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A COMPARISON OF UV INDUCED DNA PHOTOPRODUCTS FROM ISOLATED AND NON-ISOLATED DEVELOPING BACTERIAL FORESPORES

J.A.Lindsay and W.G.Murrell

CSIRO, Division of Food Research, North Ryde Australia 2113

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UV-induced photoproduct formation has been compared in non-isolated and isolated developing forespores. We have found that levels of spore type photoproducts are greatly affected by mother cell DNA. We have also observed the presence of the photoproduct 6-4'-(pyrimidin-2'-one)-thymine in developing forespores. We conclude from these and other data in the literature that the degree of hydration around the forespore DNA is reduced by the presence of dipicolinic acid which influences photoproduct formation without causing a change in conformational state.

Bacterial spores differ from their vegetative counterparts in metabolic activity, resistance to heat and resistance to various types of irradiation. UV irradiation, induces the formation of several photoproducts in bacterial spores, one of which is mainly responsible for spore death (1). The chemical structure of this product, 5-thyminyl-5,6-dihydrothymine (TDHT) is the same as that found during irradiation of thymidine films (2). Although additional spore type photoproducts have been observed and characterized (3), they are thought to have little effect on viability. Several authors believe that, during sporulation, DNA encapsulated within the developing forespore undergoes a change in state, and that this change is reflected in the production of 'spore' type photoproducts (4,5). Evidence exists that the (mother cell) sporangium contains DNA additional to that of the developing forespore (6). If the metabolic activity of the developing forespore is different from that of the mother cell, examination of photoproducts from sporulating cells may provide misleading data as to any changes in DNA state within the forespore.

The problem of masking actual UV photoproducts from the developing spore may be resolved by isolation of the forespore itself. We recently developed a method for large

Abbreviations: TDHT, 5-thyminyl-5,6-dihydrothymine; PO-T, 6-4'-(pyrimidine-2'-one) -thymine; DPA, dipicolinic acid(2,6-pyridine dicarboxylic acid); sp, spore type photo-product; U-T, uracil-thymine; T-T, thymine-thymine; BUT, n-butanol; ACET.A, acetic acid;

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scale purification of stable, intact and viable forespores (7), and this communication reports our use of this method in analysing the UV-induced DNA photoproducts from developing forespores of Bacillus megaterium QMB 1551 and Bacillus subtilis 052.

Our results indicated that forespores produce different quantities of spore type photoproducts when compared to intact sporulating cells. We have also identified a previously unobserved photoproduct in bacterial spores, and we question the validity that TDHT accounts for the majority of killing due to UV irradiation. We also conclude that dipicolinic acid (DPA) influences the production of bacterial photoproducts.

MATERIALS AND METHODS

Preparation of samples

Bacillus megaterium QMB 1551 and Bacillus subtilis 052 (thy$^-$) were grown for 6 hours in nutrient broth (Oxoid) at 37^{o}C and were then subcultured in modified G/2 medium (8). Yeast extract was omitted and replaced by 2% casamino acids (Difco vitamin free) previously steamed for I hour with activated charcoal to remove residual thymine. For subsequent photoproduct analysis, sporulating cells were labelled by supplementing the G/2 growth medium with [3H] thymine (1uCi/ml: 1Ci/mmol, New England Nuclear). Cells were harvested at various sporulation stages as determined microscopically and washed twice with 0.05M phosphate buffer pH 6.8. Forespores were prepared by the method of Lindsay and Murrell (7). For examination of refractile forespores only, the cell suspension was centrifuged at 10,000g for 20 min. at 25^{o}C and osmotically shocked by resuspension in 0.1M, pH 7.2 potassium phosphate buffer. This suspension was then centifuged as previously described and the stable forespore pellet resuspended in 0.05M phosphate buffer pH 6.8 for UV irradiation. The percent refractility of the culture was determined from the proportion of cells with refractile forespores before osmotic shock.

