# Construction of *umu-fhuA* Operon Fusions to Detect Genotoxic Potential by an Antibody–Cell Surface Reaction

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For the environmental monitoring of genotoxic effects of chemicals, a new test system was developed using immunochemical detection of a cell surface reporter protein of *Escherichia coli*. A *umu-fhuA* operon fusion was constructed on plasmid pST12, which is inducible via the SOS system by the mutagenic agent methyl–methane–sulfonate (MMS). The expression of the outer membrane protein FhuA as a reporter was detectable on the cell surface with a monoclonal anti-FhuA antibody. Enzyme-linked immunosorbent assay tests and immunofluorescence microscopy revealed that the operon fusion of PST12 led to specific and strong induction of the reporter protein FhuA after genotoxic treatment with MMS. © 1994 John Wiley & Sons, Inc.

## INTRODUCTION

Environmental pollution and the increased occurrence of genotoxic chemicals in soil and water is a pertinent fact. To monitor mutagenic potentials in the environment, different test systems have been developed, some of which measure genotoxic potentials by the activation of the SOS system of bacteria (Kwan et al., 1990, Quillardet and Hofnung, 1985). The genetically complex SOS regulatory system of bacteria is induced by DNA damage, probably by single-stranded DNA, via RecA-mediated cleavage of LexA. This inducing cascade initiates the expression of at least 16 genes involved in DNA repair, mutagenesis, and other SOS functions (for reviews, see Little and Mount, 1982, Walker, 1984). SOS-dependent test systems detect genotoxic potential by measuring the induction of reporter genes fused to SOS-regulated genes such as *umu*C or *sul*A. These tests are able to detect mutagenic

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**TABLE I. List of plasmids**

| Plasmid | Characteristics | Source or Reference |
|---|---|---|
| pGC01 | *fhu*A, *bla*, 6.3 kb | Carmel and Coulton (1991) |
| pSK1002 | *umu*C-*lac*Z, *bla*, 15 kb | Shinagawa et al. (1983) |
| PST01 | *fhu*A, linker inserted, *bla*, 5.8 kb | This study |
| pST12 | 4.5 kb *umu* fragment Before *fhu*A, *bla*, 10.3 kb | This study |
| pST201–pST224 | 1.1 kb *umu* fragment Before *fhu*A, *bla*, 6.9 kb | This study |

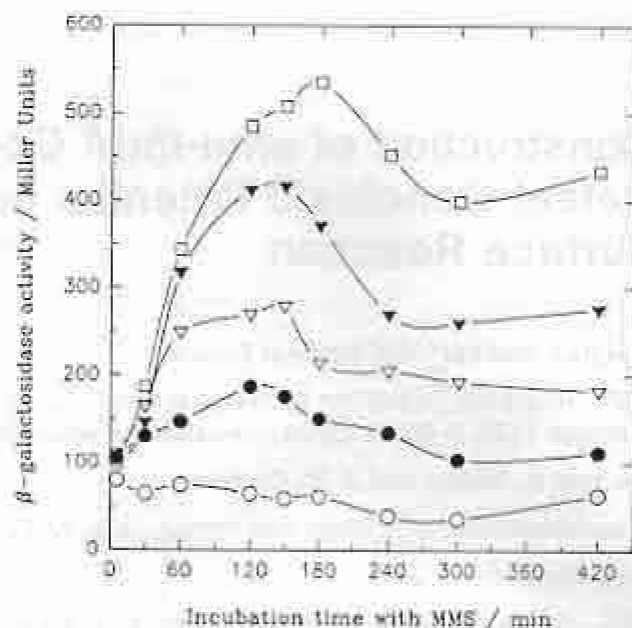


**Fig. 1.** Time course of induction of the *umuC-lacZ* fusion with MMS. Symbols: (○) negative control; (●) 30 ppm MMS; (▽) 67 ppm MMS; (▽) 170 ppm MMS; (□) 670 ppm MMS.

effects in aqueous solution. Therefore sediment or soil samples have to be extracted or suspended in water (Kwan and Dutka, 1992). This implies that the bioavailability of genotoxins could be dramatically altered before the test. The true mutagenic effects of chemicals in undisturbed soil or sediment are not yet possible to detect. It is therefore of basic ecotoxicological importance to measure in situ the effects of genotoxins in soils or sediments (Ahlf et al., 1993).

