Tolerance to Bromodeoxyuridine in a Thymidine-requiring Strain of *Bacillus subtilis*

By J. G. COOTE* AND C. BINNIE†

Microbiology Department, University of Glasgow, Garscube Estate, Bearsden,
Glasgow G61 1QH, UK

(Received 7 May 1985; revised 4 September 1985)

Mutations which allow tolerance to 5-bromo-2'-deoxyuridine (BUdR) in a thymidine (TdR)-requiring strain of *Bacillus subtilis* have been examined. Differences in sensitivity to BUdR existed between isogenic strains harbouring the mutations. Those mutations originally isolated as BUdR-tolerant also bestowed tolerance to 5-bromouracil and vice versa. The strain exhibiting the greatest tolerance to BUdR maintained a normal rate of replication in the presence of BUdR whereas the parent strain did not, but the tolerant strain incorporated less analogue into DNA than the parent strain. The basis of the tolerance mutation appeared to lie at the point of uptake of the analogue into the cell as the tolerant mutant preferentially took up TdR over BUdR into whole cells. DNA polymerase activity measured *in vitro* did not distinguish between TdR and BUdR in either the parent or the mutant strain and although TdR kinase activity showed a preference for TdR over BUdR as a substrate, the extent of discrimination was similar in both strains.

INTRODUCTION

5-Bromo-2'-deoxyuridine (BUdR) and 5-bromouracil (BU), analogues of thymidine (TdR) and thymine (T) respectively, can be incorporated into DNA with a consequent increase in buoyant density of the DNA. This can be used to discriminate between newly synthesized and template DNA during chromosome replication. This approach has been used extensively in eukaryotes, but in bacteria wild-type strains do not take up the analogues to any significant extent, and T- or TdR-requiring strains grown in the presence of analogues show a reduced growth rate and loss of viability (Cohen & Barner, 1956; Huang *et al.*, 1968).

In an attempt to overcome this problem, Bishop & Sueoka (1972) isolated several BU-tolerant mutants of *Bacillus subtilis* 23 (*thyA thyB* His⁻). These were considerably less sensitive than the parent T-requiring strain to the toxic effects of BU during growth, and were used in density transfer experiments concerned with replication of the *B. subtilis* chromosome (Quinn & Sueoka, 1970; O'Sullivan *et al.*, 1975). Coote (1977) isolated a BUdR-tolerant derivative of *B. subtilis* 168 (*thyA thyB trpC2*) and a derivative isogenic with the parent strain, apart from the tolerance mutation (*but-32*), was used to examine DNA replication during sporulation (Coote & Binnie, 1981; Binnie & Coote, 1983). In this report, the growth characteristics of four BUdR-tolerant and two BU-tolerant mutations in an isogenic *thyA thyB* background are compared. In addition, data are presented for one of the strains which suggest that the physiological basis of the tolerance mutation lies primarily at the point of uptake of the analogue into the cell.

---

† Present address: Genetics Department, University of Glasgow, Church Street, Glasgow G11 5JS, UK.

Abbreviations: T, thymine; TdR, thymidine; dTTP, thymidine triphosphate; BU, 5-bromouracil; BUdR, 5-bromo-2'-deoxyuridine; dBUTP, 5-bromo-2'-deoxyuridine triphosphate; U, uridine; dC, deoxycytidine; dCTP, deoxycytidine triphosphate; MM, minimal medium; CH, casein hydrolysate.

METHODS

Strains. *B. subtilis* 168 (*thyA thyB trpC2*) is referred to as the parent strain. A Trp⁺ derivative of this strain was isolated after transformation with DNA prepared from *B. subtilis* BD112 (*cysA14*) and selection of Trp⁺ transformants on minimal medium (MM) agar supplemented with TdR (10 µg ml⁻¹) (see below). A BUdR-tolerant derivative (BUt32) of *B. subtilis* 168 (*thyA thyB trpC2*) was isolated by Coote (1977). A BU-tolerant derivative (VUB112) of the same parent strain was supplied by H. de Lencastre (Instituto Gulbenkian de Ciencia, Oeiras, Portugal) and a BU-tolerant derivative (BUt23.1) of *B. subtilis* 23 (*thyA thyB His⁻*) was supplied by N. Sueoka (University of Colorado, CO 80309, USA). Strains BD112 (*cysA14*) and Mu8u5u16 (*purA16 leu-8 metB5*) were from P. Piggot, NIMR, Mill Hill, London, UK).

Media. MM was the resuspension medium of Sterlini & Mandelstam (1969) without the excess MgSO₄ and supplemented with 0.2% (w/v) D-glucose. When necessary, this medium was solidified with 1.5% (w/v) purified agar (Oxoid). CH medium was the casein hydrolysate medium of Sterlini & Mandelstam (1969) supplemented with auxotrophic requirements at 20 µg ml⁻¹ except where stated.

Growth conditions. A 0.2 ml sample from an overnight culture in 20 ml CH medium supplemented with TdR (8.5 µg ml⁻¹) was passed through a membrane filter (Oxoid, 0.45 µm pore size), washed with prewarmed (35 °C) CH medium and resuspended in 20 ml fresh CH medium plus the necessary additions. Growth was measured spectrophotometrically at 600 nm using a 1 cm light path.

Isolation of mutants. *B. subtilis* 168 (*thyA thyB trpC2*) growing exponentially in CH medium supplemented with L-tryptophan and TdR (8.5 µg ml⁻¹) was treated with ethyl methanesulphonate (Sigma) as described by Coote (1972). BUdR-tolerant mutants were selected by plating mutagenized cells on MM agar supplemented with L-tryptophan, TdR (1 µg ml⁻¹) and BUdR (20 µg ml⁻¹). The parental strain did not grow at this TdR : BUdR ratio.

Uptake of thymidine into whole cells. A cell sample (0.5 ml) containing [2-¹⁴C]TdR [54 Ci mmol⁻¹ (1 Ci = 37 GBq), Amersham] was collected on a membrane filter (Oxoid, 0.45 µm pore size) and washed twice with 1.0 ml of the same medium without added radiolabel. The filter was dried and radioactivity counted in a Packard Tricarb 300C scintillation counter.

