Genetic Analysis of Fusion Recombinants and Presence of Noncomplementing Diploids in *Bacillus megaterium*

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We have attempted to undertake genetic analysis in *Bacillus megaterium* using the technique of protoplast fusion that has been successfully applied in *Staphylococcus* and *Streptomyces*. Efficient production of protoplasts, fusion and regeneration techniques have been established. However, variability in numbers and types of recombinants using two-, three-, and four-factor crosses was observed throughout these studies. No linkages were detected, even between loci known to be linked by cotransduction with bacteriophage MP13. These results were similar to those reported by Alföldi and coworkers using *B. megaterium* strain 216, even though the experimental design was significantly changed. During initial subculturing, segregants were observed in a 1:2:2 ratio of noncomplementing diploids:parental-1:parental-2. The ratio changed dramatically after seven subcultures. Double recombinants appeared after nine subcultures. These results corroborate those reported in *B. subtilis* and suggest that there is a locus-inactivation phenomenon present in *Bacillus* which is not evident in *Streptomyces* or *Staphylococcus*. Until the mechanism is elucidated, protoplast fusion should not be used for chromosomal mapping in *B. megaterium*. However, it can be used to transfer plasmids among the bacilli at a frequency of 10^{-5} – 10^{-6} per regenerated protoplast.

INTRODUCTION

Polyethylene glycol-mediated fusion of bacterial protoplasts was first reported in the genus *Bacillus*. *Bacillus subtilis* (Schaeffer *et al.*, 1976) and *Bacillus megaterium* (Fodor & Alföldi, 1976) were both shown to produce recombinants when multiply auxotrophic parental cells were stripped of their cell walls, then fused in the presence of polyethylene glycol. Recombinants exhibiting all possible combinations of parental markers could be recovered following regeneration of bacillary forms on hypertonic media. Both electron-microscopic analysis and phage complementation studies further demonstrated that fusion occurs by a two-step process involving multiple cell fusions (Frehel *et al.*, 1979; Sanchez-Rivas & Garro, 1979). Unfortunately, variability in the recombination frequencies was soon reported in both species (Hotchkiss & Gabor, 1980; Fodor & Alföldi, 1979), and the latter investigators observed that in *B. megaterium* the physiological state of the cells prior to protoplast formation seemed greatly to influence the number and types of recombinants recovered. Sanchez-Rivas *et al.* (1982) demonstrated that in *B. subtilis* up to 50% of prototrophs recovered after fusion were unstable complementing diploids. In addition to the unstable prototrophs, they observed a small percentage of unstable noncomplementing diploids (Ncds), also called biparentals, in which one or the other chromosome in a diploid fusion product is inactivated. The silent chromosome remains unexpressed until segregation occurs (Hotchkiss & Gabor, 1980; Sanchez-Rivas *et al.*, 1982). To try to understand what was different about the two chromosomes in a Ncd cell, Guillen *et al.* (1982) gently isolated the nucleoids from Ncd colonies and observed a difference in

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Abbreviation: Ncd, noncomplementing diploid.

density between expressed and unexpressed chromosomes after centrifugation through CsCl gradients. They concluded from the density shift that a change in the tertiary structure of the inactivated chromosome, rather than a difference in methylation, caused it to be faster sedimenting. The unexpressed chromosome was also shown to have much reduced transforming ability (Bohin *et al.*, 1982). However, the transforming ability of the inactivated loci could be increased 20- to 50-fold by treatment of the DNA with proteases prior to transformation, implying that a protein bound to the DNA was preventing gene expression. Guillen *et al.* (1983) have further shown, by following $\phi 105$ induction in silent and expressed Ncd chromosomes, that no transcription of repressor mRNA occurs when the phage is carried on the silent chromosome.

Protoplast fusion is potentially a powerful genetic tool. It has now been successfully used for genetic mapping in *Streptomyces* (Baltz, 1978; Hopwood & Wright, 1978; Hopwood *et al.*, 1979) and in *Staphylococcus* (Stahl & Pattee, 1983). It might be possible to establish conditions for genetic analysis by protoplast fusion in *Bacillus* if the correct conditions could be found. Indeed, Dancer & Mandelstam (1981) fused *B. subtilis* strains in experiments which enabled them to observe complementation patterns among several *spo* mutants.

