5-Bromouracil-Tolerant Mutants of Bacillus subtilis

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5-Bromouracil (BU)-tolerant mutants of Bacillus subtilis 23 (thy his) have been isolated. Several classes of tolerant mutants were obtained by a sequential selection procedure. The classes can be distinguished by their relative BU tolerance as well as several other phenotypic characteristics. The mutants can grow for an extended period of time in minimal medium supplemented with amino acids and BU, in which the sensitive parental strain (BU+) undergoes rapid cell death. Both mutants But-1 and But-1310 have a greater rate of deoxyribonucleic acid (DNA) synthesis by a factor of two in the presence of BU than BU+, But-1 being somewhat faster than But-1310. The preferential incorporation of thymine to BU of But-1 is about half that of the Bu+ strain during DNA replication in minimal medium supplemented with 10 μg of BU/ml and 1 μg of thymine/ml. It is not known at what step or steps this reduction in selectivity occurs.

5-Bromouracil (BU) has been used extensively for the density labeling of deoxyribonucleic acid (DNA). Its specificity as a precursor for DNA synthesis and its relative low cost make it an ideal analogue, but its extreme toxicity can be a serious disadvantage. If one is interested in studying some aspect of DNA metabolism by density labeling, ideally the analogue should not interfere with DNA metabolism or cell viability. This is clearly not the case with BU (4, 5, 7). Growth of thymine-requiring Bacillus subtilis in the presence of BU is accompanied by a reduced rate of DNA synthesis and loss of viability. It was therefore of interest to obtain mutants which, although still incorporating BU into their DNA, were less susceptible to its toxic effects.

We have obtained several BU-tolerant mutants of B. subtilis. Two of these mutants show an improved rate of DNA synthesis in the presence of BU. One of these mutants (But-1310) has been used for a study of the symmetric nature of DNA synthesis in B. subtilis (10).

MATERIALS AND METHODS

Bacterial strains. The BU-tolerant mutants were derived from B. subtilis 23 (thy his; from F. Rothman); strains 168 Mu8u5u16 (leu8 met5 ade16) and 168 Mu8u5u6 (leu8 met5 ade6) from this laboratory were used as recipients for transformation.

Media. Medium C (Spizizen minimal salts medium) (1) contains, per liter: 14.0 g of K2HPO4, 6.0 g of KH2PO4, 2.0 g of (NH4)2SO4, 1 g of C4H7NaO7, 0.17 g of MgSO4·7H2O, and 5 g of glucose. Medium C+ is medium C supplemented with 500 μg of Casamino Acids (Difco Labs.), 50 mg of L-tryptophan, 50 mg of base requirement, and 100 mg of amino acid requirement, per liter. Medium G is medium C supplemented, per liter, with 100 mg of alanine, 500 mg of Casamino Acids, 200 mg of yeast extract, and 5 g of glucose.

Preparation of spores. Spores were prepared by plating 0.3 ml of a mid-exponential culture onto TBA (tryptose blood agar base, Difco) plates supplemented with 10 μg of thymine/ml. Spores were harvested 2 to 5 days after plating. For prelabeling spores, 0.17 μCi of 14C-thymine/ml (thymine·2-14C, 55.8 μCi/μmole, New England Nuclear Corp.) was added to TBA plates supplemented with 5 μg of unlabeled thymine/ml. The cultures used to inoculate the TBA plates contained the same concentration of 14C-thymine.

Growth experiments. A 5.0-ml overnight culture was filtered through a 55-nm membrane filter (Millipore Corp.) with a 0.22-μm pore size (GSW P05500). The cells were then washed with 95 ml of prewarmed (37°C), unsupplemented C medium. The cells were suspended from the filter and diluted into the growth medium.

The samples taken for viable counts were diluted in Penassay medium (1.2%) and plated onto minimal plates supplemented with histidine (50 μg/ml) and thymine (10 μg/ml).

Incorporation experiments. The cultures for the incorporation experiments were prepared identically to those for the growth experiments with the addition
of thymine-2-14C (55.8 μCi/mmole, New England Nuclear Corp.) and 5-bromouracil-6-3H (10.55 Ci/mmole, New England Nuclear Corp.). Samples (0.2 ml) were taken at the desired intervals and pipetted into 1.0 ml of 1 N KOH, and the samples were then kept on ice. After the last sample was taken, all the samples were incubated at 37°C for 120 min. This treatment completely hydrolyzes the ribonucleic acid. After KOH treatment, the samples were precipitated with two volumes of cold 10% trichloroacetic acid containing 100 μg of thymine/ml and 100 μg of 5-bromouracil-6-3H per ml. The samples were filtered onto glass filters (Whatman GF/A; 2.7 cm) which were presoaked in 10% trichloroacetic acid containing BU and thymine. The filters were washed with 10 to 15 ml of 10% trichloroacetic acid and then washed with 5 to 10 ml of 50% ethanol, dried, and placed in scintillation vials containing 5.0 ml of scintillation fluid. The samples were counted in a Packard Tri-Carb scintillation counter.

