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Activation and Detection of (Pro)mutagenic Chemicals Using Recombinant Strains of *Streptomyces griseus*

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ABSTRACT

Two recombinant strains of *Streptomyces griseus* have been developed to report on the activation of promutagenic† chemicals. This activation is monitored by reversion of the bacterial test strains to a kanamycin-resistant phenotype. Strain H69 detects point mutations and was reverted at an increased frequency by acetonitrile, 2-aminoanthracene, 1,2-benzanthracene, benzidine, benzo(a)pyrene, 9,10-dimethyl-1,2-benzanthracene, and glycine. The second strain, FS2, detects frame shift mutations and was reverted at an increased frequency by 1,2-benzanthracene, benzidine, and glycine. Compounds such as butylated hydroxytoluene, catechol, chlorobenzene, hydroquinone, potassium chloride, phenol, *cis*-stilbene, *trans*-stilbene, and toluene did not elicit positive responses in either strain. In addition, these strains are capable of detecting direct-acting mutagens such as *N*-methyl-*N'*-nitrosoguanidine and ICR-191, providing further evidence

†(pro)mutagenic = promutagenic/mutagenic

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of their promise for detecting a wider range of mutagens. To our knowledge, this is the first report of bacterial strains capable of activating promutagenic compounds and detecting their mutagenic metabolites without the benefit of an exogenous activation system such as the rodent liver homogenate (S9).

Index Entries: *Streptomyces griseus;* cytochrome P-450; activation of promutagens; detection of mutagens.

INTRODUCTION

The prolific action of industrial communities releases vast quantities of man-made chemicals into our environment. Some of these chemicals are directly toxic, and others are nonhazardous in their original form. Metabolism of this latter class by mammalian cells sometimes leads to the formation of mutagenic chemicals. Such chemicals are called promutagens, and their metabolism to mutagenic metabolites is termed *activation*. This particular capability of mammalian cells has been exploited in current short-term genotoxicity assays such as the Salmonella/Ames assay, in which the enzymatic system of rodents is harnessed for activation of a variety of promutagenic xenobiotics (*1*).

Microorganisms have long been recognized for their abilities to oxidize xenobiotic compounds using either their constitutive or inducible enzymatic systems. Xenobiotic oxidation by microorganisms can lead to detoxification and/or toxification of various chemicals. An example of detoxification of xenobiotics was shown by Cerniglia et al. (*9*) with the filamentous fungus *Cunninghamella elegans,* which oxidizes promutagenic polycyclic aromatic hydrocarbons such as benzo(a)pyrene [B(a)P] to nonmutagenic metabolites.

We have shown that the soybean flour-induced cytochrome P-450_{soy} of *S griseus* resembles its mammalian counterparts in oxidizing a variety of structurally diverse xenobiotics (*25*) and also in activating a variety of promutagenic chemicals by replacing the rat liver (S9) fractions in the conventional salmonella/Ames gene-mutation assay (*23*).

Exploiting this capability, we have constructed genetically engineered strains of *S griseus* containing a suitable phenotypic marker that can concomitantly activate (pro)mutagenic chemicals and monitor their mutagenic potentials.

MATERIALS AND METHODS

Microorganisms, Growth Media, and Buffers

Streptomyces griseus ATCC 13273 was obtained from the American Type Culture Collection (Rockville, MD). *Streptomyces lividans* JI1326 (*13*)

and *Escherichia coli* MC1061 (*8*) have previously been described. Streptomyces strains were typically stored as spores frozen in 20% glycerol at −80°C. *Escherichia coli* MC1061 was stored at −80°C in 25% glycerol/50% growth medium. *Escherichia coli* strains were grown on LB medium consisting of tryptone (1%), NaCl (1%), and yeast extract (0.5%) at 37°C. Ampicillin or kanamycin (Kan) (25 μg/mL) were added as required. Streptomyces strains were grown at 28°C in soybean-glycerol (SBG) medium (*22*) and one-half Yeme (*13*) as described before. Alternatively, these organisms were grown on M7 agar that contained (per L) glucose (10 g), NZ amine type A (casein hydrolysate, 2 g), yeast extract (1 g), $CoCl_2$ (10 mg), and agar (22 g) with thiostrepton (Ts, 10 μg/mL) or Kan (50 μg/mL). P buffer was prepared as described before (*13*).