Our laboratory has been undertaking genetic mapping in *B. megaterium* for approximately five years using a generalized transducing phage MP13 (Vary, 1979; Vary *et al.*, 1982; Callahan *et al.*, 1983; Garbe *et al.*, 1984). We are also interested in developing other methods of genetic analysis to augment our transductional studies and decided to try protoplast fusion as a possible method for ordering mutations on the *B. megaterium* chromosome. Ncds had not been reported in *B. megaterium*, and Hopwood (1981) suggested that some of the variability in recombinants observed by Fodor & Alföldi (1979) in *B. megaterium* might have been because of their use of selective regeneration media. Therefore, we tried to undertake genetic mapping in *B. megaterium* by protoplast fusion using non-selective regeneration media. This paper reports that the difficulties in recombinant variability encountered in both *B. megaterium* and *B. subtilis* are not solved by the use of a non-selective regeneration medium, and that Ncd clones are also produced in *B. megaterium*. These Ncd clones show similar segregation patterns to those observed in Ncd clones arising from protoplast fusion in *B. subtilis*.

METHODS

Strains and culture conditions. The strains used in this study were derived from *Bacillus megaterium* QM B1551 (ATCC 12778) or from *B. subtilis* 168 and are shown in Table 1. Routine culturing was done as reported previously (Garbe *et al.*, 1984) using supplemented nutrient broth (SNB; Shay & Vary, 1978) with selection for prototrophic markers on minimal glucose medium (MC; Lammi & Vary, 1972). Regeneration media included; DM3 (Chang & Cohen, 1979), 10% (w/v) sucrose nutrient agar (10% SNA; B. C. Carlton, personal communication), R medium (Schaeffer *et al.*, 1976), and minimal hypertonic medium (HGM; Fodor *et al.*, 1975). Sucrose/magnesium/maleate buffer (SMM; Wyrick & Rogers, 1973), was used for protoplast formation and stabilization, and SMMP (equal volumes of 2 × SMM and 4 × Penassay broth; Chang & Cohen, 1979) was used both to dilute PEG-treated cells and as a post-PEG incubation medium. PEG solution (40%, w/v; mol. wt 8000; Sigma) contained 40% (w/v) PEG in 2 × SMM.

Protoplast fusion. Several published methods were tested and modified to improve fusion in our species. The highest yield of recombinants was found using the method of Gabor & Hotchkiss (1979) with mid-exponential phase cells (OD_{660} 0.5, measured in a Perkin-Elmer $\lambda 3$ spectrophotometer, equivalent to 2×10^7 c.f.u. ml⁻¹) and 40% (w/v) PEG. Unless otherwise indicated, 100 ml of each parental culture was grown in SNB to OD_{660} 0.5, and the cells were pelleted at 5000 g at room temperature, resuspended in 0.1 vol. SMMP with 250 μ g lysozyme ml⁻¹ and gently shaken for 30 min at 30 °C. The protoplasts were washed twice with, and then resuspended in 1 ml of, SMMP. Equal amounts (0.1 ml) of each parental strain were mixed, and 0.8 ml 40% (w/v) PEG was added; after 2 min incubation the protoplasts were diluted with 5 ml SMMP, pelleted by centrifugation at 2600 g for 5 min, resuspended in 1 ml SMM, diluted 10⁶-fold and plated on regeneration medium. Controls included in each protocol were: (1) parental protoplasts without PEG treatment, and (2) a parallel culture of unprotoplasted parental cells treated with PEG. After incubation for 24–36 h, well-separated colonies (<100 per plate) were picked at random to master plates of minimal medium complete with all required amino acids. These master plates were replicated after 16–24 h incubation at 30 °C to appropriate selective media and were scored after 24 h. Colonies that showed growth on each parental medium and on complete medium, but did not grow on minimal medium, were designated as Ncds (Guillen *et al.*, 1982).

