EPA, 1979). These negative monitoring results are expected because animals in the field are not generally exposed to measurable amounts of TCDD. TCDD is not ingested by most animals because of the combined effects of very low application rate, rapid photodecomposition rate (Crosby and Wong, 1977; Getzendaner and Hummel, 1975; Nash and Beall, 1978), and very strong binding to soil, k_{oc} = 486 000 (Kenaga, 1980).

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Pesticide Mutagenicity in *Bacillus subtilis* and *Salmonella typhimurium* Detectors

S. Y. Shiau, R. A. Huff, and I. C. Felkner*

Four pesticides, captan [*N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide], folpet [*N*-[(trichloromethyl)thio]phthalimide], naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate), and triallate [*S*-(2,3,3-trichloroallyl) diisopropylthiocarbamate], were evaluated for their ability to induce mutations in *Salmonella typhimurium* mutants (*TA1535*, *TA1536*, *TA1537*, *TA1538*, *TA98*, and *TA100*) and *Bacillus subtilis* mutants (*TKJ5211* and *TKJ6321*) with and without a rat liver microsomal activation system. These pesticides were more mutagenic in *TKJ6321* or *TKJ5211* than in *TA100* or *TA1535* (base pair substitution mutants). None of the pesticides required metabolic activation, but they were significantly detoxified by this metabolism.

Captan [*N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide) and its isomeric relative, folpet [*N*-[(trichloromethyl)thio]phthalimide), possess valuable antifungal properties and have been used commercially on a large scale in agriculture and horticulture. They are widely applied as a spray, both to control various fungal diseases and to prevent spoilage of fruit and vegetables during storage and transit. Naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate) is used on numerous crops, also against flies in barns, poultry houses, and kennels, and in and around food processing plants. Triallate [*S*-(2,3,3-trichloroallyl) diisopropylthiocarbamate] is used for preemergence application control of wild oats in barley, drum wheat, spring wheat, winter wheat, green peas, field-dried peas, and lentils. Because of the widespread application of these pesticides, humans may be exposed to these chemicals through their occupation or by consuming food containing residues of these chemicals.

Although procedures for establishing risk criteria for such things as acute or chronic toxicity can be readily determined, the risk for long-term effects such as cancer and deleterious human or animal heritable defects are much more difficult to assess. In recent years, simple, rapid, and economical short-term microbial mutagenicity

Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409.

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assays have been employed as mutagenesis/carcinogenesis tests. The most widely used bacterial assay system was developed by Ames et al. (1973). Our previous report (Shiau et al., 1980b) has indicated that *Salmonella typhimurium* (Ames et al., 1973) may not be a sensitive detector for some chemical categories. We, therefore, applied *S. typhimurium* and *Bacillus subtilis* mutants simultaneously to a screening program for the above pesticides. We show in this study that *B. subtilis* is superior to *S. typhimurium* in the detection of mutagenic activity of several pesticides.

MATERIALS AND METHODS

Bacterial Strains. *B. subtilis* strains *TKJ5211* and *TKJ6321*, provided by Dr. H. Tanooka, National Cancer Center Research Institute, Japan, were used in the mutagenesis assays. *TKJ5211* is an Exc$^-$ strain (*uvrA10*) that has the suppressible nonsense mutations *his** and *met**. *TKJ6321*, developed by Munakata from *TKJ5211*, has the additional mutations *polA151* and *spp-1*. It not only has a higher sensitivity to agents such as X-rays than does *TKJ5211* but also has a higher spontaneous background mutation rate. *S. typhimurium* strains *TA1535*, *TA1536*, *TA1537*, *TA1538*, *TA98*, and *TA100* used in the intial spot tests were obtained from Dr. B. Ames, University of California at Berkeley. All the strains are histidine dependent and are induced to mutate back to histidine independence by active mutagens. Strains *TA100* and *TA98* were derived from *TA1535* and *TA1538* by introduction of an R-factor plasmid. The presence of the plasmid enhances the sensitivity of these strains to most mutagens (McCann et al., 1975b).

Preparation of Liver Microsomal Fraction (S-9). The S-9 fraction was prepared from rat livers as described by Ames et al. (1975). For induction of microsomal enzymes, Sprague–Dawley male rats weighing ~250 g were injected intraperitoneally with Aroclor 1254 dissolved in corn oil. On the fifth day after injection the rats were sacrificed, and the S-9 liver microsomal fraction was prepared and stored at −80 °C.