Materials

Soybean flour was purchased from Natural Sales Co. (Pittsburgh, PA); yeast extract was from Oxoid (Colombia, MD); and antibiotics and TES buffer were from Sigma Chemical Co. (St. Louis, MO). NZ amine was obtained from ICN Biomedicals (Cleveland, OH).

Plasmid Constructions

Plasmid pIJ702-pBR322 was made by ligating BamHI-digested pBR322 (*6*) with BamHI-digested pIJ702 (*14*). This plasmid was used for subsequent manipulations in *E coli* of the streptomyces vector pIJ702. A 1.3 kb HindIII-Xmal DNA fragment containing the Tn5 Kan resistance gene was inserted into the BgIII site of pIJ702-pBR322 using BcII linkers (New England Biolabs, Beverly, MA), forming pIJ702-Kan^r-pBR322. Next, pIJ702-Kan^r-pBR322 or its derivatives was cut with BamHI, separating the pBR322 DNA from the pIJ702-Kan^r DNA, and recircularized under dilute conditions (< 3.0 μg/mL) to favor intramolecular ligation before transformation into *S lividans*.

Plasmid pFS, a Kan-sensitive plasmid similar to pIJ702-Kan^r, was constructed by inserting, via site-directed mutagenesis (*7,21*), a guanine nucleotide between bases four and five of the open reading frame of the Kan resistance gene. Kan-sensitive derivatives of pIJ702-Kan^r (pH 60–71) were made by hydroxylamine mutagenesis of this plasmid as described before (*18*), followed by transformation and selection of *S griseus* for Ts resistance and Kan sensitivity. The nature of these mutations is unknown.

Genetic Transformations

Streptomyces lividans was transformed as described by Hopwood and colleagues (*13*); *S griseus* protoplasts were obtained using 0.7% glycine and were incubated with shaking (250 rpm) at 28°C for an additional 24 h. Protoplasts were resuspended in 200 μL of modified P buffer and were either used immediately for transformation or stored at −80°C.

Mutation Frequencies

Mutation (revertant) frequencies for Kan resistance of *S griseus* strains containing Ts^r-Kan^s (FS2, H60–H71) plasmids were determined as follows. Cultures were streaked onto M7 agar plates containing Ts and incubated aerobically for 5 d at 28°C. Spores were harvested as described previously (*13*), titered by dilution in sterile distilled water, and plated on M7 plates containing either Ts or Kan. Plates were then incubated aerobically for 4 d at 28°C, and the number of colonies on each plate was counted. Mutation frequencies were calculated as the number of colonies growing on plates containing Kan divided by the number of colonies growing on plates containing Ts multiplied by the dilution factor.

Mutagenicity Assessment

Two *S griseus* strains, FS2 and H69, were used in all assays. One control flask and one test chemical flask of each strain were used per assay. Approximately 10^5–10^6 spores were inoculated into 125mL DeLong flasks containing 25 mL of SBG medium and Ts (10 μg/mL). Organisms were first grown in this medium at 28°C for 72 h (stage I). A 10% inoculum was then made in another flask containing fresh SBG medium and Ts (stage II). Various concentrations of the test chemicals [in 1500 μL of dimethylsulfoxide (DMSO) or in a minimal vol of a suitable solvent plus 1500 μL of DMSO] were added to 24-h-old stage II cultures. An equal aliquot of solvent, without the test chemical, was added to the control flask. The cultures were then incubated with shaking at 28°C for 24 h. Aliquots were then removed from each flask, washed three times with sterile 100 m*M* potassium phosphate buffer (pH 6.7), resuspended in a small quantity of the same buffer, and spread (in triplicates) onto M7 agar plates containing either Ts or Kan.