Table 1. *Bacterial strains*

Organism*	Genotype	Source/Reference†
<i>Bacillus megaterium</i>		
QM B1551	prototrophic	1
PV30	<i>pheA20</i>	2
PV33	<i>argO25</i>	6
PV57	<i>leuC4 trp-27 str-3</i>	6
PV59	<i>gly-21</i>	6
PV104	<i>cys-20 erm-3</i>	6
PV143	<i>ilv-4 thr-22 str-3</i>	6
PV160	<i>ilv-4 leuA25 str-3</i>	2
PV170/pCM194 (Cm ^R)	<i>cys-20 erm3</i>	
JV26	<i>str-1</i>	3
JV78	<i>leuC4 str-3</i>	1
<i>Bacillus subtilis</i>		
BR151/pCM194 (Cm ^R)	<i>trpC2 lys-3 metB10</i>	4
BR151/pUB110 (Km ^R)	<i>trpC2 lys-3 metB10</i>	4
<i>Staphylococcus aureus</i>		
1SP479/pI258 (Em ^R)	<i>pig-131/bla-401 mer-14 repA36</i> <i>Tn551 (ermB)</i>	5

* Strains with a PV prefix originated in this laboratory. Strains with a JV prefix are from the laboratory of J. C. Vary. All *B. megaterium* strains are derived from QM B1551.

† 1, Vary (1972); 2, Garbe *et al.* (1984); 3, J. C. Vary, University of Illinois, Chicago, USA; 4, P. Lovett, University of Maryland, Catonsville, USA; 5, P. Pattee, Iowa State University, Ames, Iowa, USA; Luchansky & Pattee (1984); 6, this study.

Subcloning Ncd colonies. Colonies classified as Ncd were picked with sterile toothpicks from different media, inoculated separately into SNB, shaken at 30 °C until they reached an OD₆₆₀ of 0.4–0.8, and then were streaked on SNB agar or minimal medium with appropriate supplements. After 16–24 h at 30 °C, well-isolated single colonies were transferred with toothpicks to master grids on the same medium. After a further 16–24 h growth at 30 °C, the master grids were replica plated to selective media and the colonies were reclassified as parental, recombinant, or Ncd.

Transfer of plasmids by fusion. Cultures of the desired plasmid-bearing *Bacillus* strain and plasmid-less strain were grown in Penassay broth (Difco) with all requirements and fused according to Dancer (1980). After diluting the PEG-treated protoplasts in 5 ml SMMP and centrifuging at 2500 *g* at room temperature, the pellet was gently resuspended in 1 ml SMMP and given 4 h post-PEG incubation at 30 °C to allow for phenotypic expression. Samples were then removed, diluted 10⁶-fold, and plated on DM3 regeneration medium supplemented with the appropriate selective antibiotics. The plates were incubated for 48 h at 30 °C for *B. megaterium*, or 37 °C for *B. subtilis*, then scored for antibiotic resistance. The frequency of plasmid transfer was calculated by dividing the number of resistant colonies per ml by the number of colonies per ml that were obtained in the absence of selection.

Plasmid isolation and agarose gel electrophoresis. Plasmids were isolated by the rapid method of Birnboim & Doly (1979) and analysed by electrophoresis through 0.7% agarose as previously described (Kieselburg *et al.*, 1984).

Chemicals and reagents. PEG, chloramphenicol, streptomycin, lincomycin, kanamycin, erythromycin, lysozyme and BSA were obtained from Sigma. Cadmium nitrate was purchased from Mallinckrodt Chemical Works (St Louis, MO., USA), SDS from Eastman Kodak and agarose from Bio-Rad. All other chemicals were of reagent grade.

RESULTS

Determination of optimum conditions for protoplast production and regeneration of bacillary forms

Starting with the procedure of Gabor & Hotchkiss (1979), several conditions, including phase of growth, lysozyme concentration, pH and temperature, were varied to establish the most efficient method of producing protoplasts. Efficiency of protoplasting (*A*) was calculated as $A = 1 - a/b$ (equation 1), where *a* is the colony count following osmotic shock of protoplasts in water, and *b* is the colony count of the unprotoplasted culture. When each condition was varied separately, the highest efficiencies of protoplasting were obtained with cells grown to OD₆₆₀ 0.5 (99.2%), lysed in 250 µg lysozyme ml⁻¹ in SMM (99.3%) at pH 6.5 (96.2%) for 30 min (99.1%) at 32 °C (99.3%). These conditions were combined and used in all subsequent fusions.

