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Spontaneous Auxotrophic and Pigmented Mutants Occurring at High Frequency in *Bacillus pumilus* NRRL B-3275

PAUL S. LOVETT

Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228

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Broth cultures of *Bacillus pumilus* NRRL B-3275 (BpB1) grown at 25, 30, or 37 C contain 1 to 2% spontaneous auxotrophic mutants in both the exponential and stationary phases of growth. Of 70 such mutants isolated from cultures grown at 37 C, approximately two-thirds reverted at such a high frequency as to preclude their study. Of the remaining 22 mutants, 18 required a single amino acid, 1 required adenine, and 1 required uracil. Two of the auxotrophs each required two unrelated amino acids resulting from two independent mutations. All of the mutations reverted spontaneously. Enhanced reversion of approximately one-third of the mutations was obtained with nitrosoguanidine, ethyl methane sulfonate, or diethyl sulfate, or with more than one of these mutagens. The reversion of one mutation was enhanced by 2-aminopurine. The reversion of the remaining mutations was not enhanced by the above mutagens, nor by mutagens known to induce (and revert) frameshift mutations in other bacterial systems. Nine of 10 mutants examined did not show a selective growth advantage over the parents. All but three of the mutations could be linked by PBS1 transduction to one of the previously described auxotrophic markers in strain BpB1. No evidence was obtained for clustering of the mutations on the BpB1 genome. Six of the mutations conferred a requirement for serine. One linked by transduction to *trp-2*, three linked to *argA1*, and two (*ser-2*, *-3*) linked to *argO1*. Pigmented mutants (containing a carotenoid-like pigment), which occur spontaneously in BpB1 cultures at a frequency on the order of 1 to 5 mutants per 10^4 cells, link by transduction to *ser-2*, *-3*. Spontaneous mutants of strain BpB1 resistant to rifampin, streptomycin, erythromycin, 5-fluorouracil, or 5-methyltryptophan occur at a frequency similar to that of strains of *B. pumilus* which do not exhibit a high rate of spontaneous mutation to auxotrophy. It is suggested that certain sites or regions of the BpB1 genome exhibit a high rate of spontaneous mutation.

Bacillus pumilus NRRL B-3275 (BpB1) has the ability to adapt to the "L-form" type of growth during cultivation in liquid media containing 7% sodium chloride (4). With respect to several other physiological properties, strain BpB1 behaves as a typical strain of *B. pumilus* (15). The adaptation of strain BpB1 to the L-form state occurs without prior mutagenesis, and the L-forms revert to the bacillary form after a reduction of the sodium chloride concentration of the medium to 3% (Lovett, *unpublished data*). The reversion is prevented by including penicillin in the growth medium (*unpublished data*). Since several passages are required for both the "induction" of the L-form and its reversion to the bacillary form, it is not clear whether the medium containing a high salt concentration selects a class of spontaneous mutant that outgrows the bacillary form or whether the high salt concentration causes a phenotypic alteration of the cells.

During the isolation of mutants from strain BpB1 for use in genetic studies (16, 17), it became evident that a variety of spontaneous mutations arose in this strain at a frequency significantly higher than is observed in other strains of *B. pumilus*. The present study was undertaken to examine the properties of two

types of spontaneous mutants arising at high frequency in strain BpB1. The possible relationship between the apparent high mutation rate of certain regions of the BpB1 genome and the ability of this organism to adapt to the L-form state is discussed.

MATERIALS AND METHODS

Bacteria. *B. pumilus* strain NRRL B-3275 (BpB1) and several auxotrophic mutants of BpB1 obtained after mutagenesis (Table 1) were used as starting material for the isolation of spontaneous mutants and for the genetic mapping experiments. The physiological and genetic properties of strain BpB1 and of most of these mutants have been described (15–17). The remainder were isolated from strain BpB1 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) or ethyl methane sulfonate (EMS) as previously described (16, 17). The source of other bacteria used in this work has been reported (14).

Growth media. Antibiotic medium no. 3 (Penassay broth) and Tryptose blood agar base (TBAB), both from Difco, were prepared according to package directions. L-broth is composed of tryptone (1%), yeast extract (0.5%), and glucose 0.5%, *p*H 7.2. The minimal medium was that previously reported (16). MGGN medium is minimal medium supplemented with glutamic acid (50 μg/ml) and liquid nutrient broth (1%).