Germination

Isolated forespores were germinated by heat treatment at 60^{o}C for 10 min. and shaking gently in 5mg/ml L-alanine, 2mg/ml glucose in sucrose salts buffer pH 7.2 at 30^{o} (7). Germination was monitored by change in refractility to phase dark by microscopic examination. Usual germination time depending on the concentration of forespores was 15-30min.

Irradiation and chromatography

Irradiation of constantly stirred 5ml samples in plastic Petri dishes with 1.8 x 10^5 ergs of 254nm UV light (Sylvania G 30T8 30W Germical lamp) was followed by sequential washing and digestion in 2ml 99% formic acid at 175^{o}C for 30 min (5). The digest was dried under a stream of dry nitrogen and resuspended in 0.5ml sterile deionised water. 50µl samples were subjected to thin layer chromatography on silica gel (Merck). Chromatograms were sectioned into 0.5cm strips and the radioactivity counted using a Packard Scintillation 2660 counter (Packard Instrument Co., Ill. U.S.A.).

Photoproduct Purification

Individual photoproducts were purified chromatographically using 0.5ml digest samples on 2mm thick, 20x20cm silica gel plates using n-butanol/ acetic acid/water (80/12/30). A 0.5x20cm strip from the plate was sectioned and counted, the region containing the required photoproduct was sectioned and the silica gel washed with 10ml deionised water. The supernatant was collected and concentrated under dry nitrogen and rechromatographed as previously. The concentrated eluant was applied to a 0.9x20cm column of Dowex 20w-X12 H^+ (100-200 mesh) and eluted with deionised water, the radioactive fractions being collected and freeze dried. Isolated photoproducts were subjected to analysis by mass spectroscopy using a Varian MAT Mass Spectrometer (Varian MAT Bremen, Germany).

RESULTS AND DISCUSSION

Fig. 1 shows a comparison of spore type photoproduct (sp) levels for both B. megaterium and B. subtilis isolated and non-isolated forespores. The patterns for both species reveal the same general trend, ie. for isolated forespores the level of spC (R_f 0.52) increases during sporulation to a maximum at free spore stage while spB (TDHT,

