Control of Sporulation in Fused Protoplasts of *Bacillus subtilis* 168

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Complementable *spo* mutants of *Bacillus subtilis* could be 'rescued' to form heat-resistant spores by fusing them with sporulating cells of the wild-type strain (Dancer & Mandelstam, 1981). The latter strain acted as a 'rescuer'. Rescuer protoplasts had to be derived from cells grown in a medium that allowed rapid and synchronous sporulation subsequently. Even protoplasts made from vegetative cells growing in such a medium (t_0 protoplasts) acted as rescuers. Therefore, it was not necessary for the rescuer cells to have initiated sporulation before they were converted to protoplasts.

The genetic requirement for t_0 rescuer protoplasts was that they were *spo*⁺ for the gene to be complemented. Strains in the same complementation group did not rescue one another. Protoplasts of *spo* strains blocked at stages O, IV and V of sporulation acted equally well as rescuers whether they were converted to protoplasts at t_0 or t_3 (i.e. 3 h after transfer to sporulation medium). However, t_3 protoplasts of stage II and stage III mutants were much less effective rescuers than their t_0 counterparts. This difference was not due to a dominance effect expressed only in t_3 protoplasts.

INTRODUCTION

Protoplasts of bacilli are able to complete sporulation if they are made from cells that have reached stage III of sporulation (completion of prespore engulfment) or beyond (Fitz-James, 1964). *Bacillus subtilis* protoplasts containing forespores complete sporulation even when subjected to a fusion procedure involving treatment with 40 % (w/v) polyethyleneglycol 6000 (Dancer & Mandelstam, 1981). This has been used to establish complementation relationships between asporogenous strains blocked at late stages of sporulation (stages III, IV and V), i.e. the defective forespores of some strains can be 'rescued' to form heat-resistant spores by fusion with sporogenous strains or with asporogenous strains in other complementation groups.

Having established that complementation by fusion was possible and that complementation groups could be distinguished, it was of interest to investigate the conditions for obtaining rescue of complementable strains and, in particular, the requirements for the 'rescuer' strain to act best in fusions. The experiments described in this paper show that t_3 protoplasts of complementable strains can be rescued by t_0 protoplasts of strains in any other complementation group provided that growth took place in a sugar-free medium. Rescue was also effective when t_3 protoplasts of the *spo*⁺, *spoO*, *spoIV* and *spoV* strains were used as rescuers but was poor when *spoII* or *spoIII* strains were used.

METHODS

Organisms. The strains of *Bacillus subtilis* used are listed in Table 1. Strain 168 is the wild-type strain from which all of the others are derived. Strains 168 and MY2016 sporulate equally well in the presence of their respective auxotrophic requirements and either was used, as convenient, as a *spo*⁺ parent in fusion experiments. The nomenclature for *spo* mutants is that of Piggot & Coote (1976).

Table 1. *Strains of Bacillus subtilis* used

Strain no.	<i>spo</i> allele isolation no.*	Genotype	Complementability†	Source
168	—	<i>trpC2</i>	—	Stock
MY2016	—	<i>hisA lys rif tal sul</i>	—	Stock
34.3	34(3F1)	<i>metC3 spoOA34</i>	—	Stock
485	14UL	<i>trpC2 spoOG14</i>	—	P. J. Piggot
17	E22	<i>trpC2 spoOH17</i>	—	Stock
69.2	NG18.6	<i>metC3 spoIIA69</i>	—	Stock
20	N25	<i>trpC2 spoIIE20</i>	—	Stock
55.1	NG12.12	<i>leu spoIIG55</i>	—	Stock
59.2	NG14.7	<i>metC3 spoIIIA59</i>	NC	Stock
2	A3	<i>trpC2 spoIIIB2</i>	NC	Stock
IS39	94U	<i>trpC2 spoIIID94</i>	NC	BGSC‡
36.1	NG1.67	<i>metC3 spoIIIE36</i>	NC	Stock
133.1	Z7	<i>trpC2 spoIVC133</i>	C	Stock
133.2	Z7	<i>phe spoIVC133</i>	C	Stock
149.3	X2	<i>hisA spoIVC149</i>	C	Stock
23.1	E31	<i>trpC2 rif spoIVC23</i>	C	Stock
92.1	92	<i>metC3 spoIVD92</i>	C	Stock
IS40	11T	<i>trpC2 spoIVE11</i>	C	BGSC‡
88	88	<i>trpC2 metC3 spoIVF88</i>	C	Stock
5	A8	<i>trpC2 spoIVG5</i>	C	Stock
156.2	W10	<i>trpC2 spoVD156</i>	NC	Stock
224	DG47	<i>trpC2 spoVF224</i>	C	Stock
224.1	DG47	<i>trpC2 rif spoVF224</i>	C	Stock

* See Piggot & Coote (1976).