Table 2. Results of fusion of *B. megaterium* strains of PV57 (*trp-27 leuC4 str-3*) and PV143 (*ilv-4 thr-22 str-3*)

Fusion of protoplasts was done using optimum conditions. Parental cultures (about 2×10^7 c.f.u. ml⁻¹) were converted to protoplasts and fused as described in Methods. The mixture was diluted and plated for single colonies on nonselective regeneration media. After 36 h incubation, colonies were replica plated to all necessary media to test for phenotype. Conditions in all three experiments were identical.

Phenotype*					No. of colonies†		
Trp	Leu	Ilv	Thr		Expt 1	Expt 2	Expt 3
1	1	1	1	Trp ⁻ Leu ⁻ (parental)	479	491	500
0	0	0	0	Ilv ⁻ Thr ⁻ (parental)	622	376	321
1	0	1	1	Trp ⁻	0	4 (3.0)	22 (12.4)
0	1	1	1	Leu ⁻	16 (4.0)	13 (9.8)	5 (2.8)
0	0	0	1	Ilv ⁻	79 (19.8)	3 (2.2)	39 (22.0)
0	0	1	0	Thr ⁻	29 (7.3)	5 (3.8)	17 (9.6)
1	0	0	1	Trp ⁻ Ilv ⁻	63 (15.8)	16 (12.0)	23 (13.0)
1	0	1	0	Trp ⁻ Thr ⁻	68 (17.0)	25 (18.8)	6 (3.4)
0	1	0	1	Leu ⁻ Ilv ⁻	76 (19.0)	17 (12.8)	16 (9.0)
0	1	1	0	Leu ⁻ Thr ⁻	20 (5.0)	3 (2.3)	14 (7.9)
1	1	0	1	Trp ⁻ Leu ⁻ Ilv ⁻	7 (1.8)	5 (3.8)	1 (0.6)
1	1	1	0	Trp ⁻ Leu ⁻ Thr ⁻	1 (0.3)	3 (2.3)	0
0	1	0	0	Leu ⁻ Ilv ⁻ Thr ⁻	13 (3.3)	8 (6.0)	2 (1.1)
1	0	0	0	Trp ⁻ Ilv ⁻ Thr ⁻	12 (3.0)	2 (1.5)	6 (3.4)
1	1	0	0	Trp ⁻ Leu ⁻ Ilv ⁻ Thr ⁻	2 (0.6)	0	6 (3.4)
0	0	1	1	Prototrophic	13 (3.3)	29 (21.8)	20 (11.3)
Total recombinants					339	133	177
Total percentage of recombination					26.6%	13.3%	17.9%

* Donor 1 allele = 1; donor 2 allele = 0.

† Numbers in parentheses are the percentage of total recombinants observed.

The regeneration of protoplasts was also tested on several hypertonic media including 8%, 10% and 12% SNA ± BSA, DM3, R and HGM (see Methods). Regeneration efficiencies were measured by diluting lysozyme treated cells in either water or hypertonic buffer, plating on hypertonic media, and comparing the two plate counts. The regeneration efficiency (RE) was given by $RE = [(e - a)/b] \times 1/A$, where e is the colony count after dilution in hypertonic buffer and A , a and b are the same as in equation 1. The 10% SNA, which is very simple to prepare (in contrast to DM3 and HGM) gave the highest regeneration efficiency (98.2%) and was used for all subsequent tests.