DNA synthesis *in vivo*. This was measured by incorporation of [2-¹⁴C]TdR (54 Ci mmol⁻¹, Amersham) into acid-insoluble material. A cell sample (0.2 ml) was mixed with 2 ml ice-cold 10% (w/v) TCA containing unlabelled TdR (100 µg ml⁻¹) and left on ice for 20 min. The sample was filtered through Whatman GF/A glass fibre filters pre-soaked in ice-cold 10% (w/v) TCA plus TdR (100 µg ml⁻¹), washed with 10 ml ice-cold 10% (w/v) TCA followed by 10 ml 95% ethanol. The filter was dried and radioactivity counted.

DNA synthesis *in vitro*. Toluene-treated cells were used to measure DNA replication *in vitro* by a modification of the method of Winston & Matsushita (1975). A 50 ml culture was grown to an OD₆₀₀ of 0.6 to 0.7, centrifuged and the cells resuspended in 1/200 volume of MM without added glucose. Toluene (1%, v/v) was added and the suspension mixed vigorously for 1 min followed by agitation at 60 r.p.m. for 15 min at room temperature in a Gallenkamp rotary incubator.

Toluene-treated cells (0.1 ml) were added to an incubation mixture which contained in a total volume of 0.5 ml; 100 mM-KH₂PO₄ (pH 7.4), 1.3 mM-ATP, 13 mM-MgSO₄, 10 mM-dithiothreitol, 100 µM-dATP, dCTP and dGTP (deoxyribosyladenine, cytidine and guanosine triphosphates) together with either 60 µM-dTTP (thymidine triphosphate) or 4 µM-dTTP + 56 µM-5-bromo-2'-deoxyuridine triphosphate (dBUTP) and 5 µCi [methyl-³H]dTTP (50 Ci mmol⁻¹, Amersham). The mixture was incubated at 37 °C and at intervals 50 µl was withdrawn into 0.5 ml 0.15 M-NaCl, 0.1 M-EDTA (pH 8.0) containing lysozyme (1 mg ml⁻¹). After 15 min incubation at 37 °C, 2.0 ml ice-cold 10% (w/v) TCA containing dTTP (100 µg ml⁻¹) was added, the sample mixed and left on ice for 20 min. Samples were filtered through Whatman GF/A glassfibre filters pre-soaked in ice-cold 10% (w/v) TCA and washed twice with 5 ml 10% (w/v) TCA. The filters were dried and radioactivity counted.

Thymidine kinase assay. Toluene-treated cells (0.1 ml) prepared as above were added to an incubation mixture which contained, in a total volume of 0.4 ml; 70 mM-Tris/HCl (pH 7.5), 500 µg bovine serum albumin ml⁻¹ (Sigma), 5 mM-ATP, 2.5 mM-MgCl₂, 0.6 mM-EDTA, 1 mM-β-mercaptoethanol together with either 10 µM-TdR or 0.66 µM-TdR + 9.34 µM-BUdR and 5 µCi [methyl-³H]TdR (47 Ci mmol⁻¹, Amersham). The mixture was incubated at 37 °C and at intervals 50 µl was withdrawn into 0.5 ml 0.15 M-NaCl, 0.1 M-EDTA (pH 8.0) containing lysozyme (1 mg ml⁻¹). After 15 min incubation at 37 °C the samples were placed on ice for 30 min. They were then diluted with 5 ml distilled water containing TdR (100 µg ml⁻¹), filtered slowly through Whatman DE81 filters to bind phosphorylated TdR and washed with 5 ml distilled water + TdR and 10 ml distilled water. The filters were dried and radioactivity counted.

RNA synthesis. The procedure was identical to that used for measurement of DNA synthesis *in vivo* except that [4-³H]uridine (U) (27 Ci mmol⁻¹, Amersham) was used and samples were precipitated with 10% (w/v) TCA containing unlabelled U (100 µg ml⁻¹).

DNA preparation. A 10 ml sample of cells was treated with 0.1 ml 100 mM-NaN₃ and DNA extracted as described previously (Binnie & Coote, 1983), except that after treatment with RNAase and protease, excess

protein was removed by two extractions with phenol saturated with 100 mM-Tris/HCl-5 mM-EDTA (pH 8.0). DNA was prepared from spores as described by Sargent (1980a).

Transformation. When strain Mu8u5u16 was used as recipient, competent cells were prepared by the method of Bott & Wilson (1967) and treated with purified DNA at a concentration of $1 \mu\text{g ml}^{-1}$. For the preparation of isogenic strains, recipient cells were made competent by the method of Ayad & Barker (1969) and transformed using the method of Ephrati-Elizur (1968). Here, donor strains were grown in Penassay broth (Difco) to stationary phase when they release DNA into the medium. A portion (0.5 ml) of the donor culture of a BUdR- or BU-tolerant strain (which in all cases carried an auxotrophic marker) was added to 5 ml of competent recipient cells of a Trp⁺ derivative of the parent strain. Transformants were selected on MM agar supplemented with TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($20 \mu\text{g ml}^{-1}$).

Density gradient centrifugation. A sample (0.1 to 0.2 ml) of [$2\text{-}^{14}\text{C}$]TdR-labelled DNA was loaded onto a preformed CsCl gradient consisting of three layers, each of 2 ml, of densities 1.65, 1.72, and 1.79 g ml^{-1} , together with similar volumes of two reference DNA samples prepared separately and labelled with [$\text{methyl-}^3\text{H}$]TdR (47 Ci mmol^{-1} , Amersham). Reference DNA substituted with BUdR in both strands (HH form) was prepared from cells of strain BUt32 grown for at least 15 generations in CH containing TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) + [$\text{methyl-}^3\text{H}$]TdR ($0.5 \mu\text{Ci ml}^{-1}$). Unsubstituted reference DNA (LL form) was isolated from BUt32 cells grown to mid-exponential phase in CH + TdR ($8.5 \mu\text{g ml}^{-1}$) + [$\text{methyl-}^3\text{H}$]TdR ($0.5 \mu\text{Ci ml}^{-1}$). Gradients were centrifuged at $110\,000 \text{ g}$ for 24 h at 20°C and 0.1 ml fractions collected from the bottom of the tubes. The linearity of the CsCl gradient was monitored by refractometry. ^3H and ^{14}C radioactivity were counted separately in a Packard Tricarb 300C scintillation counter and corrections made for the contribution of ^{14}C -derived emissions in the ^3H energy spectrum.