† C, Complementable by fusion; NC, not complementable by fusion (Dancer & Mandelstam, 1981). —, Not applicable in this system.

‡ Bacillus Genetic Stock Center, University of Ohio, U.S.A.

Growth and sporulation. To obtain sporulating cultures, cells were grown in a hydrolysed casein medium (CH) at 37 °C to a density of about 0.25 mg dry wt ml⁻¹ and transferred to a sporulation medium containing glutamate, inorganic salts and any auxotrophic requirements (Sterlini & Mandelstam, 1969). Tryptophan was supplied at 20 µg ml⁻¹ and other amino acids at 100 µg ml⁻¹. With the wild-type strain, continued agitation at 37 °C results in the formation of fully heat-resistant spores after 8–10 h. In some experiments cells were grown either in the casein hydrolysate medium supplemented with 0.2% (w/v) sucrose (CHS) or in brain heart infusion broth (BHIB; Oxoid). Cell density was measured turbidimetrically at 600 nm with a Unicam SP600 spectrophotometer and was related to dry weight by a standard curve. Times (h) after resuspension are referred to as *t*₀, *t*₁, etc., and the stages of sporulation are those suggested by Ryter (1965) except that stages O and I are not distinguished.

Nomenclature. For clarity, there is a need to define the terms used to describe strains involved in protoplast fusions. When two parent protoplast preparations are mixed and treated with polyethyleneglycol (PEG), some individual protoplasts fuse with one another and thus give rise to 'homokaryons' (where fusion is between individuals of one strain) and 'heterokaryons' (where fusion is between individuals of both strains). PEG treatment of the parent strains individually (i.e. 'fusing a strain with itself') serves as a control as only homokaryons can be generated. In the experiments described here, there was always at least one culture that was converted to protoplasts at *t*₃ to be tested for complementation by recovery of genetically *spo*, heat-resistant spores at *t*₁₀. This strain is referred to as the 'complemented' strain. The strain used to provide the missing component(s) and which itself may or may not be able to sporulate is referred to as the 'rescuer'. In some experiments reciprocal rescue was possible so that each strain was both a rescuer and was complemented. When vegetative cells were used as a source of rescuers the protoplasts were prepared, as usual, in the sporulation medium supplemented with 20% (w/v) sucrose and 2 mg lysozyme ml⁻¹; these protoplasts were thus, formally, prepared at *t*₀ of sporulation.

Protoplast formation and fusion. Usually cells were converted to protoplasts and fused as described previously (Dancer & Mandelstam, 1981). Briefly, cells from two 30 ml cultures each at *t*₃ in sporulation medium were concentrated tenfold and converted to protoplasts. The two preparations were mixed, centrifuged and the pellets were treated with 40% (w/v) polyethylene glycol (PEG) 6000 to promote fusion. After 1–2 min incubation the PEG mixture was diluted sixfold with protoplasting medium, agitation at 37 °C was continued until *t*₁₀, and then samples were taken for determination of heat resistance.

In some experiments the method was modified in the following ways. (a) Cells grown in CHS or BHIB medium required 30–40 min to complete protoplast formation instead of the usual 10–15 min. (b) In fusions of *t*₃

protoplasts with t_0 protoplasts, the two parent cultures were prepared in such a way that when the complementable strain was at t_3 the rescuer strain had reached 0.25–0.30 mg dry wt ml⁻¹. (c) Protoplasts from a 150 ml culture were fused with protoplasts from a 30 ml culture. The protoplasts were prepared as described above and mixed; the resulting 18 ml preparation was centrifuged and the pellet was taken up in 1 ml PEG solution as usual. Subsequent steps were the same as when 6 ml protoplast preparations were used, but allowance was made for different protoplast densities in the calculations of viable counts.