Using immunostaining techniques, it is possible to identify single bacteria in soil when antibodies against cell surface structures are used (Asanuma et al., 1985). We adapted the approach of an in situ test system by combining sensitivity of the SOS induction of the *umu* operon with a well-characterized reporter protein that is exposed on the cell surface of a indicator bacterium. Identification by immunological techniques permits highly sensitive, specific, and nondestructive measurement of the SOS induction level. We chose the ferrichrome-iron receptor of *Escherichia coli* (FhuA protein), which is detectable on the bacterial cell surface by the monoclonal antibody Fhu3.1. The FhuA protein is part of the high-affinity ferrichrome uptake system in *E. coli* and is normally induced under iron limited conditions (Braun, 1985; Carmel and Coulton, 1991).

In this article we report the construction of *umu-fhuA* operon fusions and the identification of genotoxic effects of methyl–methane–sulfonate (MMS) by immunoreactivity of the SOS-induced outer membrane reporter protein FhuA.

## MATERIALS AND METHODS

All tests were performed using *E. coli* JM83 (Viera and Messing, 1982). The plasmids are listed in Table I. Bacteria were grown in LB broth or on LB agar (Sambrook et al., 1989). Ampicillin (100 μg/mL) was added to the media to maintain the selective pressure for the plasmids.

DNA isolation, digestion with endonucleases, ligation, and transformation were done as described (Sambrook et al., 1989). The linker was synthesized on a Pharmacia Gene Assembler, Pharmacia, Germany.

Induction of the *umuC-lacZ* fusion was by adding

**TABLE II. Immunological reaction of whole cells of *E. coli* with the monoclonal antibody Fhu3.1 under different conditions in the ELISA test[a]**

| Cells | Reaction with the antibody |
|---|---|
| *E. coli* JM83 | − |
| *E. coli* JM83, grown under iron starvation conditions | + |
| *E. coli*, pGC01 | ++++ |
| *E. coli*, pGC01, grown under iron starvation conditions | ++++ |

[a] Iron starvation of the cells was achieved by growing *E. coli* for two days in LB medium that contained 40 μM EDDHA. Reaction of the antibody was tested in triplicate by ELISA against whole cells as described in Material and Methods. Symbols: minus: no reaction; plus: weak reaction; four plus signs: strong reaction of the antibody.

MMS at different concentrations to logarithmically growing cultures. Cell density was measured turbidimetrically at 600 nm. The β-galactosidase activity was tested as described by Quillardet and Hofnung (1985). Enzymatic units were calculated according to Miller (1972).

The enzyme-linked immunosorbent assay (ELISA) was performed with the antibody Fhu3.1, and the peroxidase system, according to Schloter et al. (1992), with the following modification: the ELISA plates were coated with the bacterial suspension [optical density (436) = 0.9] using polylysine for coating of the plates (Harlow and Lane, 1989). Fhu3.1 is a mouse monoclonal antibody (isotype IgG2b) raised against FhuA that was purified from the outer membrane of *E. coli* K-12. The specificity of Fhu3.1 was confirmed by (a) ELISA against total outer membrane proteins, and (b) immunoprecipitation of FhuA from radiolabeled cell extracts (data not shown). Fhu3.1 is also reactive in flow cytometry against intact cells overexpressing FhuA, indicating that its epitope on FhuA is cell-surface exposed. For the immunological detection of the bacteria, the monoclonal antibody Fhu3.1 and a fluorescein-coupled secondary antibody were used (Schloter et al., 1993). Phase contrast and immunofluorescence microscopy were performed using a Zeiss Axioplan epifluorescence microscope; the excitation wavelength was 485 nm and the emission wavelength was 538 nm.