Data Evaluation

Data (control mutation frequencies) were analyzed by One-Way Analysis of Variance (ANOVA) using standardized computerized software (*4*). The mean square error estimate from the ANOVA of all control data was used in calculating the Dunnet's *t* statistic (*24*). Statistical significance (probability of response greater than one-tailed) was judged at the 95% level of confidence ($p < 0.05$). This analysis provides for comparing each treatment individually with the control. A response was judged positive when the mean mutation frequency of the individual treatment exceeded the calculated Dunnet's *t* statistic.

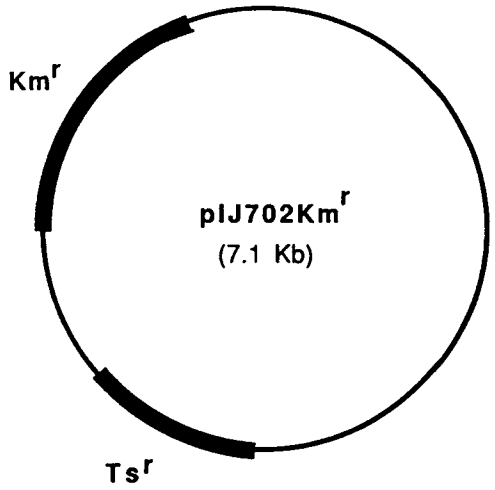

Fig. 1.

RESULTS AND DISCUSSION

Genetically manipulated strains of *S griseus* were used to activate promutagenic chemicals and report on the formation of their mutagenic metabolites. The Tn5 Kan resistance gene was chosen to provide the phenotypic marker in a reversion assay, since the wild-type *S griseus* cells are highly sensitive to this antibiotic (*5*). Two high-copy number plasmids, pIJ702-Kan^r (Fig. 1) and pFS, each containing a Kan resistance gene were transformed into *S griseus*. Plasmid pIJ702-Kan^r confers resistance to both Kan and Ts, whereas the analogous pFS plasmid contains a frameshift mutation within the Kan resistance gene.

A number of other plasmids with mutations in pIJ702-Kan^r were made by in vitro hydroxylamine treatment (*18*). *Streptomyces griseus* transformants containing pH69 and pFS were then chosen on the basis of spontaneous reversion frequencies (8×10^{-7} and 0.9×10^{-7}, respectively) (Table 1), uniformity of colonies of spontaneous mutants, and ability to be reverted to Kan^r by known (pro)mutagens. Mutagenesis in both strains was monitored by reversion of the mutated Kan resistance gene. For ease of nomenclature, these strains are referred to as strains H69 and FS2 in this communication.

Many (pro)mutagenic chemicals are hydrophobic in nature and must be dissolved in a solvent to ensure their homogeneous distribution in the aqueous growth medium. B(a)P was chosen as a model substrate, and the effect of a variety of commonly used organic solvents and their concentra-

Table 1
Spontaneous Mutation Frequencies[a] of *S griseus* Strains H69 and FS2

Strain	N[b]	Parameter		
		Ave. ($\times 10^{-7}$)	SD ($\times 10^{-7}$)	SEM ($\times 10^{-7}$)
H69	35	8.0	10.63	1.80
FS2	35	0.9	1.40	0.24

Note: Ave. = average mutation frequency; SD = standard deviation; and SEM = standard error of mean.
[a]Mutation frequency in the presence of 6% DMSO.
[b]Number of independent experiments from which data were taken.

