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Spore Germination of *Bacillus megaterium* QM B1551 Mutants

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Auxotrophic or antibiotic-resistant mutants of *Bacillus megaterium* QM B1551 are capable of initiation of germination similar to the parental (QM B1551) prototrophic strain.

Several laboratories have employed mutants as a means of studying the biochemical mechanism of initiating bacterial spore germination. Recently, McCormick, Feeherry, and Levinson (7) have reported that auxotrophic or streptomycin-resistant mutants of *Bacillus megaterium* QM B1551 produce spores with altered germination properties. Because of the numerous implications of this observation, the germination properties of 55 *B. megaterium* QM B1551 mutants from this laboratory's collection have been tested. Both auxotrophic and antibiotic-resistant mutants, isolated by several different methods of mutagenesis, were examined.

B. megaterium QM B1551, a prototroph, was used as the wild type and mutants derived from this organism are listed in Table 1. Spores were grown in supplemented nutrient broth (SNB) as previously described (6). Auxotrophic mutants were grown in SNB plus their specific growth factor at a final concentration of 16 μg/ml, except for biotin and *p*-aminobenzoic acid which were 1.6 ng/ml and 83 ng/ml, respectively.

Antibiotic-resistant mutants were derived spontaneously and auxotrophic mutants were made by three methods of mutagenesis as described in Table 1. To select for auxotrophs, mutagenized cultures were diluted and spread on SNB plates. The plates were incubated for 16 to 18 hr at room temperature, replica-plated to minimal media (MC; reference 6) plates, and presumptive auxotrophs were picked after 24 hr of incubation at 30 C. The auxotrophic requirements were identified, first using pools as described by Clowes and Hayes (1), and then checked for the single specific growth factor. From single colony isolates, each mutant was sporulated in SNB plus its specific growth factor as described above, lyophilized, and stored at room temperature. Each of these spore batches was again tested and shown to be auxotrophic for one growth factor and free of revertants.

The spores of these mutants were tested for germination in SNB, MC, glucose, or KBr. None of the mutants required their respective growth factor or antibiotic for the initiation of germination. All of the mutants required heat activation when assayed immediately after being grown. This activation requirement was slowly lost with storage. Initiation of germination by glucose or KBr was tested in 5 mM tris(hydroxymethyl)aminomethane (*p*H 8) buffer which maintained the *p*H between 7.8 and 8.0. The data for initiation by L-alanine or calcium dipicolinate (Ca DPA) is not shown because neither mutant nor wild-type spores responded to these reagents. However, with storage at room temperature, both mutant and wild-type spores gained the capacity to be initiated by Ca DPA (J. C. Vary, *unpublished results*).

Four streptomycin-resistant, four spectinomycin-resistant, and two methicillin-resistant mutants were tested for their germination properties. These are all spontaneous mutants that are prototrophic, resistant to only one of the three antibiotics, and presumably isogenic with QM B1551. All of these mutants germinated similarly to wild type as judged by the percentage decrease in optical density during the first 20 min of germination. The data for three mutants are shown in Table 1.

By using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) mutagenesis (method B or C), 23 auxotrophs were isolated. In all but three cases, the spores of these mutants initiated germination (see Table 1). His-4 and Ade-8 initiated poorly, but other histidine or adenine auxotrophs did initiate germination similarly to

TABLE 1. *Initiation of germination of Bacillus megaterium mutants*[a]

Strain	Phenotype[b]	Method of mutagenesis	Spore germination[c]				
			SNB	MC	Glucose	KBr	Buffer
QM B1551	Wild		63	64	66	48	0
JV-28	SmR-3	A[d]	57	60	65	54	9
JV-35	SpcR-2	A	56	63	61	58	10
JV-32	MethR-1	A	49	54	58	42	0
JV-47	Pur-8	B[e]	53	53	58	40	0
JV-87	His-2	B	54	44	50	27	3
JV-89	His-4	B	11	0	2	0	4
JV-39	Ura-1	B	39	45	34	27	6
JV-104	Bio-2	B	55	54	53	45	2
JV-68	Met-2	B	50	54	34	37	14
JV-52	Ade-6	B	67	63	64	50	4
JV-81	Gly-3	B	42	43	45	50	2
JV-71	Arg-2	B	17	7	0	4	2
JV-65	Thr-2	B	62	62	61	28	0
JV-54	Ade-8	C[f]	31	10	12	4	6
JV-96	His-11	C	59	53	60	22	3
JV-97	His-12	C	60	61	61	65	11
JV-105	Bio-3,SmR-3	D[g]	56	58	56	38	0
JV-95	His-10,SmR-3	D	57	57	56	51	0
JV-78	Leu-4, SmR-3	D	60	59	62	38	0
JV-112	Pab-3,SmR-3	D	60	54	60	37	4
JV-99	Phe-1,SmR-3	D	65	70	67	54	8
JV-100	Ser-2,SmR-3	D	73	74	73	70	12
JV-61	Trp-6,SmR-3	D	60	65	57	53	6

[a] Abbreviations: SmR, streptomycin-resistant; SpcR, spectinomycin-resistant; MethR, methicillin-resistant; SNB, supplemented nutrient broth; MC, minimal media.

[b] Phenotypic designations are according to the proposed rules of Demerec et al. (4) for either antibiotic-resistant or auxotrophic strains.