## RESULTS

### Induction of the *umu* Operon

To establish a molecular test system, it is necessary to know the time course and the induction level of the relevant operon. The induction of the *umu* operon was measured with a fusion of *umuC* to *lacZ* on plasmid pSK1002 (Fig. 1). Thirty minutes after addition of MMS, an increase of β-galactosidase was evident; maximum induction was achieved at 150–180 min.

Different concentrations of the mutagen yielded different induction levels, indicating that the level of induction is dependent on the amount of DNA damage to the cells. A maximum inducing value of 6 was calculated as the relative increase of β-galactosidase activity as compared to the noninduced control. Between 150 and 180 min the specific β-galactosidase activity decreased, while cell growth continued until 300 min (not shown). The induction ended about 180 min. Therefore, the measurement of a specific induction of the *umu* genes by MMS is possible under the conditions used.

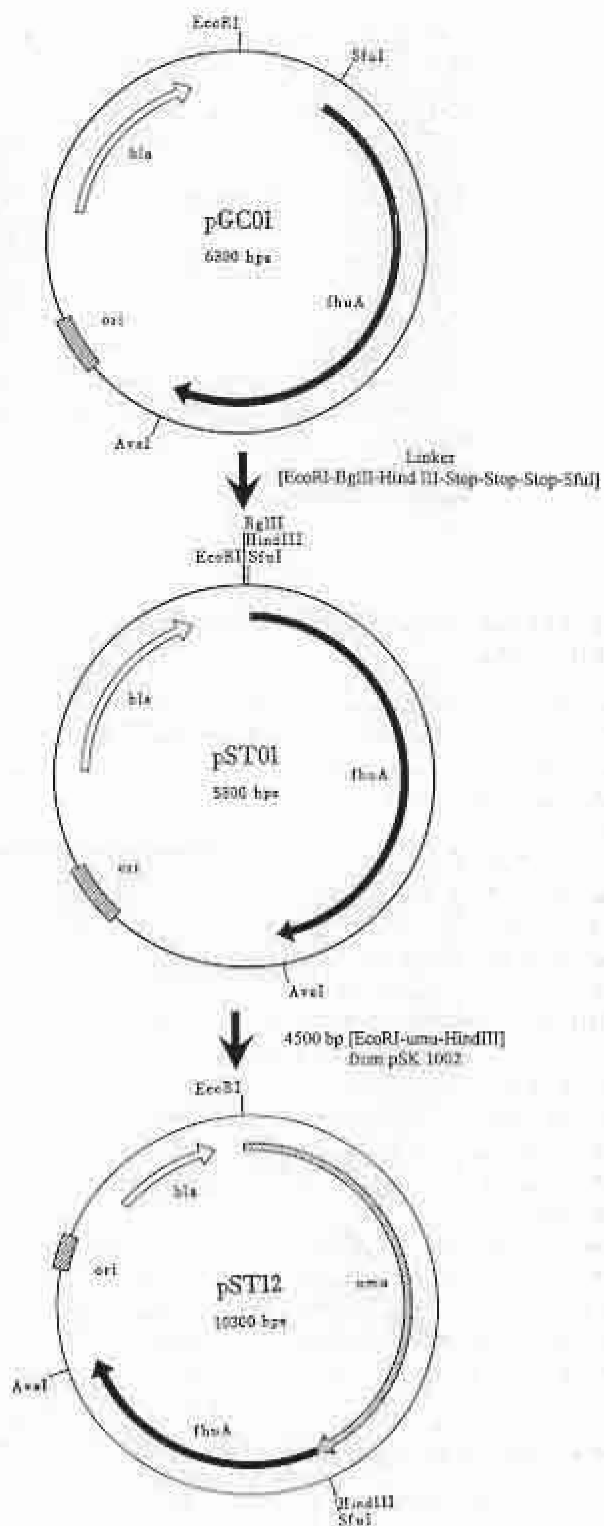


**Fig. 2.** Construction of the *umu-fhuA* operon fusions. Starting from plasmid pGC01, a linker was inserted in the *Eco*RI/*Sfu*I linearized plasmid yielding pST01. In the cloning sites of this linker a 4.5 kb *Eco*RI/*Hin*dIII fragment of pSK1002 containing the transcriptional regulation signals of the *umu* operon and parts of the coding region of *umu*C were inserted.

**TABLE III. Specificity of induction of FhuA under SOS-inducing conditions in *E. coli*/pST12[a]**

| Cells | SOS induced | Immunofluorescence | ELISA |
|---|---|---|---|
| *E. coli* JM83 | No | – | – |
| *E. coli* JM83 (negative control) | Yes | – | – |
| *E. coli* JM83/pGC01 (positive control) | No | ++++ | ++++ |
| *E. coli* JM83/pST12 | No | – | – |
| *E. coli* JM83/pST12 | Yes | ++++ | ++++ |

[a] The induction with MMS was in duplicate as described in Results. Reaction of the antibody Fhu 3.1 was measured with fluorochrome (i.e., fluorescein) coupled secondary antibody and followed by epifluorescene microscopy. ELISA using a peroxidase-coupled secondary antibody were performed. Symbols: minus: no reaction; four plus signs: strong reaction of the antibody.

## Expression Levels of the Reporter Protein FhuA

The reporter protein of an in situ test must be detectable serologically at the cell surface of an indicator bacterium. The expression of the reporter protein should be highly specific under the particular inducing conditions.