Isolation of spontaneous auxotrophic mutants. Individual colonies taken from TBAB plates incubated for 18 to 24 hr at 37 C were inoculated into Penassay broth, unless otherwise specified. The quantity of medium was 2.5 ml in 18 by 150 mm tubes or 10 ml in 250-ml nephelometer flasks adapted to fit a Klett-Summerson colorimeter. Cultures were incubated at 37 C with rotary shaking (250 rev/min). The 2.5-ml cultures were incubated for 18 to 24 hr (stationary phase; ca. 2×10^9 cells/ml). Growth in 10-ml cultures was monitored turbidimetrically, and samples were withdrawn during late exponential growth, at 5 hr after the cessation of exponential growth (t = 5), and at t = 24.

TABLE 1. *Mutant strains isolated from Bacillus pumilus NRRL B-3275 (BpB1) after mutagenesis*

Strain designation	Genotype	Origin and mutagen
BpB5	*phe-1*	BpB1; NG[a]
BpB7	*argA1*	BpB1; NG
BpB10	*trp-2*	BpB1; NG
BpB11	*gly-1*	BpB1; NG
BpB13	*his-1*	BpB1; NG
BpB16	*argO1*	BpB1; NG
BpB17	*argO2*	BpB1; NG
BpB20	*cys-1*	BpB1; NG
BpB203	*argA2*	BpB1; NG
BpB231	*lys-2*	BpB1; NG
BpB635	*cys-1 trp-3*	BpB20; EMS[b]

[a] *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.
[b] Ethyl methane sulfonate.

Cells were diluted in Penassay broth, and appropriate dilutions were spread onto TBAB plates. The plates were incubated for 18 hr at 37 C, and the resulting colonies were replicated to minimal plates. Colonies that did not grow on the minimal plates were cloned and their nutritional requirements were identified when possible. All of the spontaneous mutants identified in the present study were sensitive to the phages PBS1 and PBP1 (14), and each could be transduced to prototrophy by PBS1 propagated on strain BpB1. Whenever an auxotroph was used as starting material for the isolation of spontaneous mutants, the resulting spontaneous mutants always carried the original auxotrophic requirement.

Reversion induced by mutagens. The ability of mutagens to enhance the reversion of each of the spontaneous auxotrophs was determined by use of a procedure based on that described by Hartman et al. (11). Samples of 0.1 ml (ca. 10^8 cells) of washed early stationary-phase broth cultures were spread onto MGGN plates. A separate plate was used for each mutagen, and the following chemicals were applied in an excentric position on the plates: crystals of NG (Aldrich Chemical Co.) and 2-aminopurine nitrate (B grade, Calbiochem); single drops of EMS, DES (diethyl sulfate, Eastman), and ICR 364-OH (1 mg/ml; 2-chloro-6-methoxy-9-[2-(2-hydroxyethyl) aminoethylamino]-1-azacridine dihydrochloride). The following chemicals were applied as crystals or as single drops of 1 mg/ml solutions: ICR 191 (2-chloro-6-methoxy-9-[3-(2-chloroethyl) aminopropylamino] acridine dihydrochloride) and hycanthone monomethane sulfonate. Results were recorded after incubation of the plates at 37 C for 48 hr in the dark. Each auxotroph was tested with each mutagen on at least five separate occasions.

Transduction. The methods for the preparation of transducing PBS1 lysates and for performing transductions have been described (16).

Pigment extraction. Cells were grown to stationary phase (t = 2 or 3) in 400 ml of minimal medium containing 0.05% acid-hydrolyzed casein. The culture was chilled, washed four to six times with cold 0.1 M sodium phosphate buffer, *p*H 7.0, resuspended in 10 ml of buffer, and incubated at 37 C for 1 hr with lysozyme (200 μg/ml; EC 3.2.1.17), deoxyribonuclease (50 μg/ml; EC 3.1.3.5), ribonuclease (50 μg/ml; EC 2.7.7.16), and 10^{-3} M magnesium sulfate. The crude membranes (yellow-orange in color) were washed eight times on a centrifuge (15,000 × *g*, 30 min, 4 C) with 20-ml portions of cold water. The final membrane pellet was shaken at 4 C with acetone for 2 hr. Insoluble debris was removed by centrifugation (20,000 × *g*, 45 min), and the yellow-orange supernatant fraction was taken to dryness by flash evaporation at 40 C. The pigment was dissolved in a given solvent and scanned over the visible spectrum (300 to 600 nm) in a Cary recording spectrophotometer. The solvent was removed by flash evaporation, and the pigment was dissolved in another solvent and scanned as above. Pigment dissolved in acetone, hexane, or petroleum ether showed a similar absorption spectrum, whereas a shift was observed in chloroform.