RESULTS

Tolerance of isogenic strains

Three independently isolated BUdR-tolerant mutants were selected (BUt18, BUt21 and BUt24). The *but* mutations in these strains, together with *but-32* from strain BUt32 (Coote, 1977) and two BU-tolerance mutations from strains VUB112 and BUt23.1 (see Methods) were transferred into the *B. subtilis* 168 (*thyA thyB*) genetic background to make them isogenic. Mutations *but-112* and *but-23.1*, originally identified as BU-tolerance mutations, were selected in the presence of BUdR during construction of the isogenic strains. None of the isogenic tolerant strains was able to grow for more than a few generations with BUdR alone. This was also a feature of BU-tolerant mutants examined in a previous study (Bishop & Sueoka, 1972). A small amount of the natural nucleoside had to be present and an arbitrary concentration of $1 \mu\text{g ml}^{-1}$ was chosen, as this alone would sustain normal growth up to an OD_{600} of >1.0 . The growth rates of the Trp⁺ parent strain and each of the isogenic tolerant strains with increasing BUdR:TdR ratios were compared (Table 1). The data presented are from a single experiment, but similar experiments reproducibly indicated that differences in the sensitivity to BUdR existed between the strains. Strain BUt32 was the least sensitive to BUdR and its growth rate was only slightly affected at a TdR:BUdR ratio of 1:15 or 1:30. A comparison of the growth of strain BUt32 and that of the parent strain in CH medium with TdR ($8.5 \mu\text{g ml}^{-1}$) or TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) (Fig. 1*a, b*) showed that whereas BUt32 grew in a similar manner in both media, the parent strain showed an initial slight increase in OD_{600} in the presence of BUdR, but then growth ceased. Strain BUt18 also grew exponentially at the 1:30 ratio (Table 1), but the remaining strains failed to grow at this ratio after an initial rise in the OD_{600} of the cultures. Strains BUt24 and BUt23.1 grew exponentially at the 1:15 ratio, whereas strains BUt21 and BUt112 were unable to do so. All of the strains grew exponentially at the 1:7.5 ratio, although the growth of the parent strain was noticeably the slowest. Similar differences in growth rates between the strains were found when they were grown in MM instead of CH with TdR and BUdR at the same ratios (data not shown). In addition, the growth rates were only marginally affected by increasing the absolute concentrations of TdR and BUdR, while maintaining the same ratio between them. Thus, for example, strain BUt32 grew with a doubling time of 78 min in CH with TdR ($8.5 \mu\text{g ml}^{-1}$) + BUdR ($256 \mu\text{g ml}^{-1}$), a ratio of 1:30, and for strain BUt18 the doubling time was 150 min at the same concentrations.

Fig. 1. A comparison of the growth rates of the parent strain (a) and strain BUt32 (b) in the presence or absence of BUdR. Each strain was grown in CH medium containing either TdR ($8.5 \mu\text{g ml}^{-1}$) (○) or TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) (●).

Table 1. *Growth rates of isogenic strains at various TdR:BUdR ratios*

Cells were grown in CH medium with the additions shown. The symbol ∞ denotes zero increase in OD_{600} after an initial period of growth (see Fig. 1).

Strain	Doubling time (min)			
	TdR ($8.5 \mu\text{g ml}^{-1}$)	TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($7.5 \mu\text{g ml}^{-1}$)	TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$)	TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($30 \mu\text{g ml}^{-1}$)
BUt32	57	57	63	72
BUt18	60	81	102	120
BUt24	60	78	87	$120 \rightarrow \infty$
BUt23.1	57	63	84	$120 \rightarrow \infty$
BUt21	60	78	$120 \rightarrow \infty$	$180 \rightarrow \infty$
BUt112	69	75	$120 \rightarrow \infty$	$180 \rightarrow \infty$
Parent	57	90	$150 \rightarrow \infty$	$200 \rightarrow \infty$

Mutations *but-112* and *but-23.1* came originally from strains selected as tolerant to BU rather than BUdR. The observation that these mutations also bestowed tolerance to BUdR indicated that tolerance to the two analogues might be manifestations of the same mutation. However, whereas growth of strain BUt23.1 was more seriously affected than that of strain BUt32 in medium containing TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) (Table 1), the converse was the case when the two strains were grown in CH containing T ($1 \mu\text{g ml}^{-1}$) + BU ($15 \mu\text{g ml}^{-1}$). Here, growth of strain BUt23.1 was essentially unaffected (doubling time 48 min) whereas growth of strain BUt32 was slower (doubling time 72 min).

Strain BUt32 showed the greatest tolerance to BUdR and was compared in subsequent experiments with the parent strain in an attempt to determine the basis of the tolerance mutation.

Whole cell uptake of TdR

When cells previously grown with TdR alone were suspended in growth medium containing TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) together with $[2\text{-}^{14}\text{C}]\text{TdR}$, uptake of radiolabelled TdR by whole cells of strain BUt32 was markedly greater than that of the parent strain grown in the same medium. A representative experiment (Fig. 2) showed that, in strain BUt32, whole cell uptake of $[^{14}\text{C}]\text{TdR}$ in the presence of BUdR was similar to that of cells grown with TdR ($1 \mu\text{g ml}^{-1}$) alone (Fig. 2b). In the parent strain, uptake of $[^{14}\text{C}]\text{TdR}$ by BUdR-grown cells was noticeably slower than that of cells grown with TdR alone (Fig. 2a). This indicated that the mutant cells discriminated against the BUdR in the medium and showed a preference for uptake of the natural nucleoside.