The reactivity of the monoclonal antibody Fhu 3.1 against FhuA on whole cells of *E. coli* was tested using ELISA (Table II). The wild-type strain JM83 did not react with the antibody. Induction of wild-type FhuA under iron-starvation conditions (addition of 40 $\mu$M EDDHA) yielded cells that reacted weakly with the monoclonal antibody. In contrast, the high copy number plasmid pGC01, containing the *fhuA* gene cloned into the pBR322 replicon, led to a constitutive and strong induction of the outer membrane protein and to high signals in the serological test. Inhibition of growth of the bacteria containing pGC01 was not detected (not shown), indicating that strong expression of the reporter protein did not have adverse effects on normal cell metabolism. We therefore chose to use the *fhuA* gene on the multicopy plasmid pGC01 as the desired serologically detectable reporter function.

## Construction of *umu-fhu*A Operon Fusions

The strategy for construction of an *umu-fhu*A operon fusion had to consider that an outer membrane protein, altered through a gene fusion at the amino-terminus, would not be localized properly at the cell surface (Saier et al., 1989). To avoid the formation of protein fusions, a linker was constructed that contained stop codons in all three reading frames behind the cloning sites *Eco*RI, *Bgl*II, and *Hin*dIII. This linker was inserted into the *Eco*RI-*Sfu*I linearized pGC01 yielding pST01 (Fig. 2). A 4.5 kilobase (Kb) *Eco*RI/*Hin*dIII fragment was derived from pSK1002, which contains the promoter and part of the coding region of *umu*C, was inserted into the cloning sites of pST01, yielding plasmid pST12.

*Escherichia coli*, transformed with plasmid pST12, was tested for its ability to induce *fhu*A specifically under the control of the SOS system. To obtain good induction in 1 h, 500 ppm MMS was used in liquid culture. The appearance of the FhuA reporter protein was demonstrated by immunofluorescence labeling using a fluorescein-labeled secondary antibody (Table III). Cells containing pGC01, which express FhuA constitutively, reacted very strongly with the monoclonal antibody, but showed no filamentation. In the presence of MMS, pST12-containing bacteria showed both strong fluorescence and filamentation (Fig. 3). The latter is a well-known phenomenom of SOS-induced cells (Little and Mount, 1982). *Escherichia coli* JM83 did not express FhuA with or without SOS induction under iron-sufficient conditions. This showed that specific induction with the genotoxin MMS was achieved by pST12. In addition, the specific induction of the *umu-fhu*A fusion by MMS was confirmed by ELISA (Table III), showing the same patterns of response as the immunofluorescence microscopy.