Reconstruction experiments. The procedure followed is a modification of that described by Berg (1). Equal numbers (ca. 5×10^8) of early stationary-phase cells (grown at 37 C in Penassay broth) of the two strains to be tested were combined. The cells were diluted 1,000-fold into 2.5 ml of broth and incubated at 37 C with shaking. At 24-hr intervals, the culture was diluted 1,000-fold in broth and incubated as above. At the beginning of the experiment and at each 24-hr interval, viable counts were made (on TBAB). Between 200 and 300 of the resulting colonies were assayed for their nutritional requirements to determine the percentage contribution of each strain to the total cell population.

Bacteriophage induction. The procedure followed is that described by Huang and Marmur (12). Cells were grown at 37 C in Penassay broth to the early sequential growth phase. Mitomycin C was added (2 or 5 μg/ml), and incubation was continued for 10 min. The cells were washed once at room temperature and resuspended to their original volume in broth; incubation was continued at 37 C. Lysis of the culture began within 2 hr. After 5 hr, the culture was centrifuged at low speed, and the supernatant fraction (20 ml) was treated with deoxyribonuclease and ribonuclease (each at 50 μg/ml) at 37 C for 1 hr. The lysate was centrifuged at $40,000 \times g$ for 1.5 hr. The pellet was resuspended in 1 ml of TMA buffer (14). Electron microscopy was performed as previously described (14). Determination of the killing activity of bacteriophage-containing lysates was performed as described by Huang and Marmur (12).

Isolation of spontaneous mutants resistant to bactericidal agents. Streptomycin sulfate (Calbiochem), erthromycin (Sigma Chemical Co.), and rifampin (Calbiochem) were incorporated into TBAB plates. 5-Methyltryptophan (Sigma Chemical Co.) and 5-fluorouracil (Nutritional Biochemicals Corp.) were incorporated into minimal agar containing 0.05% acid-hydrolyzed casein. Approximately 10^8 to 10^9 stationary-phase cells washed with minimal medium were spread onto plates which were then incubated at 37 C for 48 hr.

RESULTS

Spontaneous auxotrophic mutants. Cultures of *B. pumilus* NRRL B-3275 (BpB1) grown to the late exponential or stationary phases of growth contain 1 to 2% auxotrophic mutants. These results are obtained when cells are grown at 25, 30, or 37 C in Penassay broth or L-broth. Examination of at least 1,500 colonies of each of three other strains of *B. pumilus* (NCIB 8600, BD-2002, and ATCC 6631) grown in broth at 37 C has not revealed any spontaneous auxotrophs. Seventy spontaneous auxotrophs were independently isolated from stationary-phase Penassay broth cultures of BpB1 and five auxotrophs of BpB1. After the initial isolation, approximately two-thirds of the mutants were found to revert at such a high frequency as to preclude their study (i.e., >500 revertants per 10^8 cells). Of the remaining 22 mutants, 18 carried requirements for a single amino acid, 2 required two unrelated amino acids, 1 required adenine, and 1 required uracil (Table 2). Each of the nutritional requirements appears to result from a "point" mutation based on the spontaneous reversion frequency (Table 2). Enhanced reversion of approximately one-third of the mutations was obtained in spot tests with NG, EMS, and DES. One was reverted only by 2-aminopurine. The reversion of the remaining mutations was not enhanced by the above mutagens nor by ICR 191, ICR 364-OH, and hycanthone monomethane sulfonate. All but three of the mutations were linked by PBS1 transduction to one of the previously described auxotrophic markers in strain BpB1 (Table 2). No evidence was obtained for clustering of the spontaneous mutations on the BpB1 chromosome.

The results of reconstruction experiments performed with 10 of the spontaneous auxotrophs suggest that the majority of the mutants do not possess a selective growth advantage over the parental strain. The reconstruction experiment with strains BpB1 and 1-11-3 (*lys-3*) gave results (Table 3) similar to those obtained with strains 1-1-4 (*ser-2*) and BpB1, strains 1-1-1 (*cys-2*) and BpB1, strains 113-1-1 (*his-7*) and BpB16 (*argO1*), and strains 231s-1-1 (*lys-2 ade-1*) and BpB231 (*lys-2*). In these experiments, the parent showed a significant growth advantage over the mutant. Reconstruction experiments with strains BpB1 and 1-10-2 (*thr-1*) gave results (Table 4) comparable to those obtained with strains 1-7-6 (*met-3*) and BpB1, strains 1-9-1 (*gly-4*) and BpB1, and strains 6-1-1 (*argO2 his-6*) and BpB17 (*argO2*). In these experiments, neither the parent nor the mutant showed a significant growth advantage. Only 1 of the 10 mutants examined exhibited a significant growth advantage over the parent (Table 5).