Table 2
Effect of Solvents on Mutagenicity
of Benzo(a)pyrene [B(a)P][a] on *S griseus* Strain H69

Solvent	Mutagen	Kan titer (1×10^2)	Ts titer (1×10^8)	Mut. freq. (1×10^{-7})
1.2% Acetone	none	1.40	7.23	1.94
	B(a)P	1.63	4.40	3.70
4.0% Acetone	none	3.70	12.30	3.01
	B(a)P	3.07	25.00	1.23
4.0% DMF	none	11.30	38.0	2.97
	B(a)P	4.70	15.7	2.99
4.0% Hexane	none	5.73	16.3	3.51
	B(a)P	3.27	27.0	1.21
4.0% CH_2Cl_2	none	4.07	26.3	1.56
	B(a)P	3.87	49.7	0.78
4.0% Toluene	none	2.67	21.3	1.25
	B(a)P	6.30	27.0	2.33
0.8%DMSO +	none	0.73	4.20	1.75
0.4% Acetone	B(a)P	1.30	6.90	1.88
3.0% DMSO	none	1.40	6.43	2.17
	B(a)P	5.47	5.53	9.88
6.0%DMSO	none	1.57	4.97	3.15
	B(a)P	15.1	4.27	35.5[b]

[a]B(a)P (5.0 mg) was dissolved in the solvent indicated prior to its addition to 24-h-old stage II cultures.
[b]$p < 0.05$; $> 3\times$ over control.
Note: Kan = kanamycin; Ts = thiostrepton; DMF = dimethylformamide; DMSO = Dimethylsulfoxide; and Mut. Freq. = mutation frequency.

tion on its mutagenicity on strain H69 was tested (Table 2). The data obtained indicated that B(a)P dissolved in 6% DMSO produced the highest mutation frequency in H69, presumably the result of an increase in cellular permeability. Since a DMSO requirement was observed with a number of other promutagens (Buchholz and Sariaslani, unpublished observations), it was selected as the solvent of choice for further studies. In cases

where a particular chemical was not soluble in DMSO, the chemical dissolved in a minimal vol of another suitable solvent was added to the cultures with 6% DMSO.

It was previously observed that the highest rate of xenobiotic oxidation is usually obtained with 24-h-old stage II organisms, which contain the highest level of cytochrome P-450_{soy} (*22*). Strict adherence to the two-stage fermentation protocol used in previous studies was a prerequisite for obtaining the highest mutagenic activity with various chemicals (*23*). Reduction of the duration of stage I from 72 to 48 h or addition of the chemicals at the beginning of stage II instead of after 24 h of growth consistently reduced the mutagenic activity. Therefore, the established two-stage fermentation protocol was used prior to substrate addition throughout this study.

To obtain an accurate estimate of the number of mutated cells, a sporulation step was included in this assay. Streptomyces are mycelial organisms and as such, direct plating on a selective medium can result in colonies formed from multicellular aggregates rather than from single cells. (Pro)-mutagen-treated cultures were therefore plated on a nonselective medium to allow for spore formation. Single colonies obtained from these spores were then plated onto a selective medium for an accurate estimation of the number of mutated cells.

In order to determine an optimum concentration for chemicals in each assay, B(a)P was chosen as a model, and a study was performed in which B(a)P was tested for its mutagenicity at various concentrations. Results obtained indicated that a 200 μg/mL concentration was adequate for B(a)P since these experiments were designed to determine the overall response rather than to define a clear dose–response relationship. Dose–response analysis (linear trend) was not conducted because of the permeability problems associated with the hydrophobic substrates used. This permeability problem was overcome by Ames and co-workers in the Salmonella tester strains by selecting for the deep-rough (rfa) genotype, which also confers sensitivity to crystal violet (*1,16*).

Table 3 summarizes the positive and negative responses obtained for chemicals evaluated thus far with H69 and FS2. The known rodent mutagens/carcinogens; 2-aminoanthracene, benzidine, benzo(a)pyrene, 9,10-dimethyl-1,2-benzanthracene, and *N*-methyl-*N'*-nitrosoguanidine (*2,11*), produced positive results in the *S griseus* test system.