Test Chemicals. The pesticides were obtained with the assistance of Edward Gomes, San Benito, TX, as follows: captan, folpet, and naled from Chevron Chemical Co. and triallate from Monsanto Agricultural Products Co.

Media for Mutagenesis Assays. *B. subtilis.* Glucose minimal medium (GO) described by Spizizen (1958) was solidified with 1.5% agar (Difco). Basic selective medium (BSM) was that described by Tanooka (1977), i.e., GO agar supplemented with casein (Difco) at 200 μg/mL. BSM was further supplemented with L-methionine (SMM) at 100 μg/mL. Nutrient broth and nutrient agar were products of Difco Laboratories. Soft agar contained 8 g of agar (Difco) per L of distilled water.

S. typhimurium. Volgel–Bonner Medium E as described by Vogel and Bonner (1956) with 2% glucose was solidified with 1.5% agar (Difco). Soft agar contains 7.5 g of agar (Difco) and 7.5 g NaCl per L of distilled water.

Spot Tests for Mutation. *B. subtilis.* From the overnight (O/N) cultures of *TKJ5211* and *TKJ6321*, 0.15 mL was inoculated into 15 mL of nutrient broth and incubated with shaking at 37 °C until an OD_{540} of 0.3 was reached. The cells were then diluted with fresh nutrient broth to an OD_{540} of 0.16. For detection of the His$^+$ mutation, 0.1 mL of the culture was plated with 2 mL of soft agar on SMM plates. For detecting the His$^+$, Met$^+$ forward mutation (*su his**, *met**), BSM plates were used. Solutions of each chemical were prepared at a range of 1–6 mg/mL and 50 μL of each was added to the sensitivity disks so that the final concentrations ranged from 50 to 300 μg/plate, respectively. After 3 days of incubation at 37 °C, the plates were examined for mutant colony development.

S. typhimurium. One milliliter from an O/N culture of each *S. typhimurium* tester strains was inoculated into separate flasks containing 49 mL of nutrient broth and incubated with shaking at 37 °C until an OD_{540} of 0.25 ~1.2×10^9 bacteria/mL) was reached. In contrast to the standard procedure of Ames et al. (1975) where histidine–biotin supplement was incorporated into top agar, the modification of Ercegovich and Rashid (1977) was used. Histidine–biotin supplement (12.5 mL; 0.5 mM) was added to the culture immediately prior to its addition to the minimal top agar. The bacterial culture (0.1 mL) was added to 2 mL of molten top agar at 45 °C. Solutions of each chemical were prepared at a range of 1–6 mg/mL, and 50 μL of each was added to the sensitivity disks so that the final concentrations ranged from 50 to 300 μg/plate, respectively. After 3 days of incubation at 37 °C, the plates were examined for mutant colony development. Under conditions where test chemicals reduced cell viability, His$^-$ cells could develop colonies on plates when top agar was supplemented as in the standard Ames et al. (1975) protocol. This has been shown in our laboratory to occur in both spot and quantitative assays, leading to an interpreted false positive. The reason this growth occurs is that the trace amount of histidine supplied is sufficient for colony formation by reduced population size but insufficient in the usual assay range. That His$^-$ colonies develop under conditions where the tester population is small has been confirmed by replica plating and analysis of individual colonies. With this modified assay, spontaneous revertant colonies on control plates were very close to the published values (Ames et al., 1975), and the false positives do not appear to occur so frequently.

Quantitative Assay for Mutant Yields. A pour plate method essentially like that described by Tanooka (1977) was used for the quantitative detection of captan, folpet, naled, and triallate mutagenic activity. For this procedure, overnight cultures of *B. subtilis* and *S. typhimurium* were inoculated into Difco nutrient broth, incubated, and diluted as described in the preceding section, i.e., Spot Tests for Mutation. To 1.0-mL aliquots from cultures prepared in this manner was added 0.5 mL of chemicals in minimal salts so that final concentrations ranging from 1 to 300 μg would be present in the test plates. This mixture was preincubated at 37 °C for 30 min. This was determined to be the optimum time required for mutagenic response of the tester strains. Since stock solutions of all four pesticides were dissolved in dimethyl sulfoxide (Me_2SO), it served as a solvent control. The final concentration was 4% or less, and at this concentration Me_2SO did not affect viability. For detection of mutagenesis, 0.15 mL of this incubation mixture was plated in 2.0 mL of top agar (Tanooka, 1977) onto SMM agar plates for *B. subtilis*, and Vogel–Bonner Medium E for *S. typhimurium*. Mutant colonies were scored after incubation at 37 °C for 2–4 days. Since the treatment for this quantitative response was performed in liquid, a true frequency could be established based on mutants at each survival level. Therefore, mutants per 10^8 survivors was used to standardize the response for every treatment concentration. Mutant colonies were scored after incubation at 37 °C for 2–4 days.