Six of the spontaneous mutations conferred a requirement for serine (Table 2). The *ser-4* lesion was linked to *trp-2* by transduction. When PBS1 grown on *ser-4* was used for transduction of ser-1 (16), few or no serine-nonrequiring transductants were obtained (*unpublished data*). These results suggest that the *ser-4* and *ser-1* lesions are closely linked. The remaining five spontaneous serine-requiring mutants were not linked to *trp-2* (nor to *lys-1* and *lys-2*) but were linked to either *argA1* (*ser-*

TABLE 2. *Properties of spontaneous auxotrophs isolated from B. pumilus NRRL B-3275*

Parent	Mutants[a]	Genotype	Spontaneous revertants[b]	Reversion by mutagen[c]				Linked marker[d]	Linkage group[e]
				NG	EMS	DES	2-AP		
BpB1		*wt*		—	—	—	—		
	1-1-4	*ser-7*	24	0	0	0	0	*argA1* (46)	N
	1-5-2	*ser-5*	18	0	0	0	0	*argA1* (31)	N
	1-7-1	*ser-4*	150	0	0	0	0	*trp-2* (59)	O
		his-3	49	0	0	0	0	*gly-1* (4)	L
	1-9-2	*his-4*	98	+	+	+	0	*gly-1* (3)	L
	1-11-3-1	*his-5*	1	+	+	+	0	*gly-1* (5)	L
	1-7-4	*gly-3*	1	0	0	0	0	*his-1* (4)	L
	1-9-1	*gly-4*	2	0	0	0	0	*his-1* (5)	L
	1-7-6	*met-3*	1	0	0	0	0	*argO1* (13)	M
	1-1-1	*cys-2*	5	0	0	0	0	ND[f]	
	1-5-1	*leu-2*	50	0	0	0	0	*phe-1* (55)	N
	1-11-3	*lys-3*	13	+	+	+	0	*trp-2* (50)	O
	1-10-2	*thr-1*	8	+	+	+	0	ND	
BpB231		*lys-2*	2	+	+	+	0		
	231-1-1	*ser-2*	96	0	+	+	0	*argO1* (29)	M
	231-3-5	*ser-6*	4	0	0	0	0	*argA1* (33)	N
	231-1-8	*ser-3*	2	0	+	+	0	*argO1* (30)	M
		leu-3	8	0	0	0	+	*phe-1* (64)	N
	231S-1-1	*ade-1*	54	0	0	0	0	ND	
	231-3-10	*ile-2*	13	0	0	0	0	*trp-2* (25)	O
BpB203		*argA2*	10	+	+	+	0		
	203-1-3	*gly-5*	24	0	0	0	0	*his-1* (7)	L
BpB17		*argO2*	5	0	0	0	0		
	6-1-1	*his-6*	13	0	0	0	0	*gly-1* (6)	L
	6-1-2	*met-4*	1	0	+	+	0	*argO1* (21)	M
BpB16		*argO1*	40	0	0	0	0		
	113-1-1	*his-7*	83	0	0	0	0	*gly-1* (4)	L
1-11-3		*lys-3*	13	+	+	+	0		
	1-11-3W	*ura-2*	4	0	0	0	0	*cys-1* (82)	M

[a] Spontaneous mutants isolated from an auxotroph carry the parents auxotrophic requirement.

[b] Spontaneous revertants per 10^8 cells, determined by plating washed cells in the late log phase (2×10^8 to 20×10^8) on minimal medium appropriately supplemented when the cells have multiple auxotrophic requirements (average of three experiments).

[c] NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; EMS, ethyl methane sulfonate; DES, diethyl sulfate; 2-AP, 2-aminopurine; + indicates that the mutagen enhanced the number of revertants; 0 indicates that the mutagen did not enhance the number of revertants. The following mutagens did not enhance the reversion of any of the spontaneous mutations: ICR 191, ICR 364-OH, and hycanthone monomethanesulfonate.

[d] The numbers in parentheses indicate the percent linkage, i.e., the percentage of the transductants examined which carry the donor auxotrophic marker (16). Each result was obtained by performing reciprocal two-point crosses in which a minimum of 400 transductants was examined.

[e] Four linkage groups (M, N, O, and L) have been established in strain BpB1 by PBS1 transduction (16, 17).

[f] Not determined.