Analysis of recombinants arising from protoplast fusion

Several two-, three-, and four-factor crosses were attempted, including a cross between *B. megaterium* strains PV160 and PV30 which involved three loci (*ilv*, *leu* and *phe*) that had been shown previously to be linked by cotransduction (Garbe *et al.*, 1984). There was no detectable reduction in recombination between these linked markers as compared with the frequency of recombination between unlinked markers in the cross (data not shown). Therefore, more extensive four-factor crosses were attempted, including the cross shown in Table 2. In this experiment, inheritance of the *ilv* and *leu* markers, which are about 50% cotransduced by phage MP13, was studied by fusing PV57 with PV143. Since no linkage was seen between *ilv* and *leu*, the experiment was repeated twice. The results varied widely between experiments; however, it is noteworthy that every possible recombinant type could be recovered.

The frequencies of recombination calculated from the data in Table 2 are shown in Table 3. No apparent linkages could be detected. In an attempt to determine whether the recombination events were reciprocal, the frequencies of occurrence of each crossover in experiment 1 were calculated separately in each direction. The results (Table 3) indicate inconsistency even within one experiment. For example, if one looks at the frequency of recombination between *trp* and *leu* in each direction, one finds reciprocity, since the two classes are present at almost equal

Table 3. Frequencies of recombination and reciprocal crossover derived from fusion of *B. megaterium* strains PV57 and PV143

Crossover between:	No. of crossovers			Recombination frequency		
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
<i>trp</i> and <i>leu</i>	268	88	120	0.671	0.661	0.670
<i>trp</i> and <i>thr</i>	330	97	98	0.827	0.730	0.547
<i>trp</i> and <i>ilv</i>	162	73	69	0.406	0.549	0.385
<i>leu</i> and <i>ilv</i>	208	93	90	0.521	0.699	0.503
<i>leu</i> and <i>thr</i>	191	66	126	0.478	0.496	0.704
<i>ilv</i> and <i>thr</i>	343	77	116	0.859	0.579	0.648
Total recombinants	399	133	179			

Reciprocal crossover frequencies*				
	Trp ⁻	Leu ⁻	Ilv ⁺	Thr ⁺
Trp ⁺	x	0.313	0.145	0.461
Leu ⁺	0.358	x	0.276	0.388
Ilv ⁻	0.210	0.246	x	0.564
Thr ⁻	0.208	0.090	0.296	x

* Data from experiment 1. Frequencies of occurrence of reciprocal crossovers between two markers were determined by dividing the number of recombinants resulting from a crossover in each direction, e.g. Trp⁺ Leu⁻ vs Trp⁻ Leu⁺, by the total number of recombinants.

frequencies (0.313 and 0.358). However, the frequencies of the recombinant classes arising from crossovers in the two directions between *trp* and *thr* and between *leu* and *thr* differ by more than two-fold and four-fold, respectively.

Analysis of a *Ncd* colony

Colonies that grew on both media selecting for either parental type, but that failed to grow on minimal medium, were detected at a frequency of 1–4% in almost every cross. One such *Ncd* colony resulting from a cross between *B. megaterium* strains PV33 (*argO*) and PV59 (*gly-21*) (which grew when replicated to MC + glycine or MC + arginine but did not grow on MC) was successively subcloned and analysed for the persistence of this trait (Table 4). A ratio of 1:2:2 *Ncd*:*Arg*⁻:*Gly*⁻ was observed through six subclonings, after which the ratio became skewed toward recovery of *Arg*⁻ and *Ncd* colonies. When all five *Ncd* colonies isolated from subcloning *Ncd*8 were each subcloned (*Ncd*9:1–5), it became clear that all five had shifted from the previous 1:2:2 segregation pattern, since well over 50% of all the segregants were *Arg*⁻. Subclones *Ncd*9:3 and *Ncd*9:5 also yielded prototrophic segregants. Since *Ncd*9:3 was the only one of the five which segregated out all four genotypes, it was subcloned again and 250 colonies were analysed. The rare *Arg*⁻ *Gly*⁻ recombinants were then detected (Table 4).