All t_3 protoplast preparations were derived from CH-grown cells; t_0 protoplasts were from CH-grown cells unless otherwise stated.

Resistance determinations. Heat resistance was determined by heating preparations in a water bath at 80 °C for 40 min and plating serial dilutions on selective minimal medium (Dancer & Mandelstam, 1981). For determination of toluene resistance, 1 ml portions of culture were emulsified with 0.05 ml toluene for 1 min. The toluene was then blown off with sterile compressed air and the culture was plated as above.

RESULTS

*Rescue of complementable *spo* strains by t_0 and t_3 protoplasts of *spo*⁺ cells*

Cells of complementable *spo* strains were converted to protoplasts at t_3 and fused with t_0 or t_3 protoplasts of strain MY2016 (*spo*⁺) that had been grown in CH medium. The subsequent yields of heat-resistant spores of the complemented strains were at least 3×10^2 -fold more than those obtained when the *spo* protoplasts were fused with themselves (Table 2). In fact, the yield of spores of the complementable strain was about the same whether rescue had been effected by t_0 or t_3 protoplasts of the rescuer strain. Complementation of strain 133.2 was about the same whether strain 168 or strain MY2016 was used, i.e. rescue by t_0 protoplasts was not a peculiarity of the *spo*⁺ rescuer used. Strains carrying complementable *spoIV* or *spoV* mutations were rescued by the t_0 protoplasts (Table 2) whereas *spoIII* and *spoV* mutants that were not rescued by t_3 protoplasts of *spo*⁺ strains (Dancer & Mandelstam, 1981) were also not rescued by t_0 protoplasts (data not shown).

*Effect of growth medium on the capacity of t_0 protoplasts of *spo*⁺ cells to act as rescuers*

Cells that have been grown in CH medium have a high potential for rapid and relatively synchronous sporulation (Sterlini & Mandelstam, 1969). If they have been grown in media containing sugars, sporulation takes much longer to begin and is less synchronous. The implication is that cytoplasmic metabolites are present that inhibit the induction of sporulation. It was therefore of interest to see whether t_0 protoplasts of cells grown in rich media were as effective in rescuing complementable strains as were protoplasts of cells grown in CH medium.

Cells of the *spo*⁺ strains were grown in BHIB, CHS or CH medium, converted to protoplasts at t_0 and fused with t_3 protoplasts of complementable strains (133.2, *spoIVC*; 88, *spoIVF*; 224, *spoVF*). No heat-resistant spores of the complementable strain were obtained after fusion with rescuers derived from BHIB or CHS cultures, whereas when the rescuer was obtained from a CH culture, the yields of complemented, heat-resistant spores were about 10^4 ml⁻¹ (Table 2). The failure of t_0 protoplasts from BHIB and CHS cultures to rescue complementable strains is presumably due to some biochemical inhibition of *spo* genes by a cytoplasmic constituent of the t_0 protoplasts. If this is true, sporulation in wild-type (t_3) protoplasts ought to be reduced by fusing them with protoplasts of BHIB- or CHS-grown cells. This was confirmed in experiments in which t_3 protoplasts of strain MY2016 that had been grown in CH medium were fused with t_0 protoplasts of strain 168 that had been grown in BHIB, CHS or CH medium. In order to decrease the number of homokaryons and unfused protoplasts of strain MY2016 the proportions of the parents in the fusion mixture were changed. A ratio of five protoplasts of strain 168 to one of strain MY2016 was used (see Methods). When the t_0 protoplasts were from CH-grown cells the yield of spores of strain MY2016 was about 5×10^6 ml⁻¹. This is about 20-fold less than the number obtained when t_3 protoplasts were fused with themselves. This result is attributable to a dilution effect since a

Table 2. *Rescue of complementable spo strains by fusion with either t_0 or t_3 protoplasts of spo^+ cells*

Complementable *spo* strains were grown in CH medium and transferred to sporulation medium. At t_3 the cells were converted to protoplasts and mixed with an equal number of either t_0 or t_3 protoplasts of strain MY2016 prepared in parallel. These preparations were treated with 40% (w/v) PEG to promote protoplast fusion, after which the PEG was diluted out and agitation at 37 °C was continued. At t_{10} samples were heat-treated and plated with selection for each participating strain. Each strain was fused with itself as a control. The yield of spores of strain MY2016, whether fused alone or mixed with protoplasts of a complementable strain, was the same. For t_3 protoplasts of strain MY2016 the yield of heat-resistant spores at t_{10} was about $5 \times 10^7 \text{ ml}^{-1}$ and for t_0 protoplasts the yield was about $1 \times 10^2 \text{ ml}^{-1}$.