Synchronous DNA replication during spore germination and outgrowth. Germination and outgrowth of spores were monitored by following the absorbance of the culture with a colorimeter (Klettfilter no. 66). Samples (20–30 ml) were taken at the times indicated in Table 2 and Fig. 2 and immediately killed by heating at 70°C for 10 min. Cell lysates were prepared as previously described (9). CsCl (1.91 g) was added to 1.5 ml of each lysate, and 1.5 ml of this solution (density = 1.704) was used as the middle layer of a three-layered, preformed gradient (3). CsCl solutions of density 1.646 and 1.762 were used as top and bottom layer, respectively, (1.5 ml each). The gradients were centrifuged at 35,000 rev/min in a Beckman model L2 ultracentrifuge in either a Spincno SW39 or SW50.1 rotor for 20 to 30 hr at 25°C. After centrifugation, the bottoms of the tubes were punctured, and the fractions were collected in either sterile screw-cap tubes (for transformation) or 10-mm test tubes (for precipitation). A 1.0-ml amount of sterile SSC (0.15 M NaCl, 0.015 M C6H5Na3O7·7H2O, pH 7.0) was added to the fractions collected for transformation. Those in test tubes were precipitated with cold 10% trichloroacetic acid.

Transformation. The Bott and Wilson method (2) was used for transformation. A 0.1-ml sample of the diluted gradient samples was added to 1.0 ml of competent recipient cells. A 0.1-ml amount of this culture was plated onto selective plates.

Caffeine resistance. Overnight cultures were diluted and plated onto TBA plates supplemented with varying concentrations of caffeine (Eastman Kodak Co.). The plates were incubated at 37°C, and colonies were counted within 24 hr.

UV experiments. A 5.0-ml overnight culture in C medium supplemented with 5 μg of thymine/ml was centrifuged, and the pellet was suspended in 15 ml of C salts. The cells were irradiated in a glass petri dish which was rotated at 90 cycles per min on an Eberbach Rotator table. The ultraviolet light (UV) source was an unfiltered germicidal G8T5 lamp (General Electric) 60 cm from the surface of the petri dish. Samples were diluted in Penassay broth and plated on TBA plates. The plates were incubated at 37°C in the dark.

**RESULTS**

Selection of BU-tolerant mutants. The initial tolerant mutants were obtained by UV irradiation of *B. subtilis* 23 (thy his) on minimal plates supplemented with 50 μg of histidine/ml, 25 μg of BU/ml, and 1 μg of thymine/ml. The parental strain (Bu+) will not grow at this BU/thymine ratio. This treatment produced a class of mutants that will grow on plates with a BU to thymine ratio of 40 μg to 1 μg per ml. Successive selections on BU-histidine plates produced several additional mutants exhibiting improved growth on plates in the presence of BU. There are at least three distinct classes of mutants which differ not only in their ability to grow in the presence of BU, but in several other phenotypic characteristics. None of the mutants can grow for more than a few generations in the presence of BU alone if their pools of thymine are washed out beforehand. Two of the mutants (But-1 and But-1310) isolated by this procedure will be discussed.

Growth in the presence of BU. The BU-tolerant mutants are capable of growth in the C+ medium supplemented with 10 μg of BU/ml and 1 μg of thymine/ml (Fig. 1A). In the same medium, the BU-sensitive parental strain (Bu+) undergoes rapid cell death (accompanied by lysis) after a slight increase in cell number.

How well these strains grow in the presence of BU is dependent upon the BU to thymine ratio and the absolute concentration of thymine. All the strains tested grow better in the C+ medium supplemented with 50 μg of BU/ml and 5 μg of thymine/ml than in the C+ medium supplemented with 10 μg of BU/1 μg of thymine. Although mutant But-1310 has a lower doubling time and grows to a higher cell density than Bu+, Bu+ does increase in cell number and shows no loss of viability up to a 10-hr period. But-1 was not tested under these conditions.