Fig. 2. Uptake of $[2-^{14}\text{C}]\text{TdR}$ by whole cells during growth of the parent strain (a) and strain BUt32 (b). Parallel cultures (60 ml) of both strains were grown to mid-exponential phase in CH medium supplemented with TdR ($8.5\text{ }\mu\text{g ml}^{-1}$). Each culture was then divided into three 20 ml portions, centrifuged and the cells suspended in CH medium + TdR ($1\text{ }\mu\text{g ml}^{-1}$) (○), CH + TdR ($8.5\text{ }\mu\text{g ml}^{-1}$) (●) or CH + TdR ($1\text{ }\mu\text{g ml}^{-1}$) + BUdR ($15\text{ }\mu\text{g ml}^{-1}$) (▲). $[2-^{14}\text{C}]\text{TdR}$ was added to each culture at $0.025\text{ }\mu\text{Ci ml}^{-1}$ and 0.5 ml samples removed at intervals to measure uptake of radiolabel into whole cells. TdR incorporated was calculated from the specific activity of TdR in the medium.

In media where TdR was present alone, but at different concentrations (1 and $8.5\text{ }\mu\text{g ml}^{-1}$), cells of both the parent and the mutant strain exhibited a noticeably higher rate of uptake of $[^{14}\text{C}]\text{TdR}$ at the higher concentration. Similar observations by Rinehart & Copeland (1973) indicated that T was not actively transported into *B. subtilis* and its internal concentration was dependent on the concentration in the medium.

DNA synthesis in the presence of BUdR

When incorporation of $[2-^{14}\text{C}]\text{TdR}$ into acid-insoluble material was monitored during growth in medium containing TdR ($1\text{ }\mu\text{g ml}^{-1}$) + BUdR ($15\text{ }\mu\text{g ml}^{-1}$), strain BUt32 again exhibited a greater rate of incorporation of radiolabel than the parent strain grown in the same medium (results not shown). This again indicated that TdR was being incorporated into DNA in preference to BUdR more strongly in the tolerant strain than in the parent strain. However, the parent strain grew poorly at a TdR : BUdR ratio of $1 : 15$ (Table 1), and the lower observed rate of incorporation of radiolabelled TdR may have been due to an overall fall in the rate of DNA synthesis (see also Table 3). To minimize the adverse effect of BUdR on growth of the parent strain, cells of both strains were grown to mid-exponential phase in CH medium with TdR alone and then transferred to BUdR medium with radiolabelled TdR. Samples were taken 15, 30 and 45 min after transfer; lysates were then prepared and centrifuged to equilibrium on CsCl density gradients. Strain BUt32 progressively incorporated more $[2-^{14}\text{C}]\text{TdR}$ into its DNA than the parent strain, but the buoyant density of the DNA increased more slowly. Thus, by 45 min (Fig. 3) peak fractions showed a density increase of 17 mg ml^{-1} and a radioactivity count of 4700 c.p.m. for the parent strain compared to 7 mg ml^{-1} and a count of 13250 c.p.m. for strain BUt32. The degree of BUdR substitution can be estimated from the difference in buoyant density between poly[d(A-T)] and poly[d(A-BU)] which is 200 mg ml^{-1} (Wake & Baldwin, 1962). *B. subtilis* 168 DNA contains 28% TdR residues (Schildkraut *et al.*, 1962) compared to 50% in poly[d(A-T)] and complete substitution of TdR by BUdR would give a density increase of 112 mg ml^{-1} . Thus, after 45 min, 15% and 6% of TdR residues were substituted by BUdR in the parent strain and strain BUt32 respectively. Although there may have been slight inhibition of growth of the parent strain after 45 min in the presence of BUdR, it seemed clear that strain BUt32 incorporated less BUdR into its DNA than the parent strain. More detailed density

Fig. 3. Density gradient analysis of DNA replication during growth of the parent strain (a) and strain BUt32 (b). Both strains were grown to mid-exponential phase in CH medium containing TdR ($8.5 \mu\text{g ml}^{-1}$). A 30 ml sample from each culture was centrifuged and the cells resuspended in CH medium containing TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) + $[2\text{-}^{14}\text{C}]\text{TdR}$ ($0.025 \mu\text{Ci ml}^{-1}$). The gradients show DNA prepared 45 min after resuspension of cells with radiolabelled TdR. —, ¹⁴C-labelled test DNA; ---, ³H-labelled reference DNA; ○, refractive index of selected fractions.

Fig. 4. Incorporation of [³H]dTPP into acid-insoluble material by toluene-treated cells. Parallel cultures of the parent strain (a) and strain BUt32 (b) were grown in CH + TdR ($8.5 \mu\text{g ml}^{-1}$) to mid-exponential phase. Cells were treated with toluene and assayed for DNA polymerase activity as described in Methods. ○, 60 μM-dTTP in the incubation mixture; ●, 4 μM-dTTP + 56 μM-dBUTP; △, 60 μM-dTTP, ATP omitted. Each point represents the average of duplicate samples.

gradient analysis of DNA replicated during growth and sporulation of strain BUt32 established average buoyant densities of 1.704 , 1.715 and 1.729 mg ml^{-1} for light, intermediate and heavy DNA respectively (Binnie, 1982; Binnie & Coote, 1983). The latter two values correspond to 10% and 22% substitution respectively of BUdR for TdR residues. As the intermediate form has one unsubstituted strand, it is assumed that during replication in TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) strain BUt32 substituted approximately 20% of its TdR residues with BUdR.

Measurement of DNA replication *in vitro* using toluene-treated cells indicated that DNA polymerase activity showed little discrimination against the analogue (Fig. 4). Toluene-treated cells exhibited an ATP-dependent incorporation of radiolabelled dTTP into acid-insoluble material. In four experiments (the data in Fig. 4 represent one such experiment) the kinetics of incorporation of radiolabelled dTTP into acid-insoluble material was reproducibly the same for both the parent strain and strain BUt32 whether the system contained 60 μM-dTTP or 4 μM-dTTP + 56 μM-dBUTP. This indicated that the polymerase reactions in both strains used

Table 2. *Thymidine kinase activity in strain BUt32 and the parent strain*

Parallel cultures were grown in CH + TdR ($8.5 \mu\text{g ml}^{-1}$) to mid-exponential phase. Cells were treated with toluene and assayed as described in Methods. Activities are expressed as pmol TdR phosphorylated (min^{-1} (OD_{600} unit) $^{-1}$), and are the mean of three determinations ($\pm$ SD). No activity was observed if ATP was omitted from the assay mixture.