5, *-6*, *-7*) or *argO1* (*ser-2*, *-3*). The latter two genetic classes of serine-requiring mutants have not been described in a species of *Bacillus*. A detailed examination of the chromosomal location and the enzymatic deficiencies of these and several other serine-requiring mutants is in progress. Preliminary results indicate that independently isolated serine-requiring mutations all lie in one of three regions on the BpB1 genome: in linkage group O, N, or M. The order of markers in group N appears to be *ser argA1 leu-1 phe-1*. The order

of markers in group M appears to be *ser argO1 met-1 ura-1 cys-1* (16, 17; *unpublished data*).

The high frequency of occurrence of spontaneous auxotrophic mutants in strain BpB1 suggested the possibility that certain of the mutants previously isolated from this organism after mutagen treatment were of spontaneous origin rather than the result of induced mutagenesis. Mutants isolated from *Escherichia coli* after treatment with NG are enhanced to revert by NG (10). Similarly, of 10 amino acid auxotrophs isolated from *B. pumilus* BD-2002 after mutagenesis with NG, all were revertible in spot tests with NG. By contrast, the reversion of eight amino acid auxotrophs of strain BpB1 (*phe-1, argA1, ilv-1, trp-2, cys-1, his-1, argO2, argO2*; 16, 17) isolated from NG-treated cultures of strain BpB1 was not enhanced by NG. These results suggest that the latter mutants were of spontaneous origin.

Spontaneous pigmented mutants. BpB1 colonies are white to beige on TBAB or minimal medium. Yellow-orange pigmented mutants occur spontaneously in stationary-phase BpB1 cultures at a frequency estimated to be on the order of 1 to 5 mutants per 10^4 cells. Several pigmented mutants have also been isolated during transductions between nonpigmented parents. All *pig* mutants examined (those shown in Table 6) form spores at a frequency comparable to that observed with the nonpigmented BpB1 (approximately 60% of the viable cells are heat-resistant [70 C, 15 min] after 48 hr of incubation at 37 C in Schaeffers medium [20]). Twelve independently isolated *pig* mutants are linked by transduction to *ser-7* (Table 6). Linkage to other auxotrophic markers in our present collection could not be demonstrated. The visible absorption spectrum of the pigment extracted from strain P1 is shown in Fig. 1. The solubility properties in conjunction with the spectral properties suggest that the pigment is a complex carotenoid (8). Pigment extracted from three other mutants (P6, P9, and P11) has spectral properties virtually identical to those of the pigment extracted from strain P1.

Pigmented mutants similar to those described above have not been reported in *B. subtilis*. To determine whether the occurrence of pigmented mutants was unique to strain BpB1, two other strains of *B. pumilus* were examined. Although no spontaneous pigmented mutants were observed during screening of over 60,000 colonies of *B. pumilus* strains BD-2002 and NRS 576, pigmented mutants (yellow-orange) were isolated from

TABLE 3. *Reconstruction experiment with strains BpB1 and 1-11-3*

Day	No. of colonies			
	Total	WT[a]	Lys$^-$[b]	UID[c]
0	312	217	92	3
1	312	296	10	6
2	208	204	0	4
3	208	205	0	3
4	208	205	0	3
5	208	205	0	3
6	208	205	0	3

[a] Wild type; no auxotrophic requirements.
[b] Requires lysine.
[c] Colonies possessing unidentified auxotrophic requirement.

TABLE 4. *Reconstruction experiment with strains BpB1 and 1-10-2*

Day	No. of colonies			
	Total	WT[a]	Thr$^-$[b]	UID[c]
0	208	114	83	11
1	208	110	94	4
2	208	82	123	3
3	208	49	153	6
4	208	55	151	2
5	208	49	150	9
6	208	82	121	5
7	208	45	120	43
8	208	87	115	6

[a] Wild type; no auxotrophic requirement.
[b] Requires threonine.
[c] Colonies possessing unidentified auxotrophic requirement.

TABLE 5. *Reconstruction experiment with strains BpB231 and 231-3-5*

Day	No. of colonies				
	Total	WT[a]	Lys$^-$[b]	Lys$^-$Ser$^-$[c]	UID[d]
0	208	0	106	100	2
1	208	0	63	143	2
2	208	0	27	180	1
3	208	0	11	195	2
4	205	0	1	202	2
5	208	0	1	204	3
6	208	0	1	203	4
7	208	0	0	206	2
8	208	0	3	199	6
9	208	0	2	203	3

[a] Wild type; no auxotrophic requirement.
[b] Requires only lysine.
[c] Requires both lysine and serine.
[d] Possesses unidentified auxotrophic requirement.