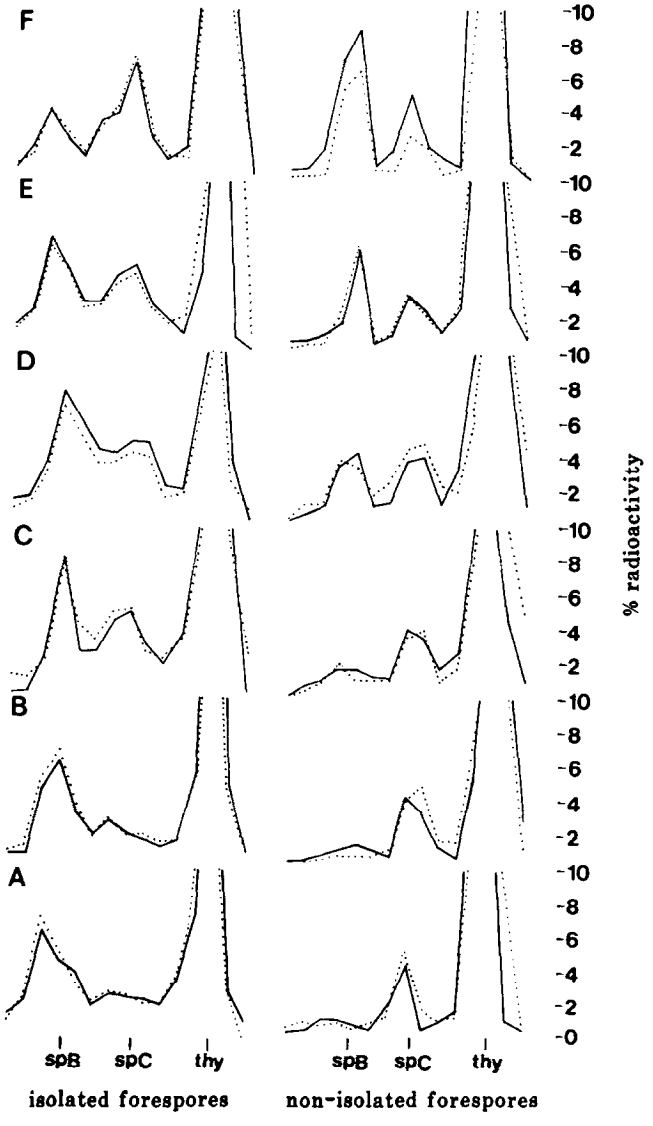


Fig. 1 A comparison of spore type photoproducts produced during sporulation of B. megaterium QMB1551 and B. subtilis 052 in isolated and non-isolated forespores. (——) B. megaterium (· · · · ·) B. subtilis, spB (spore photoproduct B), spC (spore photoproduct C), thy (thymine). Sporulation stages A (stage iv,), B (phase dark forespores,), C,D,E (1,20,50% refractility of culture), F (5% free spores in culture,).

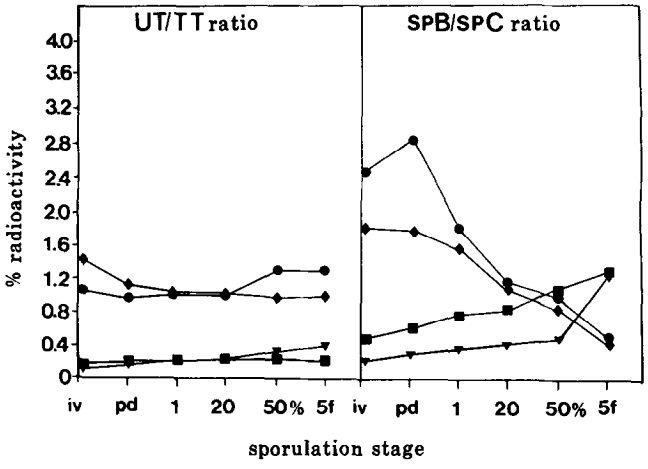


Fig. 2 Comparison of U-T (uracil-thymine)/ T-T (thymine-thymine) and spB/spC photoproduct ratios for isolated and non-isolated forespores from B. megaterium and B. subtilis during sporulation. B. megaterium: ● isolated, ■ non-isolated. B. subtilis ◆ isolated, ▼ non-isolated. Sporulation stages, iv (stage iv), pd (phase dark forespores), 1,20,50 (% refractility of culture), 5f (5% free spores in culture).

R_f 0.4) remains relatively constant. Conversely, for non-isolated forespores the level of spB increases during sporulation while spC remains relatively constant.

In Fig. 2 we have examined the ratio of U-T (R_f 0.11) and T-T (R_f 0.25) and spB to spC during sporulation. The U-T/T-T ratio varies slightly but this is insignificant since the individual curves for U-T and T-T (data not shown) reveal that these photoproduct levels increase slightly during sporulation then fall due to lysis (4,5). The spB/spC ratio, however, shows the distinct differences observed in Fig.1, for both B. megaterium and B. subtilis, where the spB/spC ratio for isolated forespores decreases during sporulation while the same ratio for non-isolated forespores increases. The low spB/spC ratio could, however, be reversed upon germination of isolated forespores, as shown in Fig.3.

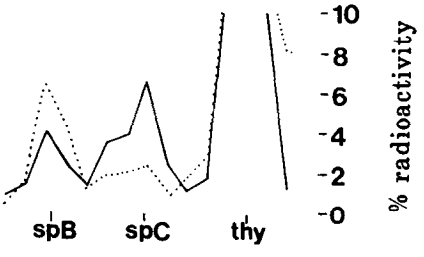


Fig 3 Comparison of spore type photoproducts produced from germinated (• • • •) and non-germinated (——) isolated forespores of B. megaterium QMB 1551.