Substrate	Strain BUt32	Parent strain
TdR ($10 \mu\text{M}$)	7.74 ± 1.5	5.54 ± 1.95
TdR ($0.66 \mu\text{M}$) + BUdR ($9.34 \mu\text{M}$)	9.84 ± 1.4	7.21 ± 2.67
TdR:TdR + BUdR ratio	0.79	0.77

dBUTP as efficiently as dTTP. A preference for dTTP over dBUTP would have been demonstrated by an increased incorporation of radiolabelled dTTP in the presence of unlabelled dBUTP.

Toluene-treated cells also exhibited an ATP-dependent TdR kinase activity (Table 2). The specific activity with TdR alone as substrate was similar in both strain BUt32 and the parent strain. If BUdR was included with TdR as a substrate in the assay at a ratio of 14:1, a higher specific activity was observed in both cases. This demonstrated preferential phosphorylation of the radiolabelled TdR over the unlabelled BUdR in the reaction mixture. There was no indication, however, that kinase activity in strain BUt32 discriminated against BUdR to any greater extent than that in the parent strain.

RNA synthesis in the presence of BUdR

The rate of incorporation of [^3H]U into acid-insoluble material was monitored in parallel cultures of strain BUt32 and the parent strain. Cells were grown to mid-exponential phase in CH + TdR ($8.5 \mu\text{g ml}^{-1}$). At this point the cultures were halved, the cells collected on a membrane filter and resuspended in an equal volume of either CH + TdR ($8.5 \mu\text{g ml}^{-1}$) or CH + TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) containing [$4\text{-}^3\text{H}$]U ($0.5 \mu\text{Ci ml}^{-1}$) together with unlabelled U ($25 \mu\text{g ml}^{-1}$). The rate of RNA synthesis, measured over the following 60 min, was unaffected by the presence of BUdR in either strain (results not shown). It was unlikely therefore that BUdR inhibited growth of the parent strain by primarily interfering with RNA synthesis.

Effect of deoxycytidine (dC) on BUdR inhibition of growth

In some eukaryotic cells dC has been shown to overcome the toxic effect of BUdR, probably by relieving the cells of starvation of dC residues caused by dBUTP inhibition of ribonucleotide reductase (Meuth & Green, 1974). However, inhibition of growth of the parent strain by BUdR was not relieved by addition of an equal concentration of dC (Fig. 5a). Indeed, growth was further inhibited if dC was added in excess. Similarly, the presence of dC ($50 \mu\text{g ml}^{-1}$) slightly decreased the growth rate of strain BUt32 in the presence of BUdR (Fig. 5b) although the effect was not as marked as that seen with the parent strain. Cells of both strains grew at the same rate with TdR ($8.5 \mu\text{g ml}^{-1}$) + dC ($50 \mu\text{g ml}^{-1}$) as they did with TdR ($8.5 \mu\text{g ml}^{-1}$) alone (results not shown).

Marker frequency analysis of DNA replication

It was reported that BU induced multi-forked (dichotomous) replication in a TdR-requiring strain to compensate for an overall reduction in the rate of DNA replication in the presence of the analogue (Nagley & Wake, 1969). It was possible, therefore, that BUdR slowed the rate of DNA synthesis and that strain BUt32 overcame this by allowing a greater degree of multi-forked replication. This was investigated by marker frequency analysis. DNA was prepared from growing cells of both strains and used to transform a recipient strain Mu8u5u16 separately to Pur⁺ and Met⁺ (Table 3). The *purA16* marker is located near the origin of replication and the *metB5* marker near the terminus. The ratio of transformants for these markers will give an indication of the average number of replication positions per chromosome (Sueoka & Yoshikawa, 1965). Spores of *B. subtilis* contain completed chromosomes (Oishi *et al.*, 1964;

Fig. 5. The effect of dC on BUdR inhibition of growth. The parent strain (a) and strain BUt32 (b) were grown in parallel 20 ml cultures of CH containing TdR ($8.5 \mu\text{g ml}^{-1}$) (○), TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) (●), TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) + dC ($15 \mu\text{g ml}^{-1}$) (△), TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$) + dC ($50 \mu\text{g ml}^{-1}$) (▲).

Table 3. Marker frequency analysis of DNA replicated during growth of strain BUt32 and the parent strain

Cells were grown in CH medium with the additions shown to mid-exponential phase and samples were then removed for preparation of DNA. Strain Mu8u5u16 was used as recipient for transformation and Pur⁺ and Met⁺ transformants were selected separately after addition of DNA to competent cells. For strain BUt32 four DNA preparations were made from separate cultures and three preparations from separate cultures were made for the parent strain. Transformation was done at least in duplicate and generally in triplicate for each DNA preparation and at least 200 transformants were counted in each case. The mean Pur⁺/Met⁺ ratios were standardized with respect to the Pur⁺/Met⁺ ratio obtained from spore DNA (see text).

Strain	Additions to medium	Mean doubling time (min) ± SEM*	Mean Pur ⁺ /Met ⁺ ratio ± SEM*	Standardized Pur ⁺ /Met ⁺ ratio	Mean no. of replication positions per chromosome†	Chromosome replication time (min)‡
BUt32	TdR ($8.5 \mu\text{g ml}^{-1}$)	37 ± 1.0 (3)	5.16 ± 0.77 (11)	3.74	1.9	70
	TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$)	48 ± 3.46 (3)	3.95 ± 0.48 (11)	2.86	1.5	72
	TdR ($8.5 \mu\text{g ml}^{-1}$)	34.5 ± 1.51 (2)	6.27 ± 1.31 (9)	4.82	2.3	79
Parent	TdR ($8.5 \mu\text{g ml}^{-1}$)	34.5 ± 1.51 (2)	6.27 ± 1.31 (9)	4.82	2.3	79
	TdR ($1 \mu\text{g ml}^{-1}$) + BUdR ($15 \mu\text{g ml}^{-1}$)	102 ± 12.1 (2)	3.15 ± 0.19 (6)	2.42	1.3	133

* No. of determinations given in parentheses.