<i>spo</i> strain (genotype)	Time of <i>spo</i> ⁺ protoplast formation	Spore yields of <i>spo</i> strain (ml^{-1})		Rescue factor (b/a)
		(a) <i>spo</i> strain fused alone	(b) <i>spo</i> and <i>spo</i> ⁺ strains fused together	
133.2 (<i>phe spoIVC133</i>)	t_0	<5	3×10^3	$>6 \times 10^2$
133.2 (<i>phe spoIVC133</i>)	t_3	<5	1×10^4	$>2 \times 10^3$
5 (<i>trpC2 spoIVG5</i>)	t_0	<5	2×10^4	$>4 \times 10^3$
5 (<i>trpC2 spoIVG5</i>)	t_3	<5	1×10^4	$>2 \times 10^3$
88 (<i>trpC2 metC3 spoIVF88</i>)	t_0	<5	4×10^3	$>8 \times 10^2$
88 (<i>trpC2 metC3 spoIVF88</i>)	t_3	<5	4×10^3	$>8 \times 10^2$
IS40 (<i>trpC2 spoIVE11</i>)	t_0	<5	3×10^4	$>6 \times 10^3$
IS40 (<i>trpC2 spoIVE11</i>)	t_3	<5	6×10^3	$>1 \times 10^3$
92.1 (<i>metC3 spoIVD92</i>)	t_0	2×10^2	8×10^4	4×10^2
92.1 (<i>metC3 spoIVD92</i>)	t_3	2×10^2	6×10^4	3×10^2
224 (<i>trpC2 spoV224</i>)	t_0	1×10^2	5×10^4	5×10^2
224 (<i>trpC2 spoV224</i>)	t_3	1×10^2	3×10^4	3×10^2

similar reduction in spore yield was observed when t_0 and t_3 preparations of protoplasts of strain MY2016 were fused together. By contrast, when the t_0 protoplasts of strain 168 were from BHIB or CHS cultures, the yield of heat-resistant spores of strain MY2016 was reduced to 10^3 ml^{-1} , i.e. by about five orders of magnitude. Thus, protoplasts from cells grown in medium containing sugar are not merely ineffective in the rescue of *spo* protoplasts but actively inhibit sporulation in protoplasts of *spo*⁺ strains.

Rescue of complementable strains by t_0 protoplasts of asporogenous mutants grown in CH medium

The fact that protoplasts of t_0 wild-type cells are effective rescuers suggested that the sporulation genes of the *spo*⁺ chromosome could be 'switched-on' by the cytoplasm of the t_3 protoplasts without undergoing a dependent sequence of expression. If this were so, rescue by stage O and stage II mutants should occur. When the complementable strain 133.2 (*spoIVC*) was used in fusion with t_0 asporogenous rescuers, it was rescued by the three *spoO* strains and the three *spoII* strains that were tested. The yields were about the same as those obtained when a *spo*⁺ strain was the t_0 rescuer (Table 3*a*). Thus, strains that become blocked in sporulation before stage III (completion of engulfment) complemented genes functionally expressed after stage III.

Similar experiments to those described above were then done in which the t_0 protoplasts were obtained from CH cultures of late-blocked mutants (*spoIII*, *spoIV* and *spoV*). As expected, no rescue was obtained when the t_0 rescuer was a strain carrying the same *spo* mutation (strain 133.1) or a mutation in the same complementation group (strain 149.3; Dancer & Mandelstam, 1981) (Table 3*a*). However, in every other case tested the t_0

Table 3. Rescue of t_3 protoplasts of complementable strains by fusion with either t_0 or t_3 protoplasts of asporogenous rescuer strains

t_3 protoplasts of complementable strains were fused with either t_0 or t_3 protoplasts of rescuer strains by the procedure described in Table 2. The yields of heat-resistant spores at t_{10} were determined. Strain MY2016 (included as a control) yielded 3×10^2 spores ml^{-1} when fused with itself at t_0 . With every other rescuer <5 spores ml^{-1} were obtained when each was fused with itself and no enhanced yield was obtained in the complementation tests (except for strain 88, see footnote). The complementable strains fused with themselves yielded spores at t_{10} as follows: strain 133.2, $<5 \text{ ml}^{-1}$; strain 88, $<5 \text{ ml}^{-1}$; strain 224, $1 \times 10^2 \text{ ml}^{-1}$.