Acetonitrile, 1,2-benzanthracene, hydroquinone, and ICR-191, which have exhibited mutagenicity in mammalian in vitro gene-mutation assays (*2*), have unknown carcinogenicity. All of these chemicals, except hydroquinone, were identified as positive by the *S griseus* test system. Available data indicate that hydroquinone is not active in the Salmonella/Ames mutagenicity assay (*20*). Nonmutagenic chemicals such as butylated hydroxytoluene, toluene, and *trans*- and *cis*-stilbene, which are inactive in the Salmonella/Ames assay, did not give rise to increased mutation frequencies in this system either. However, the nonmutagenic compound

Table 3
Summary of Mutagenic Responses for *S griseus* Strains H69 and FS2 for Chemicals Tested at 200 μg/mL Concentration

Chemical	Source	H69	FS2
Acetonitrile	Fisher	+ + +	–
2-Aminoanthracene	Sigma	+ +	–
1,2-Benzanthracene	Aldrich	+ +	+ +
Benzidine	Sigma	+ +	+
Benzo(a)pyrene	Sigma	+ + +	–
Butylated hydroxytoluene	Sigma	–	–
Catechol	Sigma	–	–
Chlorobenzene	Aldrich	–	–
9,10-Dimethyl-1,2-benzanthracene	Sigma	+ +	–
Glycine	Fisher	+ +	+
Hydroquinone	Sigma	–	–
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	Sigma	+	–
Mutagen ICR-191	Polysciences	+ + +	+ + +
Phenol	Sigma	–	–
Potassium chloride	EM Sciences	–	–
cis-Stilbene	Aldrich	–	–
trans-Stilbene	Aldrich	–	–
Toluene	Aldrich	–	–

Note: + = < 2× control, and $p < 0.05$; + + = > 2× control, and $p < 0.05$; and + + + = > 3× control, and $p < 0.05$.

glycine was identified as a mutagen by *S griseus*. The response elicited by glycine is caused by its effect on streptomyces cell walls (*13*). It should be noted that H69 and FS2 show different responses to the same compound, e.g., *N*-methyl-*N'*-nitrosoguanidine. This is expected, since FS2 detects frame-shift mutagens and H69 strain detects point mutations.

CONCLUSIONS

Since the development of the *Salmonella typhimurium* plate-incorporation assay by Ames and co-workers (*1*), numerous investigators have sought to exploit the use of bacteria in short-term genetic toxicity assessments (*3,17,19*). Of particular note is the recent effort to develop bacterial test systems that are sensitive to a wider range of genotoxins, e.g., chlorinated hydrocarbons and metals, than the *S typhimurium* strains used in the Ames test (*16*). Additionally, efforts have been made to develop test systems that require a single organism rather than the four or five strains of bacteria commonly used in the Salmonella/Ames gene-mutation assay (*15*).

Although progress has been made in the above areas, one important area of development remains to be rigorously pursued. Currently, a mammalian-derived enzymatic system (rodent liver S-9 preparation), induced by a polychlorinated biphenyl (Aroclor® 1254), is employed for activation of promutagens in all of these test systems (*10*). Considering today's concern about the use of animals in routine toxicology assessments and the safety issues on handling hazardous chemicals such as Aroclor, an alternative activation system, probably from a bacterial source, would be highly desirable. Furthermore, it would be most convenient if both the promutagen activation and mutagen detection systems were harbored within a common biological system. This could be achieved by constructing an appropriately modified bacterium that could simultaneously activate promutagens while providing a reporter function.

In an earlier publication (*23*), we reported that *S griseus* cells grown in a soybean flour-enriched medium could replace the (S9) fraction for activaing promutagenic chemicals in a modified Salmonella/Ames assay. In this communication, we have exploited the enzymatic capabilities of *S griseus* for activation of (pro)mutagenic chemicals and have developed two unique recombinant strains of *S griseus* that can activate (pro)mutagens and assay for the presence of potentially mutagenic chemicals. The data obtained in this study, in terms of the overall response, underline the potential of these recombinant strains for further development and use in genetic toxicology studies. Further studies are in progress to simplify the induction and growth requirements of these strains.