† Calculated from the equation $\text{Pur}^+/\text{Met}^+ \text{ ratio} = 2^n$, where n is the mean number of replication positions per chromosome (Sueoka & Yoshikawa, 1965).

‡ Calculated from the equation $n = C/r$, where C is the chromosome replication time and r is the mean generation time of the bacteria (Ephrati-Elizur & Borenstein, 1971).

Sargent, 1980b) and the Pur⁺/Met⁺ ratio obtained with spore DNA can be used as a correction factor to standardize the ratios obtained during growth to a value of 1.0 for spore DNA. DNA prepared from *B. subtilis* 168 *trpC2* gave a Pur⁺/Met⁺ ratio of 0.98, whereas DNA from *thyA thyB* and *thyA thyB but-32* spores gave ratios of 1.30 and 1.38 respectively (mean of three determinations in each case). The discrepancies between the ratios from the wild-type and TdR-requiring strains is probably due to a lack of isogenicity in the *metB* region of the latter strains which lowers transformation efficiency in this region (Callister & Wake, 1974). DNA preparations from BUt32 cells grown for several generations with BUdR produced a lower average Pur⁺/Met⁺ ratio than DNA preparations from the same cells grown with TdR alone (Table 3). This meant that the cell population had, on average, slightly fewer replication positions per chromosome. The chromosome replication time was essentially the same in both cases, however, as the cells had a slightly longer mean doubling time in the BUdR medium.

There was no evidence to suggest that BUdR induced a greater degree of dichotomous replication in the tolerant strain to compensate for a slowing down in the rate of DNA replication. Instead, an essentially normal rate of replication was maintained by the mutant strain in the presence of BUdR. DNA from cells of the parent strain grown with BUdR again produced a lower Pur⁺/Met⁺ ratio and a lower average number of replication forks per chromosome than DNA from TdR-grown cells. In this case though, BUdR-grown cells exhibited a markedly longer chromosome replication time.

It should be stressed that the Pur⁺/Met⁺ ratios varied from one transformation experiment to another, even when the same DNA sample was used. Thus, a higher Pur⁺/Met⁺ ratio was previously reported with DNA from BUdR-grown cells when compared with DNA from TdR-grown cells (Coote & Binnie, 1981). This difference was found from an average of four determinations obtained from one DNA preparation. The more thorough examination reported here (Table 3) does not support the earlier observation and highlights the relative inaccuracy of the marker frequency method for analysis of chromosome replication. Relatively small differences in the marker ratios lead to large differences in the calculated chromosome replication time. Nevertheless, in spite of the inaccuracy of the method, the magnitude of the differences in chromosome replication times between the parent strain and strain BUt32 in BUdR medium indicated that the BUdR tolerant strain, unlike the parent strain, was able to maintain essentially a normal replication time in the presence of the analogue.

DISCUSSION

The different growth rates of the isogenic strains in the presence of BUdR suggested that classes of tolerance to the analogue might exist. Genetic mapping would be necessary to determine whether the various mutations were located in the same area of the genome, but this would not be a straightforward task as the But⁺ phenotype is only expressed in strains bearing the *thyA* and *thyB* mutations.

It was previously reported that a TdR-requiring strain of *B. subtilis* showed a preferential incorporation into DNA of T or TdR over the analogues BU or BUdR (Laird & Bodmer, 1967). The pattern of uptake of radiolabelled TdR by the TdR-requiring strain and the BUdR-tolerant derivative used here indicated that the latter showed an even greater discrimination against BUdR, both during uptake of the analogue into the cell and during DNA synthesis *in vivo*. TdR-requiring strains lack thymidylate synthase activity and in order to grow on exogenous T or TdR need TdR kinase as a first step in the phosphorylation of these compounds before their incorporation into DNA (O'Donovan & Neuhaud, 1970; Rinehart & Copeland, 1973). The fact that TdR kinase and DNA polymerase showed similar activities towards BUdR in both strains indicated that the reduced incorporation of BUdR into DNA in the tolerant strain was a consequence of a smaller pool of the analogue within the cell. Thus, by virtue of greater discrimination against the analogue, the tolerance mutation might be expected to lower the BUdR : TdR ratio within the cell such that the amount of BUdR available was not sufficient to interfere with the rate of replication when cells were grown with a TdR : BUdR ratio of 1 : 15. In the parent strain, the BUdR concentration in the cell would be increased and the TdR concentration decreased relative to that in the tolerant strain with a consequential effect on the rate of DNA synthesis. This interpretation is substantiated by, firstly, the buoyant density data, which indicated that after 45 min of exposure to a TdR : BUdR ratio of 1 : 15, 15% of TdR residues in the parent strain were substituted by BUdR as opposed to only 6% in the tolerant strain. Secondly, marker frequency analysis showed that strain BUt32 maintained a normal rate of replication at a TdR : BUdR ratio of 1 : 15 whereas the rate of replication in the parent strain was seriously affected.

If this was the only effect, then simply lowering the TdR : BUdR ratio should eventually allow the parent strain to grow normally, yet still allow a significant incorporation of BUdR into the DNA. This does not seem to be the case, however, because the parent strain would only grow normally when TdR and BUdR were each present at 1.0 µg ml⁻¹ (data not shown). Because of the preferential incorporation of TdR over BUdR into DNA (Laird & Bodmer, 1967), very little

of the analogue (much less than the 20% substitution of TdR for BUdR observed for the tolerant strain) would be incorporated into DNA at this ratio. It would seem that the tolerance mutation in strain BUt32 is not simply associated with a reduction in the amount of available analogue within the cell, but has some other effect in reducing the overall toxicity of BUdR during DNA replication. The other effect may be associated with the observation that starvation for T or TdR during growth of *thyA thyB* cells induces the lytic cycle of SP β and PBSX phages, resulting in the synthesis of cell wall hydrolases (Yasbin *et al.*, 1980; Ward *et al.*, 1982). Indeed, both of these phages can be induced in the wild-type strain by agents that interfere with DNA replication (Seaman *et al.*, 1964; Warner *et al.*, 1977). Haas & Yoshikawa (1969) noted that a TdR-requiring strain was more sensitive than the wild-type to such agents and that the defective phage PBSH could be induced by germination of spores of a TdR-requiring strain during TdR starvation or in the presence of BUdR. If the effect of BUdR on DNA replication in the parent strain led to phage induction it would account for the initial slow growth of the cells in the presence of BUdR followed by a cessation of growth and eventual lysis of most of the cell population. The mutation in strain BUt32, by preferentially allowing uptake of TdR over BUdR, perhaps overcomes this effect by preventing too severe a reduction in the TdR pool in the cells.