RESULTS AND DISCUSSION

Table I shows the results of the spot tests for mutagenic activity after treatment of various *B. subtilis* and *S. typhimurium* mutants with and without added S-9 Mix. All the pesticides showed positive responses in *B. subtilis* strain *TK6321* both with and without the S-9 fraction, but

Table I. Spot Test for Captan, Folpet, Dibrom, and Far-go

chemical	concn, μg/plate	mutagenicity assay[a]															
		TA1535		*TA1536*		*TA1537*		*TA1538*		*TA98*		*TA100*		*TKJ5211*[b]		*TKJ6321*[b]	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
captan	50	+	±	−	−	+	±	+	±	+	±	++	+	+++	+++	+++	+++
	100	+	±	−	−	+	±	+	±	+	±	++	+	+++	+++	+++	+++
	300	+	+	−	−	+	±	+	+	+	±	++	+	+++	+++	+++	+++
folpet	50	−	−	−	−	−	−	−	−	−	−	+	−	+	±	++	+
	100	−	−	−	−	−	−	−	−	−	−	+	−	+	±	++	+
	300	−	−	−	−	−	−	−	−	−	−	+	−	+	±	++	+
Dibrom (naled)	50	+	±	−	−	±	−	±	±	±	±	±	±	±	±	+	±
	100	+	+	±	−	±	−	±	±	±	±	±	±	±	±	++	±
	300	+	+	±	±	±	±	±	±	±	±	+	+	+	±	++	+
Far-go (triallate)	50	±	−	−	−	−	−	−	−	−	−	±	±	−	−	+	±
	100	±	−	−	−	−	−	−	−	−	−	+	±	−	−	++	±
	300	±	±	−	−	−	−	−	−	−	−	+	+	−	−	++	±

[a] (−) Equal to or less than the spontaneous rate; (+) almost identical with the background, but with an inhibition zone; (+) has an inhibition zone and a few more revertants than the background; (++) has an inhibition zone and more revertants than the background; (+++) has an inhibition zone and too numerous to count revertants. [b] His^+ revertants.

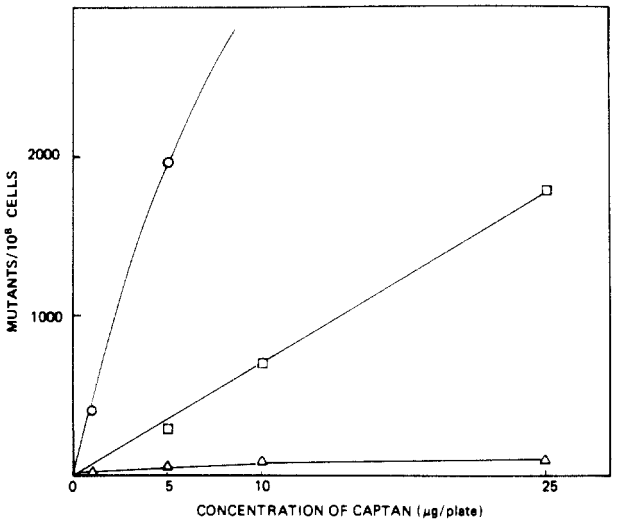

Figure 1. Comparison of *B. subtilis* strains *TKJ6321* (○) and *TKJ5211* (□) and *S. typhimurium* strain *TA100* (△) for their mutagenic response to captan. Mutants per 10^8 survivors was used to standardize the response at every treatment concentration.