Inversion of the spB/spC ratio for isolated forespores possibly reflects the presence of mother cell DNA contributing to the production of spore type photoproducts. It was not possible to determine the individual spore type photoproduct contribution of either mother cell or forespore DNA in the intact sporangia for several reasons. Firstly, some Bacillus species in particular B. megaterium have multiple genome copies in both the mother cell and forespore (6,10). Copy number per intact sporangia is not under stringent control, making comparison of individual genomes impossible. Secondly, the isolated forespore preparations were washed and concentrated and no direct comparison of cells with spores/ml was made. These factors would not, however, alter observed differences for the spB/spC ratio for intact/isolated forespores.

The production of spore type photoproducts was thought to reflect a progressive conformational change in DNA within the forespore during sporulation, from the actively transcribable B state to the inactive A (5). There is, however, some difficulty with this hypothesis since the structural state of spore DNA in vivo is not well defined (10).

With regard to sporulation, it appears that photoproducts from stage III cells are like those of vegetative cells, mainly thymine dimers, while those of the spore type are produced when the DNA becomes spore type (5) about one hour before the forespore becomes refractile ie. stage IV. As sporulation proceeds, the level of spore type photoproducts increases and paralleling this increase is the uptake of calcium ions and the synthesis of dipicolinic acid.

The influence of DPA on photoproduct type and amount has been the subject of much conjecture. Early studies (4) using E. coli DNA dried in the presence of calcium-DPA revealed that both spB and spC products were produced with UV irradiation. Other workers (5,11,12) revealed an influence of DPA in vivo on photoproduct formation and suggested that its presence was required for the maintenance of DNA in the spore type configuration, and was a means of transfer of photochemical energy to the polynucleotide.

Photoproduct type and amount is believed to be independent of DNA conformation, but dependent on the degree of hydration of the molecule (13,14). Recent studies (15,16) revealed that DPA and its calcium chelate CaDPA interact with nucleic acids

in vitro, particularily DNA, influencing the molecule's thermostability, its ability to act as a template for RNA synthesis and its motion at a variety of hydration levels, without altering the molecule's conformational state. The binding mechanisms involved include intercalation, a mechanism known to involve hydrophobic forces (17). Since DPA synthesis is thought to occur only in the mother cell (18), the molecule being transported to the developing forespore without association with calcium, small amounts may interact with mother cell DNA giving rise to spore type photoproducts. Thus, UV photoproducts from intact mother cells may result from the influence of DPA on both mother cell and forespore DNA.

This suggestion has some support from recent work (15) where B. cereus T DPA$^-$ mutant spores grown in the presence of exogenous DPA produced a photoproduct which when purified was identified as DPA linked to a thymine residue. This 'photoadduct' indicates the very close proximity of DPA to DNA within the spore.

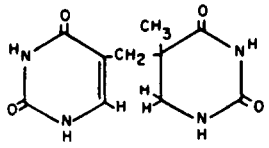
During purification both spB and spC resolved as single peaks on sequential TLC purification plates. However, in cleaning the samples for mass spectroscopy by ion exchange chromatography, spB resolved into two fractions spB1 and spB2. The isolation of an additional photoproduct co-chromatographing with TDHT (spB1) was unexpected although co-chromatography of photoproducts from biological systems has been previously observed (19). In all cases only chromatography on ion exchange resins has revealed the additional photoproduct.

UV scans of the individual fractions revealed maximum absorbances of 216nm for spB1 and 316nm for spB2. Examination of the photoproducts (Fig.4) by mass spectroscopy revealed that spB1 was TDHT, while spB2 was characterized as 6-4'-(pyrimidine-2'-one)-thymine (PO-T) (20). The isolation of PO-T agrees with the results of Patrick and Gray (13) for its isolation by formic acid hydrolysis but contrasts the results of Varghese (3) for its non-isolation. Thus, the presence of PO-T questions the proposal that TDHT is responsible for the majority of killing in spores due to UV damage since it is impossible to distinguish the independent effect of the two components.