The BU tolerance of these mutants is transient. Although the mutants initially increase in cell number in the presence of BU, they show some loss of viability upon prolonged exposure. In the experiment described in Table 1, the cells were grown at 37°C in C+ supplemented with either 10 μg of BU/ml and 1 μg of thymine/ml or 10 μg of BU/ml alone. Viable counts were determined at 0, 12, and 24 hr by plating on minimal plates supplemented with 50 μg of histidine/ml and 10 μg of thymine/ml. The number of non-thymine-requiring revertants (which are unaffected by the presence of BU) was also determined. As can be seen from Table 1, both mutants But-1
and But-1310 grown in C+ supplemented with 10 \( \mu g \) of BU/ml and 1 \( \mu g \) of thymine/ml show an increase in cell number after 12 hr, but both decrease in cell number over the next 12 hr period. Bu+ loses viability during the first 12 hr and is completely overgrown by non-thymine-requiring revertants by 24 hr. Very few non-thymine-requiring revertants appear in the But-1 and But-1310 cultures. When grown in the presence of BU alone, both tolerant mutants lose viability during the first 12 hr and continue to decrease in cell number during the next 12 hr. Again Bu+ is much more sensitive to BU and is eventually overgrown with non-thymine-requiring revertants. In both media, mutant But-1 is more tolerant than But-1310.

The BU tolerance of these mutants is also temperature sensitive. The mutants grow normally at 45 C in the presence of thymine (without BU), but they are considerably more sensitive to BU at that temperature (Fig. 1B). The BU-sensitivity of Bu+ is also increased at 45 C. The cause of the temperature sensitivity is not understood.

**DNA synthesis in the presence of BU.** Germinating spores of *B. subtilis* replicate their chromosomes synchronously (8, 9). Replication starts at a fixed origin and continues sequentially to the terminus (8). The germination of thymine-requiring spores in BU media provides a system for genetic mapping, since the order of transfer of genetic markers from light-light to heavy-light DNA (as determined by transformation) reflects their relative position on the chromosome. The success of this technique depends upon the completeness and maintenance of the synchrony of chromosome replication. The breakdown of synchrony observed when Bu+ spores are germinated in BU media limits the usefulness of BU as a density label for this type of mapping.

To characterize the DNA synthesis of the mutants in the presence of BU, spores of mutants But-1, But-1310, and Bu+ were prepared with their DNA uniformly labeled with 14C-thymine. The prelabeled spores were germi-

Fig. 1. Viable cell increase in the presence of BU. Overnight cultures in C+ medium plus 5 \( \mu g \) of thymine/ml were filter-washed with 20 volumes of prewarmed unsupplemented C+ medium. The washed cells were suspended in an equal volume of unsupplemented C+ medium and diluted 20-fold into C+ supplemented with 10 \( \mu g \) of BU/ml and 1 \( \mu g \) of thymine/ml. Samples were diluted in Penassay broth and plated on minimal plates supplemented with 50 \( \mu g \) of histidine/ml and 10 \( \mu g \) of thymine/ml to determine viable counts. The cells were grown at 37 C (A) and 45 C (B).
nated in C\(^+\) medium supplemented with 10 \(\mu g\) of BU/ml and 1 \(\mu g\) of thymine/ml. Samples were taken at 100, 120, 140, and 160 min after the initiation of germination. The samples were lysed, and the lysates were run in CsCl density gradients. The rate of DNA synthesis was measured by the rate of transfer of \(^{14}\)C-thymine from the light-light to heavy-light DNA (Fig. 2). Both But-1 and But-1310 have a greater rate of DNA synthesis in the presence of BU than Bu\(^-\), But-1 being somewhat faster than But-1310. The transfer of genetic markers from light-light to heavy-light DNA was determined for several similar germination experiments. The transfer of markers shows a similar relationship among the strains as the \(^{14}\)C-radioactivity transfer experiment (Table 2) described above. As is evident from the result, the rate of transfer of markers by the tolerant mutants is generally greater than that of Bu\(^-\).

During DNA synthesis in B. subtilis, thymine is selectively incorporated in preference to BU (6). Figure 3A illustrates that Bu\(^-\) grown in C\(^+\) medium with 10 \(\mu g\) of BU/ml and 1 \(\mu g\) of thymine/ml exhibits a preference for thymine over BU, and that this preference is significantly reduced in mutant But-1310 and more so in But-1. The thymine preference of all strains is reduced at 45 C (Fig. 3B), but the same relationship among the strains is maintained. Mutant But-1310 also has a reduced thymine preference which is intermediate between mutants Bu\(^-\) and But-1.