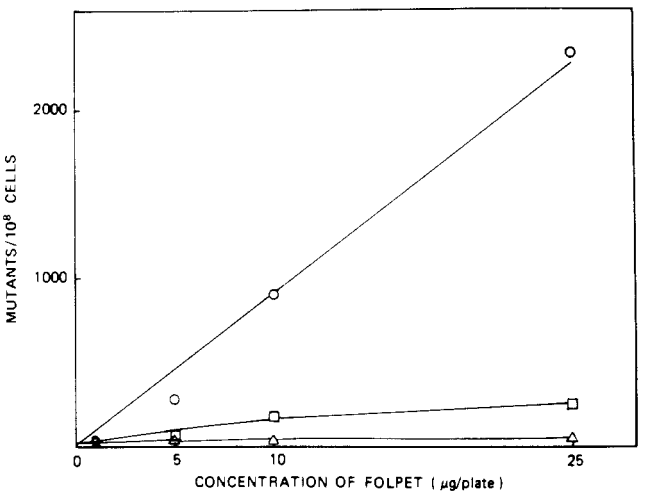

Figure 2. Comparison of *B. subtilis* strains *TKJ6321* (○) and *TKJ5211* (□) and *S. typhimurium* strain *TA100* (△) for their mutagenic response to folpet. Mutants per 10^8 survivors was used to standardize the response at every treatment concentration.

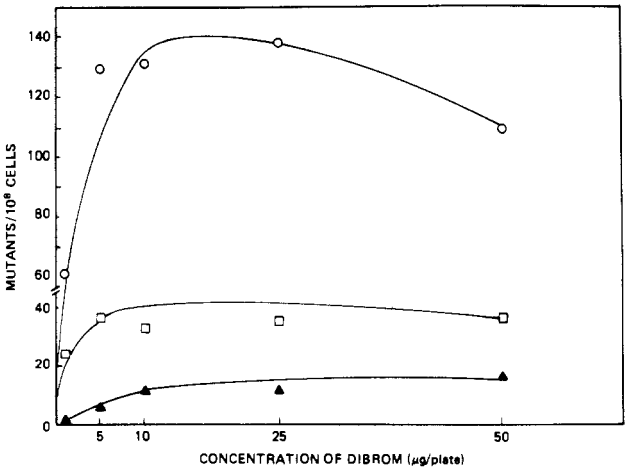

Figure 3. Comparison of *B. subtilis* strains *TKJ6321* (○) *TKJ5211* (□) and *S. typhimurium* strain *TA1535* (▲) for their mutagenic response to Dibrom. Mutants per 10^8 survivors was used to standardize the response at every treatment concentration.

the addition of S-9 Mix resulted in a reduction of the mutagenic responses for all four pesticides. Captan and folpet also showed positive responses with *B. subtilis* strain *TKJ5211*. The mutagenic response to naled with strain *TKJ5211* was weak, but it was mutagenic at 300 μg in the absence of S-9 Mix activation. Triallate did not produce a mutagenic effect with strain *TKJ5211*. For *S. typhimurium* strains, captan and naled showed mutagenic responses on all *S. typhimurium* strains except *TA1536* which failed to give a response to captan. Folpet failed to show response in all *S. typhimurium* strains except strain *TA100*. Triallate showed response in strains *TA1535* and *TA100*, but failed in the rest of *S. typhimurium* strains. *TA100* was the only *Salmonella* strain which gave a positive mutagenic response to all four pesticides. All the pesticides showed a stronger mutagenic activity in strain *TA100* than the rest of *S. typhimurium* strains with the exception of naled which showed more mutagenic activity in strain *TA1535*. The results presented in Table I are qualitative but are given because they form the basis for our decision to perform quantitative assays with the most responsive tester bacteria.

Mutation Assays. Quantitative induction of bacterial mutants with captan, folpet, naled, and triallate is shown in Figures 1–4. Since all *Salmonella* mutants but *TA100* or *TA1535* gave weak or negative results for the spot assays, either *TA100* or *TA1535* was chosen for comparison with those *B. subtilis* mutants giving a positive mutagenic response. *B. subtilis* strains *TKJ6321* and *TKJ5211* and *S. typhimurium* strain *TA100* were exposed to varying concentrations of captan and folpet. *TKJ6321*, *TKJ5211*,

