A Rapid Method for Constructing Multiply Marked Strains of *Bacillus subtilis*

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(Received 20 January 1978)

INTRODUCTION

Strains carrying multiple mutations greatly aid genetic analysis. A problem in *Bacillus subtilis* genetics has been the difficulty of constructing such multiply marked strains. Most commonly, non-selective markers are introduced by transformation with high concentrations of DNA. Under these circumstances unlinked and unselected donor markers are transferred to the selected transformants at low frequency (this phenomenon has been termed congression; Nester, Schafer & Lederberg, 1963). Any one unselected marker may be present in up to 5 % of the selected transformants. In this way different markers can be introduced into the recipient strain. The limitation is that the selective character is lost, preventing repetition of the process. Takahashi & Ikeda (1968) have described a way to overcome this limitation by alternatively selecting and screening for *ery^Rstr^S* and *ery^Sstr^R* transformants (the *str* and *ery* loci are linked by transformation). These particular markers are somewhat awkward to work with and are difficult to remove from the final strain. The method could presumably be employed with other pairs of linked markers in repulsion crosses, but still has the disadvantage of having to construct the initial donor strains. The method has not been widely used. We describe here an alternative procedure which offers a considerable advantage as special donor strains need not be constructed.

We have been studying the relationship of competence to sporulation in *B. subtilis*. We have found that it is possible to transform asporogenous mutants in a sporulation medium (that of Ramaley & Burden, 1970) so that they are able to form heat-resistant spores. This confirms the finding by Erickson & Young (unpublished results quoted by Young & Wilson, 1972) of transformation in this system. For certain mutants, some of the transformed recipients form heat-resistant spores (selected by heating to 85 °C for 20 min) and yet the colonies that grow up from these spores are genetically asporogenous. The reason for the anomalous result is being investigated. It is thought possible that the chromosome of the mother cell, but not of the forespore, is transformed and that this is sufficient for the forespore to develop into a heat-resistant spore. Whatever the explanation, the surviving asporogenous bacteria can be subjected to repeated cycles of this transformation/sporulation/heat treatment procedure. Using high concentrations of DNA, it is possible to introduce a series of unselected markers into the recipient by such repeated cycles.

METHODS

Strains. The bacterial strains used were all derivatives of *Bacillus subtilis* 168. They were: 34.1, *spoOA34 phe-12*; 91, *spoVB91 trpC2* (Hranueli, Piggot & Mandelstam, 1974; Piggot & Coote, 1976); MB8, *hisA1* (Piggot, 1973); M22, *purA16 leu-8 metB5 ile-1*; BD68, *argC4 ura-26 leu-2* (Karamata & Gross, 1970). Strains were stored as freeze-dried cultures. The phenotypes corresponding to the different symbols are: unable to

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sporulate, *spo*; requirement for arginine, *arg*; for histidine, *his*; for isoleucine, *ile*; for leucine, *leu*; for methionine, *met*; for phenylalanine, *phe*; for purine, *pur*; for tryptophan, *trp*; for uracil, *ura*.

Sporulation/transformation system. The system was based on that described by Ramaley & Burden (1970) for replacement sporulation. The media contained, in 1 litre final volume, adjusted to pH 7.2: 0.33 ml 85 % H_3PO_4 ; 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.4 g NaCl; 0.4 g KCl; 25 mg $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$; 10 mg CaCl_2 ; 6 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$. In addition the growth medium contained 20 mM-glucose and 50 mM-glutamate, while the sporulation medium contained 10 mM-glutamate. Solutions of MgSO_4 , CaCl_2 , glucose and glutamate were sterilized separately and added to a sterile solution of the other salts shortly before the medium was required. Where necessary, media were supplemented with: adenine sulphate, $20 \mu\text{g ml}^{-1}$; uracil, $20 \mu\text{g ml}^{-1}$; phenylalanine, $100 \mu\text{g ml}^{-1}$; tryptophan, $10 \mu\text{g (ml growth medium)}^{-1}$ or $1 \mu\text{g (ml sporulation medium)}^{-1}$; other amino acids, $100 \mu\text{g (ml growth medium)}^{-1}$ or $10 \mu\text{g (ml sporulation medium)}^{-1}$. The necessity for these latter shifts down was not established.