(a) Rescuer strains at t_0			(b) Rescuer strains at t_3			
Complementable strain (<i>spo</i> genotype)	Rescuer strain (<i>spo</i> genotype)	Yield of spores of complementable strain ml ⁻¹	Complementable strain (<i>spo</i> genotype)	Rescuer strain (<i>spo</i> genotype)	Yield of spores of complementable strain ml ⁻¹	
133.2 (<i>spoIVC</i>)	34.3 (<i>spoOA</i>)	3.5×10^4	133.2 (<i>spoIVC</i>)	34.3 (<i>spoOA</i>)	3.5×10^4	
	485 (<i>spoOG</i>)	1×10^4		485 (<i>spoOG</i>)	1.5×10^4	
	17 (<i>spoOH</i>)	5×10^3		17 (<i>spoOH</i>)	6×10^3	
	69.2 (<i>spoIIA</i>)	1×10^4		69.2 (<i>spoIIA</i>)	0*	
	20 (<i>spoIIE</i>)	2×10^3		20 (<i>spoIIE</i>)	0*	
	55.1 (<i>spoIIG</i>)	1.5×10^4		55.1 (<i>spoIIG</i>)	0	
	59.2 (<i>spoIIIA</i>)	1.5×10^4		59.2 (<i>spoIIIA</i>)	0*	
	2 (<i>spoIIIB</i>)	2×10^4		IS39 (<i>spoIIID</i>)	0	
	IS39 (<i>spoIIID</i>)	3×10^3		88 (<i>spoIVF</i>)	2×10^3 †	
	36.1 (<i>spoIIIE</i>)	7×10^3		156.2 (<i>spoVD</i>)	4×10^3	
	133.1 (<i>spoIVC</i>)	<5		224.1 (<i>spoVF</i>)	485 (<i>spoOG</i>)	1×10^4
	149.3 (<i>spoIVC</i>)	<5			55.1 (<i>spoIIG</i>)	5×10^1
	23.1 (<i>spoIVC</i>)	4×10^3			IS39 (<i>spoIIID</i>)	5×10^1
	88 (<i>spoIVF</i>)	5×10^3				
	156.2 (<i>spoVD</i>)	3×10^3				
	MY2016 (<i>spo</i> ⁺)	1×10^4				
88 (<i>spoIVF</i>)	485 (<i>spoOG</i>)	5×10^3				
	55.1 (<i>spoIIG</i>)	2×10^3				
	IS39 (<i>spoIIID</i>)	2×10^3				
	MY2016 (<i>spo</i> ⁺)	5×10^3				
224 (<i>spoVF</i>)	485 (<i>spoOG</i>)	5×10^4				
	55.1 (<i>spoIIG</i>)	3×10^4				
	IS39 (<i>spoIIID</i>)	2×10^3				
	MY2016 (<i>spo</i> ⁺)	1×10^4				

* In about one experiment in five a few spores ($<10^2 \text{ ml}^{-1}$) were obtained.

† This complementation test showed reciprocal rescue. The yield of strain 88 spores was $3 \times 10^3 \text{ ml}^{-1}$.

protoplasts rescued strain 133.2. Even fusions with t_0 protoplasts of strain 23.1 (also *spoIVC* but in a different complementation group to *spo-133*) resulted in successful rescue.

Additional experiments were done in which the complementable strains 88 (*spoIVF*) and 224 (*spoVF*) were rescued by t_0 protoplasts of strains 485 (*spoOG*), 55.1 (*spoIIG*) and IS39 (*spoIIID*) (Table 3a). These experiments showed that rescue by t_0 protoplasts of asporogenous strains was not restricted to a single complementable strain and probably occurs generally.