A detailed analysis of BUdR resistance in Chinese hamster ovary cells (Bradley *et al.*, 1982) indicated that resistance occurred via three discrete steps. Resistance to high concentrations of BUdR was correlated with partial or complete reduction in TdR kinase activity, whereas resistance to low concentrations of BUdR was associated with an altered ribonucleotide reductase activity. The latter enzyme controls deoxyribonucleotide pool sizes and both dTTP and dBUTP control conversion of CTP to dCTP by inhibition of this enzyme in mammalian cells (Meuth & Green, 1974). At high concentrations, these effectors induce a 'deoxycytidine-less' state with consequent inhibition of DNA synthesis and cell death. This can be reversed by addition of exogenous dC in the presence of BUdR (Meuth & Green, 1974; Rogers *et al.*, 1975). It is unlikely, however, that BUdR has this effect in *B. subtilis* as addition of an equal quantity of dC with BUdR did not relieve the inhibitory effect of BUdR on growth of the parent strain. Furthermore, when dC was added in excess, the relative inhibitory effect of BUdR on growth of both strains was enhanced. This effect may be related to the observation that TdR kinase is activated by dCTP (Okazaki & Kornberg, 1964). Addition of dC may increase the dCTP concentration within the cell, which in turn increases TdR kinase activity. Greater activity of this enzyme would increase the intracellular pool of phosphorylated BUdR and thus enhance the toxic effect of BUdR. It has been shown that exogenous dC is converted to thymidine nucleotides in *B. subtilis* (Rima & Takahashi, 1978) via initial deamination to deoxyuridine nucleotides which then serve as substrates for thymidylate synthase. In the absence of this enzyme activity in *thyA thyB* strains, the deoxyuridine derivatives would presumably accumulate, but this apparently has no adverse effect on growth because the parent strain and strain BUt32 grew equally well with TdR alone or with TdR plus an excess of dC (data not shown).

As TdR-requiring strains lack thymidylate synthase activity, resistance to BU or BUdR could not be achieved by loss of TdR kinase activity as has been observed in eukaryotic systems (Ostertag *et al.*, 1973; Lunn *et al.*, 1977; Bradley *et al.*, 1982). The increased preference for the natural nucleoside over the analogue in strain BUt32 apparently originated at the point of uptake of the compounds into the cell. As neither T or TdR appear to be actively transported into *B. subtilis* (Rinehart & Copeland, 1973), tolerance to the analogues could simply have arisen from an unspecified alteration in the membrane or surface properties of the cell. This might explain why only partial resistance to these analogues has been found in TdR-requiring strains, both in this study and in a previous report (Bishop & Sueoka, 1972). In their study of BU-tolerant strains of *B. subtilis*, Bishop & Sueoka (1972) described three classes of mutants which differed in their ability to grow in the presence of BU. In two mutants analysed in detail, the rate of DNA synthesis was maintained in the presence of BU while that of the parent strain was much slower. DNA polymerase activity, measured *in vitro*, showed no discrimination against the analogue (S. Winston, personal communication). This behaviour is similar to that of the BUdR-tolerant mutant described here. However, the data of Bishop & Sueoka (1972) indicated that the BU-

tolerant mutants had a decreased preference for T over BU during DNA replication. This is the reverse of the behaviour exhibited by strain BUt32 which tended to incorporate TdR in preference to BUdR. This difference, and the apparent existence of classes of BU- and BUdR-tolerant mutants, might suggest that more than one type of mutation can create tolerance to the analogues, but the precise nature of the mutation(s) requires further investigation.

We thank M. Achison for excellent technical assistance. The work was supported by the Science and Engineering Research Council and by a University of Glasgow postgraduate studentship.