**Table 1. The growth of mutants Bu\(^+\), But-1, and But-1310 in C\(^+\) medium**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Strain*</th>
<th>Viable cells/ml</th>
<th>BU alone</th>
<th>BU/thymine (10/1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thymine histidine plates</td>
<td>Histidine plates (Thy(^+) revertants)</td>
<td>Thymine histidine plates</td>
</tr>
<tr>
<td>0</td>
<td>Bu(^-)</td>
<td>1.3 \times 10^7</td>
<td>5</td>
<td>1.5 \times 10^7</td>
</tr>
<tr>
<td></td>
<td>But-1</td>
<td>2.6 \times 10^7</td>
<td>5</td>
<td>2.1 \times 10^7</td>
</tr>
<tr>
<td></td>
<td>But-1310</td>
<td>2.9 \times 10^7</td>
<td>5</td>
<td>2.8 \times 10^7</td>
</tr>
<tr>
<td>12</td>
<td>Bu(^-)</td>
<td>2.1 \times 10^4</td>
<td>6.9 \times 10^3</td>
<td>3.1 \times 10^3</td>
</tr>
<tr>
<td></td>
<td>But-1</td>
<td>5.6 \times 10^7</td>
<td>0</td>
<td>6.7 \times 10^7</td>
</tr>
<tr>
<td></td>
<td>But-1310</td>
<td>2.4 \times 10^8</td>
<td>0</td>
<td>3.6 \times 10^7</td>
</tr>
<tr>
<td>24</td>
<td>Bu(^-)</td>
<td>5.4 \times 10^7</td>
<td>5.2 \times 10^7</td>
<td>1.8 \times 10^8</td>
</tr>
<tr>
<td></td>
<td>But-1</td>
<td>5.6 \times 10^5</td>
<td>25</td>
<td>2.1 \times 10^7</td>
</tr>
<tr>
<td></td>
<td>But-1310</td>
<td>4.3 \times 10^3</td>
<td>0</td>
<td>1.0 \times 10^7</td>
</tr>
</tbody>
</table>

*Overnight cultures in C\(^+\) medium plus 5 \(\mu g\) of thymine/ml were filter-washed with 20 volumes of prewarmed unsupplemented C\(^+\) medium. The washed cells were then resuspended in an equal volume of unsupplemented C\(^+\) and diluted 20-fold either in C\(^+\) supplemented with 10 \(\mu g\) of BU/ml and 1 \(\mu g\) of thymine/ml or 10 \(\mu g\) of BU/ml. At 0, 12, and 24 hr, samples were diluted in Penassay and plated on minimal plates supplemented with 50 \(\mu g\) of histidine/ml and 10 \(\mu g\) of thymine/ml. Samples were also plated on minimal plates supplemented with histidine alone to determine the number of non-thymine-requiring revertants.

*All these strains carry thy and his markers.

**Fig. 2. The percentage of \(^{14}\)C-thymine counts per minute in heavy-light DNA versus time after initiation of germination.** Spores with their DNA uniformly labeled with \(^{14}\)C-thymine were germinated in C\(^+\) medium supplemented with 10 \(\mu g\) of BU/ml and 1 \(\mu g\) of thymine/ml. At 100, 120, 140, and 160 min after the initiation of germination, samples were withdrawn, lysed, and run in CsCl equilibrium gradients. The gradient fractions were assayed for cold trichloroacetic acid precipitable \(^{14}\)C counts per minute (see Materials and Methods).

Other phenotypic characteristics of BU-tolerant mutants. The BU-tolerant mutants exhibit several phenotypic characteristics other than BU tolerance. Both mutants But-1 and But-1310 are resistant to caffeine, and...
Table 2. Summary of transformation data from density transfer experiments of germinating spores of Bu* and But-1310 in BU and bromodeoxyuridine (BUdR) media

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Time* (min)</th>
<th>Percent total transformants*</th>
<th>ade16</th>
<th>ade6</th>
<th>leu</th>
<th>met</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bu*</td>
<td>C+ BUdR (10/0)</td>
<td>250</td>
<td>HH 4 16 80 0 10 90 3 97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>But-1310</td>
<td>C+ BUdR (10/0)</td>
<td>250</td>
<td>HH 21 57 22 5 26 69 2 18 80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bu*</td>
<td>BUG</td>
<td>120</td>
<td>HH 0 45 55 0 5 95 0 0 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>But-1310</td>
<td>BUG (10/1)</td>
<td>120</td>
<td>HH 1 91 8 1 76 23 0 3 97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bu*</td>
<td>BUG (10/1)</td>
<td>165</td>
<td>HH 10 63 23 0 12 88 0 0 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>But-1310</td>
<td>BUG (10/1)</td>
<td>120</td>
<td>HH 6 86 8 0 93 7 0 34 66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bu*</td>
<td>BUG (25/2.5)</td>
<td>90</td>
<td>HH 0 20 80 0 14 86 0 0 100  0 0 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>But-1310</td>
<td>BUG (25/2.5)</td>
<td>90</td>
<td>HH 0 63 37 0 21 79 0 0 100  0 0 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bu*</td>
<td>BUG (25/2.5)</td>
<td>120</td>
<td>HH 6 92 2 0 56 44 0 0 100  0 0 100</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>But-1310</td>
<td>BUG (25/2.5)</td>
<td>120</td>
<td>HH 11 84 5 0 77 23 0 0 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genetic locations of the markers are:

<table>
<thead>
<tr>
<th>ade6</th>
<th>leu</th>
<th>met</th>
</tr>
</thead>
<tbody>
<tr>
<td>ade16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>0.07</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Spores of Bu* and But-1310 were germinated in various media, and samples were taken at several times after the initiation of germination. The samples were lysed and run in CsCl equilibrium density gradients (see Materials and Methods). The transforming activity of the light-light (LL), heavy-light (HL), and heavy-heavy (HH) DNA peaks was determined for several genetic markers.

*Time after germination of spores.

C+ medium supplemented with 10 μg of BUdR/ml.

G medium supplemented with 10μg of BUdR/ml and 1 μg of TdR/ml.

G medium supplemented with 25 μg of BU/ml and 2.5 μg of thymine/ml.

stationary cultures of these mutants are more UV resistant than Bu+ (Fig. 4 and 5). The relationship among the mutants and the parental strain for caffeine and UV resistance parallels that of their BU tolerance (But-1 > But-1310 > Bu+).

DISCUSSION

This report describes the isolation and partial characterization of two BU-tolerant mutants of B. subtilis. These mutants continue to incorporate at least as much BU into their DNA as the parental strain. Although we have not obtained a mutant that has completely overcome the toxic effects of BU, the BU-tolerant mutants that we have isolated are considerably less sensitive to BU than the parental strain.

At this time, the nature of the mutation or mutations leading to BU tolerance is unknown. Of particular interest are the changes in the ratio of BU to thymine incorporation into DNA and the increased rate of DNA synthesis in the presence of BU exhibited by the mutants. The determination of the level or levels of DNA metabolism at which these changes take place is under investigation.

The BU-tolerant mutants described here should prove useful for a wide variety of experiments requiring the specific density labeling of DNA. In particular, since these mutants transfer genetic markers from light-light to heavy-light DNA more rapidly and completely

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*BISHOP AND SUEOKA J. Bacteriol.
BU-TOLERANT MUTANTS OF B. SUBTILIS

1.0
0.8
0.6
0.4
0.2
0

THYMINE/BU MOLAR RATIO

37 °C

Bu+
But-1310
But-1

INPUT RATIO

TIME (hrs)

Fig. 3. Selective incorporation of thymine over BU into DNA. Overnight cultures were filter-washed with 20 volumes of unsupplemented C+ medium. The washed cells were suspended in an equal volume of unsupplemented C- medium and diluted into C- medium supplemented with 10 μg of BU (3H)/ml and 1 μg of thymine (14C)/ml, which makes an input molar ratio (thymine to BU) of 0.15. At various times, samples were withdrawn, KOH treated, and precipitated with cold trichloroacetic acid (see Materials and Methods for details). The cells were grown at 37 C (A) and 45 C (B).

during spore germination in the presence of BU than does the BU-sensitive parental strain, they should provide a system for obtaining refined density transfer mapping data. A successful use of mutant But-1 has already been reported in a study of symmetry in chromosome initiation (10).

ACKNOWLEDGMENTS

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Fig. 4. Survival of strains Bu+, But-1, and But-1310 on caffeine plates. Overnight cultures in C- medium plus 5 μg of thymine/ml were diluted and plated directly on TBA plates supplemented with varying concentrations of caffeine. The plates were incubated at 37 C and counted within 24 hr.

Fig. 5. UV killing of stationary cultures of Bu+, But-1, and But-1310. Survivors (%) versus UV dose (sec) is given. A 5-ml overnight culture in C- plus 5 μg of thymine/ml was centrifuged, and the pellet was suspended in 15 ml of C salts (10). The cells were irradiated in a glass petri dish with an unfiltered germicidal G8T5 lamp. Samples were diluted in Penassay broth and plated on TBA plates. The plates were incubated at 37 C in the dark.
We are grateful for the gift of dBUTP by A. Kornberg. We also thank J. L. Arceneaux for his cooperation in the further synthesis of dBUTP and also ¹⁴C-dBUTP, and C. S. H. Young for his helpful discussions.

LITERATURE CITED