Bacteria were grown in growth medium at 37°C to a bacterial density of approximately $0.10 \text{ mg dry wt ml}^{-1}$. The bacteria were harvested by centrifugation and resuspended in an equal volume of prewarmed sporulation medium and again incubated at 37°C with vigorous aeration. DNA (prepared by the method of Marmur, 1961) was added at the time of resuspension to a concentration of $5 \mu\text{g ml}^{-1}$. After 16 to 20 h in the sporulation medium, cultures were heated to 85°C for 20 min in order to select heat-resistant spores, and survivors were scored by incubating samples on nutrient agar (Difco) plates for 48 h at 42°C . At this time, Spo^+ and Spo^- colonies were distinguishable by morphology and pigmentation; this distinction was confirmed by examination with a phase-contrast microscope. Growth requirements of transformants were tested by picking colonies and streaking on to minimal agar (Piggot, 1973) with appropriate supplements. Transformants that had acquired a new auxotrophic requirement were tested in detail.

RESULTS AND DISCUSSION

Bacillus subtilis mutant 91 (*spoVB91 trpC2*) was transformed with *spoVB*⁺ *phe-12* DNA (from strain 34.1), the culture was given the opportunity to sporulate then heat-treated, and survivors were identified as described in Methods. Approximately equal numbers of Spo^+ and Spo^- colonies were obtained (Table 1). No survivors of either type were obtained when the DNA had previously been degraded by DNAase, when DNA carrying the *spoVB91* mutation was used, or when no DNA was used. Of 88 Spo^- transformants tested, 10 were found to have become phenylalanine auxotrophs. One of these, SL11, was used to build up a series of multiply marked auxotrophs (Table 1). In this way, in four successive transformations up to six distinct auxotrophic mutations were introduced into strain 91 without loss of the original *spoVB91* and *trpC2* mutations.

Should a Spo^+ strain be required, the *spoVB91* mutation can be readily removed by an additional transformation. Alternatively, in a series of crosses building up a particular strain, the Spo^+ transformant could be tested for the appropriate auxotrophic requirements at the final stage.

A feature of the system was the high frequency of congression. For example, when SL20 was transformed by DNA from M22, 64 out of 216 transformants tested gained at least one auxotrophic requirement. Amongst these, 24 gained *purA16*, 28 *metB5*, 22 *leu-8* and 16 *ile-1* indicating a congression frequency of approximately 10 % for each marker. (No requirements were gained that had not been present in the donor.) The high frequency of congression necessarily meant that many of the transformants which gained donor markers also lost recipient markers; such transformants are not listed in Table 1.

Herminia de Lencastre was supported in part by a fellowship from the Instituto Gulbenkian de Ciênciã.

Table 1. Transformation of *B. subtilis* *spoVB91* derivatives

Recipient strain	Donor strain	No. of colonies resulting from incubation on nutrient agar of 1 ml heat-shocked, transformed culture		No. of Spo ⁻ transformants tested for introduction of donor mutations	Constructed strains that gained donor mutations and lost no recipient mutations		
		Spo ⁺	Spo ⁻		No. found	Genotype (all strains have <i>spoVB91 trpC2</i>)	Name
91	34.1 (<i>phe-12</i>)	2620	2705	88	10	<i>phe-12</i>	SL11
91	—	0	0				
SL11	MB8 (<i>hisA1</i>)	1120	1175	NR	NR	<i>phe-12 hisA1</i>	SL20
SL11	—	0	0				
SL20	M22 (<i>purA16 leu-8 metB5 ile-1</i>)	415	206	216	8	<i>phe-12 hisA1 purA16</i>	SL24
					4	<i>phe-12 hisA1 metB5</i>	SL25
					9	<i>phe-12 hisA1 leu-8</i>	SL26
					2	<i>phe-12 hisA1 purA16 metB5</i>	SL27
					2	<i>phe-12 hisA1 metB5 leu-8</i>	SL28
					2	<i>phe-12 hisA1 purA16 ile-1</i>	SL29
					4	<i>phe-12 hisA1 metB5 ile-1</i>	SL30
					2	<i>phe-12 hisA1 purA16 metB5 ile-1</i>	SL31
SL20	—	0	2				
SL27	BD68 (<i>argC4 ura-26 leu-2</i>)	485	425	224	5	<i>phe-12 hisA1 purA16 metB5 argC4</i>	SL32
					4	<i>phe-12 hisA1 purA16 metB5 ura-26</i>	SL33
					1	<i>phe-12 hisA1 purA16 metB5 leu-2</i>	SL34
SL27	—	0	0		3	<i>phe-12 hisA1 purA16 metB5 argC4 ura-26</i>	SL35
SL28	M22	1510	1285	308	5	<i>phe-12 hisA1 metB5 leu-8 ile-1</i>	SL36
					3	<i>phe-12 hisA1 metB5 leu-8 purA16 ile-1</i>	SL37
SL28	—	0	0		12	<i>phe-12 hisA1 metB5 leu-8 purA16</i>	SL38

NR, Not recorded.

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