TABLE 6. *Linkage between pig mutations and ser-7 determined by PBS1 transduction*

Mutant strain designation	Genotype	Parent and method of isolation[a]	Percent linkage of *pig* with ser-7[b]
P1	*pig-1*	td; BpB1 → BpB5	13 (137/1,054)
P5	*pig-5*	td; 231-1-8 → BpB20	14 (67/464)
P6	*pig-6*	td; 231-1-8 → BpB20	14 (62/445)
P8	*pig-8 argO1*	BpB16	19 (31/164)
P9	*pig-9 lys-2*	BpB231	20 (42/208)
P10	*pig-10*	BpB1	15 (58/379)
P11	*pig-11*	BpB1	24 (35/145)
P12	*pig-12 cys-1*	BpB20	20 (52/259)
P13	*pig-13*	BpB1	15 (160/1,052)
P14	*pig-14 trp-3 cys-1*	BpB635	9 (45/481)
P15	*pig-15 trp-3 cys-1*	BpB635	13 (100/770)
P17	*pig-17 thr-1*	1-10-2	8 (50/640)

[a] The designation td indicates that a *pig* mutant was isolated as a prototrophic transductant; all other *pig* mutants were isolated as spontaneous mutants arising in untreated cultures.

[b] Results of nonreciprocal crosses in which the *pig* cell was donor and 231-1-8 (*ser-7 leu-3 lys-2*) was recipient. Selection was for Ser$^+$. Pig$^+$ transductants were scored visually. Five of the *pig* mutations (1, 5, 6, 8, and 9) also link to *ser-5* (*unpublished data*). The remainder were not tested.

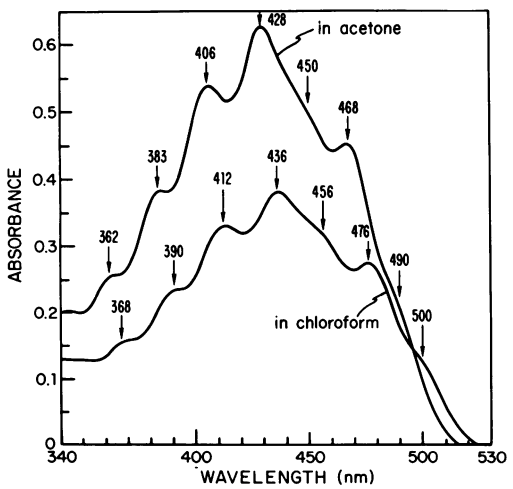

FIG. 1. *Visible absorption spectrum of the pigment extracted from strain P1 in acetone and chloroform. An extract from nonpigmented wild-type strain BpB1 had absorption properties similar to those of the extract of wild-type strain BD-2002 shown in Fig. 2.*

both strains after treatment with NG. The absorption spectrum of the pigment extracted from one of the mutants isolated from strain BD-2002 (Fig. 2) suggests that the pigment is a carotenoid.

Spontaneous mutants resistant to antibacterial agents. The frequency of occurrence of spontaneous mutants of *B. pumilus* strains ATCC 6631, BpB1, and NCIB 8600 resistant to streptomycin, erythromycin, rifampin, 5-fluorouracil, and 5-methyl tryptophan was determined. For each antibacterial agent, the frequency of occurrence of spontaneous resistant mutants was comparable for all three strains (Table 7).

Inducible bacteriophage in strain BpB1. In light of the observations that two temperate phages can cause mutations in *E. coli* (3, 21), strain BpB1 was examined for the presence of a temperate phage. Treatment of exponentially growing BpB1 cultures with mitomycin C (2 or 5 μg/ml) as described by Huang and Marmur (12) induced lysis within 2 hr (Fig. 3). Comparable results were obtained with both concentrations of mitomycin C. Electron microscopy of the lysate before and after nuclease digestion and after concentration by ultracentrifugation showed only a single morphological type of bacteriophage-like particle (Fig. 4) which resembles the defective phages of *B. subtilis* (19) and *B. licheniformis* (12). The crude lysates exhibited killing activity against strains of *B. pumilus* and *B. subtilis* (Table 8), but plaque formation could not be demonstrated. Several mutants of *B. subtilis* 168 selected for resistance to the virulent bacterio-

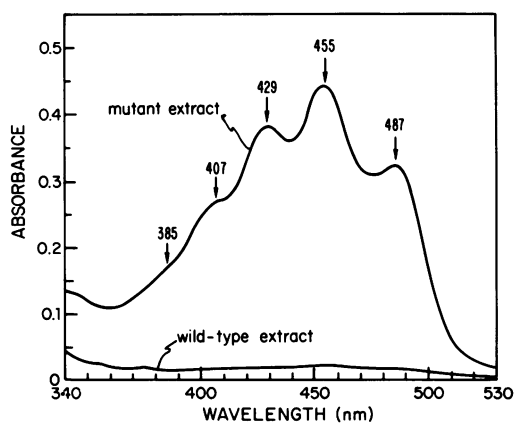

FIG. 2. *Visible absorption spectrum of the pigment extracted from a pigmented mutant of B. pumilus BD-2002 and the nonpigmented wild-type parent. The solvent was acetone.*