Rescue of complementable strains by t_3 protoplasts of other *spo* strains

The results with t_0 protoplasts described above indicate that rescue of a complementable strain requires a rescuer that is *spo*⁺ for the *spo* mutation to be complemented and that the cells have a high 'sporulation potential' as a result of the medium in which they were grown. There is apparently no need for the rescuer strain to be actually sporulating. When t_3

protoplasts of *spoIV* or *spoV* mutants are used as rescuers, complementable strains are rescued provided that the two strains are not in the same complementation group (Dancer & Mandelstam, 1981), i.e. it makes little difference whether the rescuer is subjected to fusion at t_0 or t_3 . It was of interest to see whether this was also true for mutants blocked earlier than stage IV.

Strains blocked earlier than stage III of sporulation are incapable of being rescued in the fusion system because protoplasts are unable to undergo engulfment (Fitz-James, 1964). Thus, fusions of t_3 protoplasts of strain 133.2 (*spoIVC*) and t_3 protoplasts of stage O and stage II mutants would be expected to yield only rescued spores of the stage IV mutant. This was the case. The three *spoO* strains tested at t_3 as rescuers were about as effective as t_3 protoplasts of a *spo*⁺ strain (MY2016) (Table 3*b*). Also, it made little difference to the yield of strain 133.2 spores whether the *spoO* mutants were converted to protoplasts at t_0 or t_3 (Table 3). However, when t_3 protoplasts of *spoII* and *spoIII* mutants were used as rescuers there was usually no rescue of strain 133.2 (Table 3*b*). Thus, with these complementation tests, t_0 protoplasts were much better rescuers than t_3 protoplasts. [Occasionally a few spores of strain 133.2 were obtained ($< 10^2$ ml⁻¹). This could have been due to poor initiation of sporulation so that substantial numbers of stage O rescuer cells remained at t_3 .] The failure of *spoII* and *spoIII* mutants at t_3 to act as rescuers was not confined to fusions with strain 133.2. The other complementable *spoIV* mutants also failed to be rescued in similar experiments (data not shown) as did strain 224.1 (*spoVF*) (which only requires exogenous dipicolinic acid for rescue) (Table 3*b*).

A possible explanation for the lack of rescue of complementable strains by t_3 protoplasts of stage II and stage III mutants would be a dominance effect only expressed during sporulation of the rescuer strains. This could be tested easily with strain 224.1 (*spoVF*). In sporulation medium, strain 224.1 produces about half as many spores as the wild-type strain and these are only toluene-resistant, not heat-resistant. In fusions of t_3 protoplasts a dominant defect in, say, a *spoIII* mutant should block sporulation in strain 224.1 (*spoVF*) before it gets to stage V. When t_3 protoplasts of strain 224.1 were fused with a fivefold excess of protoplasts of strain 55.1 (*spoIIG*) or IS39 (*spoIIID*) about the same number of toluene-resistant spores of strain 224.1 were recovered as when strain 485 (*spoOG*) or MY2016 (*spo*⁺) was used as the rescuer, i.e. about 2×10^6 ml⁻¹. Only the *spoO* and *spo*⁺ strains, however, rescued strain 224.1 to form heat-resistant spores. Thus, when t_3 protoplasts of a *spoII* or a *spoIII* strain were the rescuers they did not impede the development of toluene resistance by strain 224.1 spores. Therefore, no dominance relationship is shown by these experiments.

DISCUSSION

In our earlier experiments on the use of protoplast fusion for complementation of *spo* mutations (Dancer & Mandelstam, 1981) we made the assumption that the best chance for rescue of a t_3 protoplast preparation of a *spo* strain would be by fusion with rescuer strains also at t_3 . This was based on the idea that sporangia at a similar stage of sporulation to one another would be most likely to provide the necessary components for rescue. This assumption has turned out to be false. The experiments with *spo*⁺ strains reported here indicate that t_0 and t_3 protoplasts of cells grown in CH medium are equally effective as rescuers of complementable strains. It is thus unlikely that rescue of complementable *spo* strains results from mere cross-feeding. Instead, it must be concluded that *de novo* induction, transcription and translation must occur on the wild-type chromosome when it is placed, by fusion, in contact with the cytoplasm of a sporulating cell. All *spo* strains at t_0 are effective rescuers except for those in the same complementation group as the strain being complemented. The implication is that all that is necessary is that the rescuer should have a functional *spo* gene to replace the defective one in the t_3 test strain. It is not important whether