Interspecies plasmid transfer

Dancer (1980) reported interspecies transfer of plasmids by protoplast fusion. Therefore, *B. subtilis* strain BR151 (Em^S) carrying either pCM194 (Cm^R) or pUB110 (Km^R) plasmids was fused with *B. megaterium* PV104 (Em^R) in the presence of PEG and cells were regenerated on a medium containing both erythromycin and either chloramphenicol or kanamycin as appropriate. The *B. megaterium* colonies receiving the plasmids were verified by colonial and microscopic appearance, auxotrophic requirements and MP13 phage sensitivity. No evidence of chromosomal transfer was detected in the colonies receiving plasmids. Several inter- and intraspecies crosses are shown in Table 5. The first cross was done before optimal conditions had been established, and so gave a low frequency of transfer. It is shown because it generated strain PV170, which was used as donor in subsequent tests. The presence of pCM194 in PV170 was verified by agarose gel electrophoresis. Repeated attempts to transfer p1258, a plasmid in *Staphylococcus aureus* strain 1SP479, failed even though fusion pairs between protoplasts of *B. megaterium* and *S. aureus* could be seen by phase-contrast microscopy.

Table 4. *Subcloning of a Ncd colony resulting from fusion of B. megaterium strains PV33 (argO) and PV59 (gly-21)*

Colony	Phenotype (% of total)					
	Ncd	Arg ⁻	Gly ⁻	Arg ⁺ Gly ⁺	Arg ⁻ Gly ⁻	
Ncd1*	22	38	40	0	0	
Ncd2	24	40	36	0	0	
Ncd3	20	46	34	0	0	
Ncd4	14	38	48	0	0	
Ncd5	26	32	42	0	0	
Ncd6	20	36	44	0	0	
Ncd7	53	44	3	0	0	
Ncd8	10	84	6	0	0	
Ncd9	58	28	14	0	0	
Ncd10	14	84	2	0	0	
Ncd9:1†	10	90	0	0	0	
Ncd9:2	36	52	12	0	0	
Ncd9:3	18	74	6	2	0	
Ncd9:4	26	60	14	0	0	
Ncd9:5	6	90	0	4	0	
Ncd10(9:3)‡	14 (36)	65 (162)	17 (43)	3 (7)	1 (2)	

* A noncomplementing diploid colony resulting from the protoplast fusion, denoted Ncd1, was subcloned and plated for single colonies. Fifty colonies were then tested by replica plating to all necessary selective media, except for Ncd7 where 36 colonies were plated. One of the eleven Ncd colonies thus identified was denoted Ncd2 and the procedure repeated another nine times.

† Each of the five Ncd colonies isolated by subcloning Ncd8 was itself subcloned (Ncd9:1-5) and 50 colonies were analysed as described in Methods.

‡ The colony Ncd9:3 was again subcloned and 250 single colonies were analysed as described in Methods. The numbers in parentheses represent the actual number of colonies observed.

Table 5. *Plasmid transfer by protoplast fusion*

Donor and recipient cells were grown to 10^8 c.f.u. ml⁻¹ according to Dancer (1980). Protoplasts were concentrated 25-fold in SMMP and 0.5 ml of each were mixed; 1.5 ml 40% (w/v) PEG was added and after 2 min the protoplasts were diluted and washed twice in SMMP. After 4 h incubation, the protoplasts were diluted and plated on DM3 medium with and without antibiotic: chloramphenicol (Cm), 10 µg ml⁻¹; erythromycin (Em), 2 µg ml⁻¹; streptomycin (Sm), 100 µg ml⁻¹; or kanamycin (Km), 25 µg ml⁻¹. The frequency of plasmid transfer was calculated as the number of resistant colonies per regenerated protoplast.

Donor	Recipient	Counter selection against donor	Selection	Frequency
<i>B. subtilis</i> BR151/pCM194	<i>B. megaterium</i> PV104	Em ^S	Cm ^R	8.0×10^{-9}
<i>B. subtilis</i> BR151/pUB110	<i>B. megaterium</i> JV78	Sm ^S	Km ^R	6.7×10^{-6}
<i>B. megaterium</i> PV170/pCM194	<i>B. subtilis</i> BR151/pUB110	Km ^S	Cm ^R	7.6×10^{-6}
<i>B. megaterium</i> PV170/pCM194	<i>B. megaterium</i> JV26	Sm ^S	Cm ^R	2.2×10^{-5}
<i>B. megaterium</i> PV170/pCM194	<i>B. megaterium</i> JV78	Sm ^S	Cm ^R	3.8×10^{-6} 1.8×10^{-5}