TABLE 7. *Frequency of occurrence of spontaneous mutants of B. pumilus strains BpB1, ATCC 6631, and NCIB 8600 resistant to streptomycin, erthromycin, rifampin, 5-fluorouracil, and 5-methyltryptophan*

Agent	Concn (μg/ml)	Resistant mutants per 10^8 cells[a]		
		BpB1	ATCC 6631	NCIB 8600
Streptomycin sulfate	10	15	26	30
	50	3	9	11
	100	3	8	12
Erythromycin	1	0.5	1	0.5
Rifampin	1	93	106	83
	10	65	57	46
	50	58	45	39
5-Fluorouracil	10	311	294	282
	25	296	295	241
	100	187	217	222
5-Methyltryptophan	500	7	30	2

[a] Average of two or three experiments.

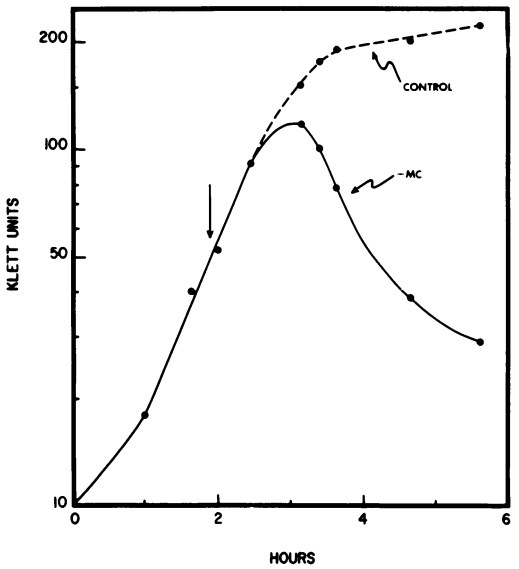

FIG. 3. *Lysis of strain BpB1 after treatment with mitomycin C. A 40-ml PB medium culture of strain BpB1 was grown at 37 C to a turbidity of 50 Klett units (Klett-Summerson colorimeter, filter no. 66) and divided in half (at arrow). One portion was treated with mitomycin C (5 μg/ml). The remainder of the procedure is described in Materials and Methods. One Klett unit equals 1 (±0.5) × 10^7 cells/ml.*

phage φ29 are resistant to the killing activity of mitomycin C-induced BpB1 cultures. This may indicate that φ29 and the bacteriophage-like killer particle induced from strain BpB1

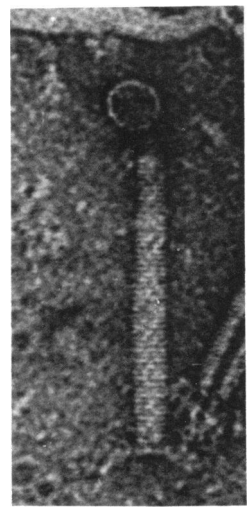

FIG. 4. *Bacteriophage induced from strain BpB1 by mitomycin C. A mitomycin C-induced culture of strain BpB1 was concentrated as described in Materials and Methods and stained with 1% uranyl acetate as previously described (14). ×197,000.*

TABLE 8. *Killing activity of a bacteriophage-like particle induced from strain BpB1*

Bacterium	Killing activity[a]
B. pumilus	
NRRL B-3275 (BpB1)	0
ATCC 6631	+
NCIB 8600	+
ATCC 1	+
ATCC 14884	0
B. subtilis	
168	+
168/29	0
W-23	+

[a] Symbols: +, killing activity observed; 0, no killing activity observed.

share a common receptor site on *B. subtilis* 168 (24).

Strains of *B. pumilus* other than strain BpB1 harbor inducible phage-like killer particles but do not exhibit a high rate of spontaneous mutation (e.g., strains ATCC 6631 and NCIB 8600; *unpublished data*). Therefore, a correlation cannot be established between the presence of an inducible phage and the high rate of mutation of strain BpB1.

DISCUSSION

During the isolation of mutants from strain BpB1 for use in genetic studies (16, 17), it became evident that a variety of spontaneous

mutations arose in this strain at a frequency significantly higher than that observed in other strains of *B. pumilus*. The present study was undertaken to examine the frequency of the occurrence and the properties of two types of spontaneous mutants arising in strain BpB1. However, it should be noted that other types of spontaneous mutants have been isolated, including sporulation-deficient variants and those with altered colonial morphology.