REFERENCES

- AYAD, S. R. & BARKER, G. R. (1969). The integration of donor and recipient deoxyribonucleic acid during transformation of *B. subtilis*. *Biochemical Journal* **113**, 167-174.
- BINNIE, C. (1982). *The effect of 5-bromo-2-deoxyuridine on growth and sporulation in Bacillus subtilis*. PhD thesis, University of Glasgow, UK.
- BINNIE, C. & COOTE, J. G. (1983). Density gradient analysis of DNA replicated during *Bacillus subtilis* sporulation. *Journal of Bacteriology* **156**, 466-470.
- BISHOP, R. J. & SUEOKA, N. (1972). 5-Bromouracil-tolerant mutants of *Bacillus subtilis*. *Journal of Bacteriology* **112**, 870-876.
- BOTT, K. F. & WILSON, G. A. (1967). Development of competence in the *Bacillus subtilis* transformation system. *Journal of Bacteriology* **94**, 562-570.
- BRADLEY, W. F. C., DINELLE, C., CHARRON, J. & LANGELIER, Y. (1982). Bromodeoxyuridine resistance in CHO cells occurs in three discrete steps. *Somatic Cell Genetics* **8**, 207-222.
- CALLISTER, H. & WAKE, R. G. (1974). Completed chromosomes in thymine-requiring *Bacillus subtilis* spores. *Journal of Bacteriology* **120**, 579-582.
- COHEN, S. S. & BARNER, H. D. (1956). Studies on the induction of thymine deficiency and on the effect of thymine and thymidine analogues in *Escherichia coli*. *Journal of Bacteriology* **71**, 588-597.
- COOTE, J. G. (1972). Sporulation in *Bacillus subtilis*. Characterization of oligosporogenous mutants and comparison of their phenotypes with those of asporogenous mutants. *Journal of General Microbiology* **71**, 1-15.
- COOTE, J. G. (1977). Interference by bromodeoxyuridine with differentiation in a prokaryote. *Nature, London* **267**, 635-637.
- COOTE, J. G. & BINNIE, C. (1981). Association between DNA replication and interference by bromodeoxyuridine with sporulation in *Bacillus subtilis*. In *Sporulation and Germination*, pp. 157-162. Edited by H. S. Levinson, A. L. Sonenshein and D. J. Tipper. Washington, DC: American Society for Microbiology.
- EPHRATI-ELIZUR, E. (1968). Spontaneous transformation in *Bacillus subtilis*. *Genetical Research* **11**, 83-96.
- EPHRATI-ELIZUR, E. & BORENSTEIN, S. (1971). Velocity of chromosome replication in thymine-requiring and independent strains of *Bacillus subtilis*. *Journal of Bacteriology* **106**, 58-64.
- HAAS, M. & YOSHIKAWA, H. (1969). Defective bacteriophage PBSH in *Bacillus subtilis*. I. Induction, purification and physical properties of the bacteriophage and its deoxyribonucleic acid. *Journal of Virology* **3**, 233-247.
- HUANG, P. C., EBERLE, H., BOICE, L. B. & ROMIG, W. R. (1968). Replication of *Bacillus subtilis* DNA during germination in 5-bromouracil containing medium and marker mapping. *Genetics* **60**, 661-672.
- LAIRD, C. D. & BODMER, W. F. (1967). 5-Bromouracil utilisation by *Bacillus subtilis*. *Journal of Bacteriology* **94**, 1277-1278.
- LUNN, A., COOKE, D. & HAUGLI, F. (1977). Genetics and biochemistry of 5-bromodeoxyuridine resistance in *Physarum polycephalum*. *Genetical Research* **30**, 1-12.
- MEUTH, M. & GREEN, H. (1974). Induction of a deoxycytidineless state in cultured mammalian cells by bromodeoxyuridine. *Cell* **2**, 109-112.
- NAGLEY, P. & WAKE, R. G. (1969). Effect of 5-bromouracil on the pattern of deoxyribonucleic acid replication in germinating *Bacillus subtilis* spores. *Journal of Molecular Biology* **43**, 619-630.
- O'DONOVAN, G. A. & NEUHARD, J. (1970). Pyrimidine metabolism in microorganisms. *Bacteriological Reviews* **34**, 278-343.
- OISHI, M., YOSHIKAWA, H. & SUEOKA, N. (1964). Synchronous and dichotomous replication of the *Bacillus subtilis* chromosome during spore germination. *Nature, London* **204**, 1069-1073.
- OKAZAKI, R. & KORNBERG, A. (1964). Deoxythymidine kinase of *Escherichia coli*. II. Kinetics and feedback control. *Journal of Biological Chemistry* **239**, 275-284.
- OSTERTAG, W., CROZIER, T., KIUGE, N., MELDERIS, H. & DUBE, S. (1973). Action of 5-bromodeoxyuridine on the induction of haemoglobin synthesis in mouse leukaemia cells resistant to 5-BUdR. *Nature New Biology* **243**, 203-205.
- O'SULLIVAN, A., HOWARD, K. & SUEOKA, N. (1975). Location of a unique replication terminus and genetic evidence for partial bidirectional replication in the *Bacillus subtilis* chromosome. *Journal of Molecular Biology* **91**, 15-38.
- QUINN, W. G. & SUEOKA, N. (1970). Symmetrical replication of the *Bacillus subtilis* chromosome. *Proceedings of the National Academy of Sciences of the United States of America* **67**, 717-723.
- RIMA, B. K. & TAKAHASHI, I. (1978). Synthesis of thymidine nucleotides in *Bacillus subtilis*. *Canadian Journal of Biochemistry* **56**, 158-160.
- RINEHART, K. V. & COPELAND, J. C. (1973). Evidence that thymine is not a normal metabolite in wild-type *Bacillus subtilis*. *Biochimica et biophysica acta* **294**, 1-7.
- ROGERS, J., NG, S. K. C., COULTER, M. B. & SANWAL, B. D. (1975). Inhibition of myogenesis in a rat myoblast line by 5-bromodeoxyuridine. *Nature, London* **256**, 438-440.

- SARGENT, M. G. (1980*a*). A procedure for isolating high quality DNA from spores of *Bacillus subtilis* 168. *Journal of General Microbiology* **116**, 511–514.
- SARGENT, M. G. (1980*b*). Chromosome replication in sporulating cells of *Bacillus subtilis*. *Journal of Bacteriology* **142**, 491–498.
- SCHILDKRAUT, C. L., MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *Journal of Molecular Biology* **4**, 430–443.
- SEAMAN, E., TARMY, E. & MARMUR, J. (1964). Inducible phages of *Bacillus subtilis*. *Biochemistry* **3**, 607–613.
- STERLINI, J. M. & MANDELSTAM, J. (1969). Commitment to sporulation in *Bacillus subtilis* and its relationship to the development of actinomycin resistance. *Biochemical Journal* **113**, 29–37.
- SUEOKA, N. & YOSHIKAWA, H. (1965). The chromosome of *Bacillus subtilis*. I. Theory of marker frequency analysis. *Genetics* **52**, 747–757.
- WAKE, R. G. & BALDWIN, R. L. (1962). Physical studies on the replication of DNA *in vitro*. *Journal of Molecular Biology* **5**, 201–216.
- WARD, J. B., CURTIS, C. A. M., TAYLOR, C. & BUXTON, R. S. (1982). Purification and characterisation of two phage PBSX-induced lytic enzymes of *Bacillus subtilis* 168. *Journal of General Microbiology* **128**, 1171–1178.
- WARNER, F. D., KITOS, G. A., ROMANO, M. P. & HEMPHILL, H. E. (1977). Characterisation of SP β : a temperate bacteriophage from *Bacillus subtilis* 168M. *Canadian Journal of Microbiology* **23**, 45–51.
- WINSTON, S. & MATSUSHITA, T. (1975). Permanent loss of chromosome initiation in toluene-treated *Bacillus subtilis* cells. *Journal of Bacteriology* **123**, 921–927.
- YASBIN, R. E., FIELDS, P. I. & ANDERSON, B. J. (1980). Properties of *Bacillus subtilis* 168 derivatives freed of their natural phages. *Gene* **12**, 155–159.