this gene is normally expressed before or after that in which the rescuer is defective, i.e. *spoO* and *spoV* mutants are both effective rescuers of *spoIV* strains. Hence, in the heterokaryons it is likely that *spo* genes are transcribed and translated from both chromosomes and that the *spo* genes on the rescuer chromosome, not normally derepressed because of an earlier mutational block in the sporulation process, are nevertheless expressed. This is particularly noteworthy in the experiments in which strain 224 (*spoVF*) was rescued. This strain is rescued by exogenous dipicolinic acid, the production of which is a late event in sporulation. Thus, defects in the latest events in sporulation can be corrected by strains carrying even the earliest mutations in the process.

Rescue by t_0 protoplasts has several advantages over the system that we have previously described. First, one does not have to arrange for two cultures to reach t_3 simultaneously. Secondly, more strains become available as rescuers. When oligosporogenous strains (i.e. 'leaky' *spo* mutants; Coote, 1972) were used as t_3 rescuers the results were ambiguous because of the background of spores of the rescuer strain which could have provided the necessary spore components for complementation. However, if t_0 protoplasts of oligosporogenous strains are used, rescue can occur without the background of *Osp* spores.

In heterokaryons obtained by fusing t_3 protoplasts of strains able to reach stage III or later in sporulation there is a possibility that sporulation-specific compounds are made in the cytoplasm of one forespore, then diffuse through the shared cytoplasm and correct a defect in the other forespore. Thus, expression of the spore genes would have been in the forespore compartment and not the mother cell. However, when t_0 rescuers are used this possibility is precluded. The fact that the strains we have previously shown to be complementable remain so in tests with t_0 protoplasts shows that it is sufficient that the *spo* genes in question be expressed in the mother cell only.

Lencastre & Piggot (1979) used a transformation method to assign the site of expression of *spo* genes to either the mother cell or the forespore compartment. Technical problems prevented them from examining some loci. However, for those loci that have been examined in both the fusion and the transformation system (*spoIVA* and *spoIVC*) the results agree that expression of the gene in the mother cell alone is sufficient to allow sporulation to occur.

When t_3 protoplasts of *spo* strains were used as rescuers in complementation tests, the results were dependent in some cases on the stage of sporulation at which they were blocked. t_3 and t_0 protoplasts of stage O, IV and V mutants were equally effective as rescuers. However, t_3 protoplasts of stage II and III mutants were ineffective as rescuers compared with their t_0 counterparts. It was possible that a dominance effect might be the cause of this. If that were so, t_3 protoplasts of the strains in question should have prevented, or at least substantially reduced, the development of spores of strain 224.1 (*spoVF*). The fact that they did not seems to rule out the possibility that these stage II and III mutations are dominant. Instead, it is likely that they are physiologically altered by t_3 in sporulation so that they no longer participate in fusions.

Protoplasts that were made from cells grown in sugar-supplemented media were ineffective rescuers. The same strain grown in sugar-free medium (CH) and converted to protoplasts at t_0 or t_3 was able to rescue complementable strains in the fusion test system. Thus, rescuers need to be derived from cells in a particular physiological state. The media containing sugar support rapid growth, but subsequent sporulation is slow and asynchronous. Conversely, growth in hydrolysed casein is comparatively slow but subsequent sporulation is rapid and synchronous. Thus, effective rescuers are derived from cells that have a high sporulation potential. A reasonable explanation for the inability of t_0 protoplasts of CHS- or BHIB-grown cells to act as rescuers is that they contain cytoplasmic inhibitors or repressors of sporulation. In support of this idea, t_0 protoplasts of *spo*⁺ cells grown in rich medium actually inhibited sporulation in t_3 protoplasts of another *spo*⁺ strain when fused with them. However, when the t_0 protoplasts as well as those at t_3 were obtained from CH-grown cells, sporulation of the latter was only affected to a relatively small extent.

As a result of this investigation it seems that the best way of establishing complementation relationships with the fusion system is to run tests initially with t_0 rescuers. This procedure is technically simpler and gives a more reliable result than experiments with t_3 rescuers. If needed, as in reciprocal rescue tests for example, fusions between t_3 protoplasts can be done subsequently.

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