# DISCUSSION

The effects of genotoxins in undisturbed soil or sediments are difficult to measure but are of great ecotoxicological importance (Ahlf, 1993). A test with a new class of reporter protein, such as a cell-surface exposed protein that can be detected in situ with immunological techniques, is required. Such in situ detection tech-

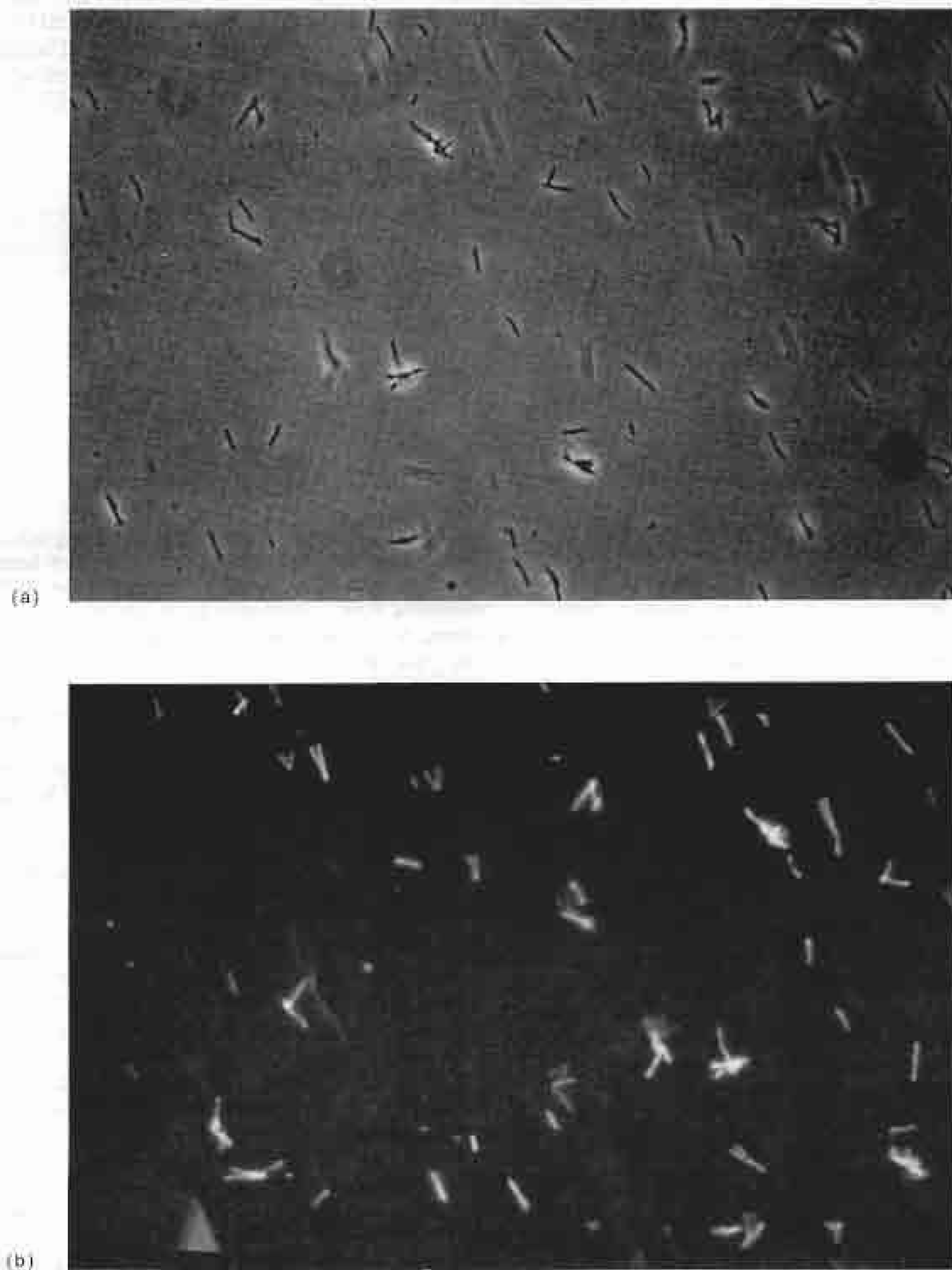


**Fig. 3.** Phase contrast and immunofluorescence microscopy of SOS-induced *E. coli*/pST12 after staining with fluorescein coupled secondard antibody. (a) The cell shape of all cells showed filamentation, a phenomenom of SOS-induced bacteria. (b) By fluorescence microscopy, all cells could be detected.

niques are already used for the identification and localization of some specific bacteria in their natural habitats (Asanuma et al., 1985, Schloter et al., 1993).

We chose the ferrichrome-iron receptor FhuA as reporter protein in a genotoxic test system that is based on the induction of the *umu* operon. The induction of the *umu* operon was dependent on genotoxins such as MMS and the level of induction correlated with sensitive detection by the enzymatic β-galactosidase assay. The detection of FhuA protein on the cell surface was possible with the monoclonal antibody Fhu3.1. Fhu 3.1 staining of negative controls was comparable to background levels of staining. These experiments showed that the inducing properties of the *umu* operon and the surface accessibility of FhuA as reporter protein are suitable for the desired ecotoxicological test system.

Since changes in the amino-terminal amino acid sequence of a membrane protein were predicted to prevent the localization of this protein to the outer membrane, a strategy to avoid protein fussions of FhuA was employed. A linker inserted in front of the *fhuA* gene contained stop codons in all three reading frames to terminate translation from the *umu*C ribosome binding site. Synthesis of the reporter protein could therefore only initiate from the original *fhu*A translation start. This ensured that expression of hybrid proteins, which might have perturbed the expression level of the reporter protein FhuA, did not occur. Furthermore, the linker contained cloning sites that facilitated the insertion of fragments of pSK1002 with the regulation signals of the *umu* operon upstream of *fhu*A.