The results of the studies with the spontaneous auxotrophs demonstrate several general properties of this class of mutations. All revert spontaneously, suggesting that each results from a single point mutation. The chromosomal location of the mutations does not indicate any clustering on the BpB1 genome. It seems more likely that the isolation of spontaneous mutants from BpB1 is due to a high mutation rate rather than to an overgrowth of a few mutants in the cultures, since 9 of the 10 mutants examined do not possess a selective growth advantage over the parents. Since spontaneous mutations conferring resistance to several antibiotics do not occur at a high frequency relative to that observed in other strains of *B. pumilus*, it appears that only certain regions or sites on the BpB1 genome exhibit a high rate of spontaneous mutation. Attempts to classify the mutations by the ability of different mutagens to enhance their reversion indicates that at least two types of mutants were isolated: those which are reverted by NG, by EMS and DES, or by all three mutagens, and those which are not reverted by these mutagens. None of the mutations was reverted by mutagens which induce (and revert) frameshift mutations in *E. coli* (2) and *Salmonella typhimurium* (11, 18).

The basis for the high mutation rate in strain BpB1 is unknown, although possible mechanisms are suggested by studies in other systems. Errors in deoxyribonucleic acid (DNA) replication appear to be responsible for the high rate of spontaneous mutation exhibited by mutants of *B. subtilis* (9) and bacteriophage T4 (6). Similarly, cultures of mutants of *E. coli* deficient in DNA polymerase 1 activity (*polA*) contain a high percentage of spontaneous auxotrophic mutants (1, 5). In the case of the *polA* mutants, however, the high frequency of occurrence of certain of the auxotrophic mutations appears to be due, at least in part, to a selective growth advantage over the parent (1). Several so-called mutator strains have been isolated from *E. coli* (7, 13, 22). The type of mutation appearing in one of these mutator strains has been identified (23), although the mechanism(s) inducing the mutations is not known. Since the elevated rate of mutation in strain BpB1 is limited to certain sites or regions of the genome, errors in the DNA replication process would have to exhibit some specificity. For example, spontaneous mutations (replication errors) might occur only in regions of a particular nucleotide sequence.

In spite of the apparent genetic instability of strain BpB1, no difficulty has been encountered in maintaining the wild-type parent or mutant cultures. It is unlikely that the mutation rate of strain BpB1 has significantly affected the results of the genetic crosses previously reported (16, 17) for the following reasons. First, the methods for selecting transductants prevent the growth of virtually all auxotrophic cells. Second, linkage values previously reported have been obtained on several occasions and reciprocal crosses show similar recombination frequencies between linked loci. Difficulty has, however, been encountered in determining the order of closely linked loci by three-factor crosses. Whether this difficulty reflects the genetic instability of strain BpB1 or is attributable to the transducing phage PBS1 is not known. If the latter is true, the availability of a second transducing phage for strain BpB1 may allow this difficulty to be resolved (14).

Of 32 strains of wild-type *B. pumilus* acquired from several sources (14), none is pigmented. Three of these strains possess the latent biosynthetic capacity to produce pigment as evidenced by the isolation of the *pig* class(es) of mutants. The mutation(s) resulting in pigmentation could involve an alteration of a single enzyme or the derepression of genes for several enzymes. At present, there are insufficient data to explain the biochemical basis of this class of mutation. The only physiological difference detected between the *pig* mutants and their nonpigmented parents is the extent of killing induced by visible light in the presence of the photosensitizing dye toluidine. The *pig* mutants appear more resistant than the nonpigmented parents (Lovett, *unpublished data*).

Without prior mutagenesis, BpB1 rapidly "adapts" to growth as osmotically fragile spherical bodies ("L-forms") during cultivation in liquid media containing 7% NaCl (4). Although apparently similar variants of *B. subtilis* have been isolated from nontreated and NG-treated cultures (25), these are more difficult to obtain than the "L-forms" of strain

BpB1 (Young, *personal communication*). Based on the observations described in the present work, it seems possible that the ease in isolation of "L-forms" from strain BpB1 may be related, in part, to the elevated rate of mutation of this organism. That is, the selective conditions of the growth medium containing a high salt concentration may allow a particular class of spontaneous mutant to proliferate. If this is true, mutants of strain BpB1 which do not exhibit a high rate of spontaneous mutation may adapt less readily to the L-form state. However, attempts to isolate such mutants have thus far been unsuccessful.

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