The characterization of spore photoproduct spC has provided some difficulties. Firstly, the mass spectrum analysis revealed a molecular ion of 252, which is similar to

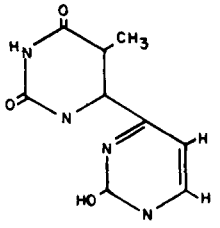
A

SPORE PHOTOPRODUCT B_1 5,THYMINYL-5-6-DIHYDROTHYMINE



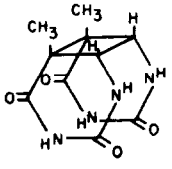
MOL. WT. 252 $C_{10}H_{12}N_4O_4$
MASS SPECT. DEGR. 252,237,179,151,126
R_F VALUE BUT/ACET.A./H_2O 0.40

B SPORE PHOTOPRODUCT B_2 6-4'-(PYRIMIDIN-2'-ONE)-THYMINE



MOL.WT. 220 $C_9H_8N_4O_3$
MASS SPECT. DEGR. 220,203,176,126,96.
R_F VALUE BUT/ACET.A./H_2O 0.40

C SPORE PHOTOPRODUCT C ISOMERIC CYCLOBUTANE THYMINE DIMER, CIS-SYN



MOL. WT. 252 $C_{10}H_{12}N_4O_4$
MASS SPECT. DEGR. 252,210,182,126
R_F VALUE BUT/ACET.A./H_2O 0.52

Fig. 4 Characterization of spB1, spB2 and spC photoproducts by mass spectrum and chromatographic analysis.

that obtained for TDHT. However, apart from spC and TDHT having a complementary fragmentation peak at 126, the thymine moiety, the fragmentation patterns are dissimilar (Fig.4). Varghese (3) points out that although UV light induces many nucleic acid photoproducts, very few are believed to survive the acid-hydrolysis step. This then poses the interesting problem of what spC is.

ACKNOWLEDGEMENTS

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REFERENCES

1. Donnellan, J.E., and Stafford, R.S. (1968) Biophys. J. 8, 17-28.
2. Varghese, A.J. (1970) Biochem. Biophys. Res. Commun. 38, 484-490.
3. Varghese, A.J. (1971) Photochem. Photobiol. 13, 357-364.
4. Donnellan, J.E., and Setlow, R.B. (1965) Science 149, 308-310.
5. Baillie, E., Germaine, G.R., Murrell, W.G., and Ohye, D.F. (1974) J. Bacteriol. 120, 516-523.
6. Johnson, G.C. (1973) Ph.D Thesis, York University, Toronto Canada.

7. Lindsay, J.A., and Murrell, W.G. (1983) Anal. Biochem. (in press).
8. Stewart, B.T., and Halvorson, H.O. (1953) J. Bacteriol. 65, 160-166.
9. Slee, A.M. and Slepecky, R.A. (1977) Spore Research 1976, pp183-194, Academic Press, London.
10. Tsuji, S., Suzuki, K., and Imahori, K. (1975) Agric. Biol. Chem. 39, 1581-1591.
11. Munakata, N.,Fitz-James, P.C., and Young, I.E. (1975) Can. J. Microbiol. 21, 1121-1132.
12. Germaine, G.R., and Murrell, W.G. (1973) Photochem. Photobiol. 17, 145-154.
13. Patrick, M.H., and Gray, D.H. (1976) Photochem. Photobiol. 24, 507-513.
14. Rahn, R.O., and Hosszu, J.L. (1969) Biochim. Biophys. Acta 190, 126-131.
15. Murrell, W.G. (1981) Sporulation and Germination, pp. 64-77, Amer. Soc. Microbiol. Washington, D.C.
16. Lindsay, J.A., Murrell, W.G., and Cornell, B.A. (manuscript in preparation).
17. Georghoiu, S. (1977) Photochem. Photobiol. 26, 59-68.
18 Andreoli, A.J., Saranto, J., Baecher, P.A., Suehiro, S., Escamilla, E. and Steiner, A. (1975) Spores V1, pp. 418-424, Amer. Soc. Microbiol., Washington D.C.
19. Meistrich, M.L., and Lamola, A.A. (1972) J. Mol. Biol. 66, 83-95.
20. Fenselau, C. (1976) Photochemistry and Photobiology of Nucleic Acids, pp. 419-481, Academic Press, New York.