DISCUSSION

Optimum conditions were established for production and regeneration of protoplasts of *B. megaterium* QM B1551 using a non-selective, simple regeneration medium. Some of the early variations observed in fusion recombinants in *B. subtilis* and *B. megaterium* had been attributed to variations in regeneration and the media used (Schaeffer *et al.*, 1976; Fodor & Alföldi, 1976). While consistent regeneration rates could be obtained by our methods, variability in recombination frequencies was still observed; this variability was thought not to be caused by variations in the regeneration rate or type of medium used. Gabor & Hotchkiss (1982) controlled

variation somewhat by freezing cells just before protoplasting, suggesting, as did Fodor & Alföldi (1979), that growth of the cells and their physiological state before formation of protoplasts may influence the recombination rates. However, a more important factor may be the persistence of segregating complementing and noncomplementing diploids within the population. A previous attempt to detect Ncd colonies in isolated clones of *B. megaterium* (Fodor *et al.*, 1983) was unsuccessful. Our experimental design could not have detected complementing diploids, but our observed frequency of Ncd colonies (1–4%) is similar to the frequencies observed by Hotchkiss & Gabor (1980) in *B. subtilis* (1–10%). The segregation patterns of the Ncd colonies reported here also followed very closely those reported by Hotchkiss & Gabor (1980); that is, the initial ratio of Ncd:parental-1:parental-2 was 1:2:2, with double recombinants emerging after several subclonings. The similarity of the segregation patterns in the two species would suggest that several complementing diploids might also be present in the population following fusion in *B. megaterium*. By using a *spo0A* mutation as an indicator, Sanchez-Rivas (1982) observed that as many as 50% of the prototrophic *B. subtilis* selected on minimal regeneration medium were complementing diploids. In addition, Bohin *et al.* (1982) have shown that the unexpressed chromosome in *B. subtilis* Ncd colonies can become biologically active in transformation if the DNA is first treated with protease. This phenomenon could not be tested in *B. megaterium* since chromosomal transformation is not presently possible.

We have shown that transfer of several plasmids between bacilli at frequencies of 10^{-5} – 10^{-6} per regenerated protoplast is possible by protoplast fusion. These data corroborate the observations of Dancer (1980), who transferred plasmid pC221 by fusion among *B. megaterium* KM, *B. licheniformis*, *B. polymyxa* and *B. subtilis* at frequencies of 1.3×10^{-5} to 7.0×10^{-6} per regenerated protoplast. Our results revealed no significant difference in frequency whether the transfer was inter- or intraspecific, suggesting that restriction of plasmid DNA did not occur between *B. subtilis* BR151 and *B. megaterium* QM B1551. All attempts to transfer a plasmid from *Staphylococcus aureus* to *B. megaterium* failed, however. Several *S. aureus* plasmids have been shown to replicate and to be expressed in our strain (Kieselburg *et al.*, 1984), but the transfer has always been from a *B. subtilis* host. The failure to transfer plasmid pI258 could be due to several factors, including restriction of foreign DNA, incompatibility with one of the seven resident plasmids in strain QM B1551, or lack of recognition by QM B1551 of the replication origin of this particular plasmid.

Although plasmids can be transferred successfully, genetic mapping cannot be done by analysis of the products of protoplast fusion in *B. megaterium* using the current methods. The data presented here and those of other *Bacillus* researchers are in sharp contrast to the successful use of fusion analysis in *Streptomyces* and *Staphylococcus*. Perhaps some property associated with *Bacillus* spore formation is involved, but this has so far not been determined. Genetic analysis of fusion recombinants in other *Bacillus* species has not been reported to our knowledge. Dancer & Mandelstam (1981) demonstrated complementation of specific *spo* genes by fusion, taking advantage of conditions unique to sporulation. Whether their method can be modified to test other markers such as auxotrophic requirements remains to be tested.

The data presented here suggest that caution should be used in interpreting all fusion results and that great care should be taken to verify that cells resulting from fusions are haploid, still contain all expected genetic markers, and are stable.

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