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REFERENCES

1. Ames, B. N., McCann, J., and Yamasaki, E. (1975), *Mutation Res.* **31,** 347.
2. Ashby, J. and Tennant, R. W. (1988), *Mutation Res.* **204,** 17.
3. Auletta, A. and Ashby, J. Workshop on the relationship between short-term test information and carcinogenicity, Williamsburg, VA (January, 1988), pp. 20–23.
4. BBN (1984), *RS/1 User's Guide, Book 2: Graphics and Statistics,* BBN Software Products Corp., Cambridge, MA, pp.
5. Beck, E., Lugwig, G., Auerswald, E. A., Reiss, B., and Schaller, H. (1982), *Gene.* **19,** 327.
6. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, J. H., and Falkow, S. (1977), *Gene.* **2,** 95.
7. Carter, P., Bedouelle, H., Waye, M., and Winter, G. (1985), *Oligonucleotide Site-Directed Mutagenesis in M13,* Anglian Biotechnology, London, pp.

8. Casadaban, M. J., Martinez-Arias, A., Shapira, S. K., and Chou, J. (1983), *Meth. Enzymol.* **100,** 293.
9. Cerniglia, C. E., White, G. L., and Heflich, R. H. (1985), *Arch. Microbiol.* **143,** 105.
10. Claxton, L. D., Allen, J., Auletta, A., Morelmanns, K., Nestmann, E., and Zeiger, E. (1987), *Mutation Res.* **189,** 83.
11. Gold, L. S., Slone, T. H., and Berstein, L. (1989), *Environ. Health Perspect.* **79,** 259.
12. Haworth, S., Lawlor, T., Mortelmans, K., Speck, W., and Zeiger, E. (1983), *Environ. Muta.* **5(Suppl. 1),** 3.
13. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Burton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., and Ward, J. M. (1985), *Genetic Manipulation of Streptomyces: A Laboratory Manual,* John Innes Foundation, Norwich, UK, pp.
14. Katz, E., Thompson, C. J., and Hopwood, D. A. (1983), *J. Gen. Microbiol.* **129,** 2703.
15. McCoy, E. C., Speck, W. T., and Rosenkranz, H. S. (1977), *Mutation Res.* **46,** 261.
16. Maron, D. M. and Ames, B. N. (1983), *Mutation Res.* **113,** 173.
17. Nestmann, E. R., Brillinger, R. L., McPherson, M. F., and Maus, K. L. (1987), *Environ. Mol. Mutagen.* **10,** 169.
18. Pugsley, A. P. and Oudega, B. (1987), *Plasmids: A Practical Approach,* Hardy, K. G., ed., IRL, Washington, DC, pp. 105–161.
19. Quillardet, P., Bellecombe, C., and Hofnung, M. (1985), *Mutation Res.* **147,** 79.
20. Rapson, W. H., Nazar, M. A., and Butsky, V. V. (1980), *Bull. Environ. Contam. Toxicol.* **24,** 590.
21. Sarkar, H. K., Viitanen, P. V., Padan, E., Trumble, W. R., Poonian, M. S., McComas, W., and Kaback, H. R. (1986), *Meth. Enzy.* **125,** 214.
22. Sariaslani, F. S. and Kunz, D. A. (1986), *Biochem. Biophys. Res. Comm.* **141,** 405.
23. Sariaslani, F. S. and Stahl Jr., R. G. (1990), *Biochem. Biophys. Res. Comm.* **166,** 743.
24. Steel, R. G. D. and Torrie, J. H. (1960), *Principles and Procedures of Statistics,* McGraw Hill, New York, pp.
25. Trower, M. K., Sariaslani, F. S., and Kitson, F. G. (1988), *Biochem. Biophys. Res. Comm.* **157,** 1417.