[c] The initiation of germination was measured by the decrease in optical density at 660 nm after 30 min. A 60 to 70% decrease represents >90% germination. Spores were heat activated in distilled water for 10 min at 60 C and then diluted into one of the following media at 30 C: SNB, MC, 5 mM tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8), 0.1 M glucose-5 mM Tris buffer (*p*H 8) or 0.04 M KBr-5 mM Tris buffer (*p*H 8). The final concentration of spores was 200 μg/ml (dry weight) which is an initial optical density of 0.7 to 0.8.

[d] Method A. Strains with spontaneous mutations to antibiotic resistance were isolated by plating about 10^9 vegetative cells of QM B1551 on SNB containing either streptomycin (100 μg/ml; Calbiochem), spectinomycin (100 μg/ml; Upjohn Co.) or methicillin (2 μg/ml; Bristol Laboratories). From single colony isolates, each mutant strain was sporulated in SNB. The spores were washed, lyophilized, and checked again for their antibiotic-resistant phenotype.

[e] Method B. Spores of QM B1551 were heat-activated, inoculated into MC to a final concentration of 300 μg/ml (dry weight) and incubated as described previously (6). After 1 to 2 hr, a 10-ml sample was centrifuged and suspended in 1.0 ml of 0.1 M sodium acetate buffer (*p*H 5.3) containing 1 mg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG; Aldrich Chemical Co.). The sample was incubated 20 min at 30 C, centrifuged, washed once with cold MC, suspended in 10 ml of SNB, and incubated overnight on the shaker. The culture was then diluted and selected for auxotrophs (see text).

[f] Method C. Vegetative cells of QM B1551 from an overnight SNB plate were inoculated into SNB and incubated as described above. After the culture reached an optical density (660 nm) of 1.0, a 10-ml sample was mixed with an aqueous solution of NTG (final concentration 100 μg/ml), incubated 20 min on the shaker, and then treated as in method B.

[g] Method D. Vegetative cells of SmR-3 from an overnight MC plate containing streptomycin (100 μg/ml) were inoculated into 50 ml of MC (without streptomycin) and shaken at 30 C. After the culture reached an optical density of 1.0, 20 ml was mixed with 0.4 ml of ethyl methanesulfonate (Eastman Kodak Co.) and incubated on the shaker for 60 min. Then 1.0 ml of 2 M $Na_2S_2O_3$ was added to stop mutagenesis (2). The cells were centrifuged, washed twice with cold MC, and incubated overnight in 10 ml of SNB. The culture (mostly spores), was washed and stored in distilled water overnight at 4 C. The spores were heat-activated 10 min at 60 C and inoculated into 5 ml of MC (final concentration of 1.7×10^8 spores/ml). After 1 hr of shaking at 30 C, the spores were washed three times with MC, suspended in 10 ml of MC, and incubated 1 hr on the shaker. Methicillin was then added (final concentration of 300 μg/ml) and incubated for an additional 2.5 hr. The culture was diluted and selected for auxotrophs.

wild-type. The third mutant with poor initiation properties in Arg-2 and additional arginine auxotrophs have not been tested.

Twenty-two auxotrophs were isolated after ethyl methanesulfonate (EMS) mutagenesis (method D). For reasons unrelated to this study, SmR-3 was used as the parental strain. All of the auxotrophs germinated similarly to wild-type and the data for seven of these are shown in Table 1.

Although 3 of the 45 auxotrophs reported here initiated germination poorly, this does not imply any unique relationship between auxotrophy and the mechanism of germination. These three mutants were made by NTG which is a powerful mutagen known to cause multiple mutations. Also, none of the antibiotic-resistant mutants, which were derived spontaneously, and none of the auxotrophs isolated after EMS mutagenesis were defective in germination.

If there were a direct relationship between auxotrophic mutations affecting growth and the ability of spores to germinate, then germination mutants (selected for their inability to germinate) might have altered growth requirements. Mutants of *B. megaterium* QM B1551 that are temperature sensitive for germination have been reported (8). Nineteen of these twenty-five mutants are prototrophic, two require a single amino acid for growth, and four have multiple requirements (J. C. Vary, *unpublished observations*). Germination mutants in *B. cereus* T (5, 9) have not been tested for their auxotrophic requirements.

One possible explanation for the difference between these results and those of McCormick et al. (7) is their selection technique. After NTG mutagenesis, McCormick et al. (7) incubated the cells overnight. The culture was starved and then treated simultaneously with glucose and methicillin in a minimal medium. Thus, the survivors from this treatment might be defective in glucose metabolism. Whether the auxotrophs that were selected from these survivors might produce spores deficient in glucose metabolism has not been tested.

In the study of revertants, McCormick et al. (7) noted that single-step revertants of a double auxotroph did not "cure" the germination lesion; only complete reversion to prototrophy restored the ability to initiate germination on glucose. This could easily have resulted from contamination by only one wild-type cell in 2×10^8 auxotrophs that were plated and not represent true reversion, since internal markers were lacking.

The results reported here show that a variety of auxotrophs or antibiotic-resistant mutations in *B. megaterium* QM B1551 have little effect on the initiation of germination of these spores. This observation agrees with that found in *B. subtilis* in which auxotrophs initiated germination similar to wild-type (3).

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