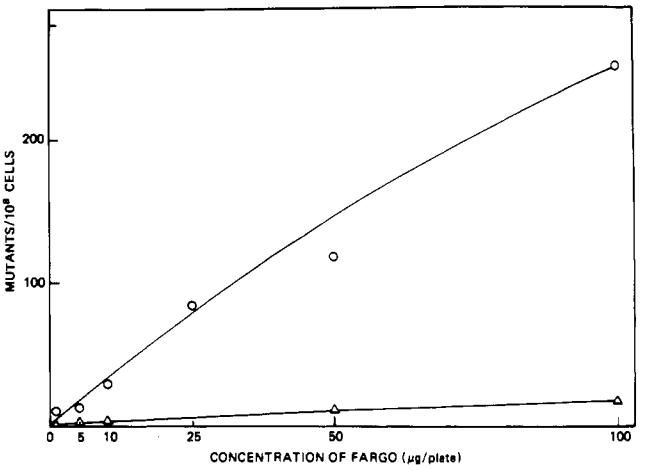

Figure 4. Comparison of *B. subtilis* strain *TKJ6321* (O) to *S. typhimurium* strain *TA100* (Δ) for their mutagenic response to Far-go. Mutants per 10^8 survivors was used to standardize the response at every treatment concentration.

and *TA1535* were exposed to varying concentrations of naled. For triallate, *TKJ6321* and *TA100* were exposed to different concentrations. His^+ mutants were induced in the absence of metabolism by liver S-9 Mix. As shown here, the *B. subtilis* mutant *TKJ6321* gave a stronger mutagenic response than could be obtained with the *Salmonella* mutant TA100 (captan, folpet, and triallate) or *TA1535* (naled). *B. subtilis* mutant *TKJ5211* also gave a stronger mutagenic response than could be obtained with *Salmonella* mutant *TA100* (captan and folpet) or *TA1535* (naled). It is concluded that captan, folpet, naled, and triallate are all base-pair mutagens based on this response.

We have made a quantitative assessment of mutagenicity for captan, folpet, naled, and triallate. The potency was expressed in mutants per nanomole (McCann et al., 1975a) so that our data could be compared with others. From these calculations based on our data, captan mutated *S. typhimurium* strain *TA100* at 26.75 revertants/nmol which is very close to the results published by McCann et al. (1975a). When *B. subtilis TKJ6321* was used, a figure of 31.87 revertants/nmol was obtained. Folpet mutated *S. typhimurium TA100* at 7.71 revertants/nmol which is lower than the result obtained by McCann et al. (1975a). A figure of 10.68 revertants/nmol with folpet was obtained when *B. subtilis TKJ6321* was used. Naled mutated *S. typhimurium TA1535* at 7.24 revertants/nmol while triallate mutated *S. typhimurium TA100* at 1.83 revertants/nmol. With *B. subtilis* values of 10.29 and 6.09 revertants/nmol were obtained with naled and triallate, respectively.

In previous reports (Felkner et al., 1979; Shiau et al., 1980b) we demonstrated that *B. subtilis* is superior to *S. typhimurium* in the detection of mutagenic activity for certain chemicals, and in this study the results substantiate this finding. Our findings strongly suggest that other pesticides that were previously reported to be nonmutagenic in *Salmonella* should be tested for mutagenicity with *Bacillus* mutants.

Finally, it is important to mention that naled and triallate have been suspected of posing a health effects risk to humans. The present report on the mutagenicity of naled and triallate is, to our knowledge, the first positive result in a prokaryotic test. Captan, folpet, and naled are all potent DNA-damaging agents in the *B. subtilis* repair assay (Shiau et al., 1980) as it is performed by Felkner et al. (1979). It seems possible, therefore, that these pesticides may present a high genetic toxicology risk because they cause damage to DNA and possess a high mutagenic potency. The evidence is even more substantial because mutagenicity is detected in both a Gram-positive and a Gram-negative bacterial species. However, caution should be taken in assuming that mutagenic potency detected in vitro will necessarily extrapolate to in vivo mammalian systems. Captan, for example, has clearly been shown to be greatly reduced in mutagenic activity when it is mixed with rat, rabbit, or human serum or blood (Ficsor et al., 1977; Bridges, 1975). This compound and its isomer, folpet, are probably inactivated by the glutathione of the erythrocytes. This decrease in activity is, as we have shown (Table I), further enhanced by incubation with microsomes prepared from rat liver.

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