*Escherichia coli*/pST12 was tested for specific induction of FhuA when subjected to genotoxic treatment. Both ELISA and immunofluorescence microscopy showed specific and strong induction under SOS-inducing conditions. Furthermore, immunofluorescence microscopy revealed a good correlation of the filamentation phenotype of SOS-induced cells with FhuA production when plasmid pST12 was presented in the cells. Nonspecific reactions of the antibody or background signals were not observed. In the ELISA test, which is more sensitive, uninduced *E. coli*/pST12 cells reacted like nontransformed *E. coli* JM 83 cells. In the immunofluorescence assay, a method that is used for in situ experiments, only nonfluorescent bacteria were detectable without mutagenic stress. These experiments demonstrated that the construction of the *umu-fhu*A fusion was successful and the *fhu*A gene in the *umu-fhu*A fusion of pST12 was specifically and strongly induced upon the addition of a mutagen such as MMS.

For a true in situ test system of soils and sediments, it is not possible to use bacteria such as *E. coli* that are not members of the natural microbial community. To create a successful in situ test means that the host for the test has to be a bacterium that can stably integrate into the soil microbial community. Therefore, test bacteria isolated from soil have to be chosen for future experiments. This could be a *Pseudomonas* strain or another gram-negative bacterium. To avoid strain-specific reactions, different strains as hosts for the test system need to be used. The *umu-fhu*A operon fusion is a prerequisite for the development of a new class of in situ ecotoxicological test system.

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