Effects of Furazolidone on the Mutation of *Vibrio cholerae* Cells to Streptomycin Resistance

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**Abstract.** Furazolidone induced the streptomycin-resistant (Str-r) forward mutation of *Vibrio cholerae* (classical) OGAWA 154 cells. The induced mutation frequency increased up to the furazolidone dose of 7.0 µg/ml × h and then gradually declined. Statistical analysis (t-test and variance analysis) revealed that the furazolidone-induced mutation of *V. cholerae* cells at any of the doses studied was highly significant.

Furazolidone or N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone, one of the members of the group of synthetic nitrofurans, displays a wide spectrum of antimicrobial activity [16, 25, 28, 31] and has useful application in human therapy [4]. The mode of action of this drug at the molecular level has been under study in our laboratory. The drug at a level of 0.5 µg/ml inhibited DNA synthesis while stimulating RNA synthesis at the same time and causing filamentation of the cells [11, 27]. DNA of cholera phage φ149 was at least ten times more sensitive to this inhibitory action of furazolidone [8, 9]. In an attempt to explain the basis of the inhibition of DNA biosynthesis, it was found that the drug underwent metabolic activation or transformation within the cell and then produced interstrand cross-links in DNA [6, 7]. No other nitrofuran has been shown so far to produce similar lesions to DNA. Formation of such DNA lesions should explain the inhibition of DNA biosynthesis in drug-treated cells. Like mitomycin C, another cross-linking agent, furazolidone exhibited radiomimetic properties, and caffeine exhibited lethal synergism with the drug [3]. The molecular mechanism of action of furazolidone revealed so far indicates that it might exhibit mutagenic and also carcinogenic activities. Furazolidone was shown earlier to induce reverse mutation in bacteria [17, 23].

Furazolidone is one of the few nitrofurans that have effective application in human medicine [4, 26, 28], and hence further investigation on the DNA-damaging and mutagenic activity of this drug should be useful and important. This communication presents the results obtained by us on the furazolidone-induced forward mutation from streptomycin sensitivity (Str-s) to streptomycin resistance (Str-r) of *Vibrio cholerae* cells.

**Materials and Methods**

**Bacterial strain and culture media.** The bacterial strain used in this study was *Vibrio cholerae* (classical) strain OGAWA 154. The bacteria were grown in nutrient broth (NB) medium containing 10 g bacto-peptone (Difco), 10 g beef extract (Oxoid), and 5 g NaCl in one liter of deionized water. Viability was assayed by the usual pour plate method on nutrient agar (NA) plates containing 15 g bacto-agar (Difco) in one liter of NB medium. The pH values of the media were adjusted to 8.0. Saline (0.85%) in deionized water was used as a diluent.

**Chemicals.** Chemically pure furazolidone was obtained as a gift from Smith Kline and French (India) Ltd., India. Streptomycin sulfate was obtained from Sigma Chemical Co., USA. All other reagents used were of analytical grade.

**Ultraviolet irradiation.** Irradiation of the bacteria by the ultraviolet light obtained from the Philips 15-W Germicidal lamp (254 nm) was done by the method described previously [2, 3].

**Assay of forward mutation.** Furazolidone-induced, streptomycin-resistant mutants were assayed as follows (Fig. 1). Appropriate amounts of furazolidone, dissolved in saline, were added to 20-ml log-phase bacterial culture in NB (~10<sup>9</sup> cells/ml), and the culture was then incubated at 37°C for 2 h in the dark. Plating was done on NA plates before (N<sub>1</sub>) and after (N<sub>2</sub>) the drug treatment so as to assess the viability. The drug-treated bacteria were then washed in saline by centrifugation in the cold, resuspended after 1:20 dilution in 20 ml of fresh NB medium, and incubated at 37°C until the stationary phase was attained to allow proper expression of the mutants to be assayed. Appropriate amounts of this culture were then assayed on NA plates supplemented without (N<sub>3</sub>) or with streptomycin (N<sub>4</sub>). The spontaneous

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Fig. 1. Protocol describing the method for assaying the furazolidone-induced, streptomycin-resistant mutants. Treatment with furazolidone (dissolved in saline) was effected by adding appropriate amounts of the drug solution directly into the log-phase cultures (~4 h) and incubating the same for 2 h at 37°C, in the dark.

streptomycin-resistant mutants were assayed by treating the bacteria similarly with saline alone (dashed lines, Fig. 1) and plating 0.1-ml aliquots of the stationary phase NB culture onto NA plates supplemented without (N5) or with streptomycin (N6).

Colony-forming units on NA plates containing no (open circles) or appropriate amounts (shaded circles) of streptomycin were counted after 72 h of incubation at 37°C in the dark. Estimations of survival and mutation were done by using the following formulae:

(i) Induced mutation frequency (MF)I = N2/N3
(ii) Spontaneous mutation frequency (MF)s = N6/N5
(iii) Mutation index (MF)I/(MF)s
(iv) Survival (%) = (N2/N0) x 100

Results and Discussion

Induction of mutation of Vibrio cholerae cells to streptomycin resistance by furazolidone was obtained in terms of mutation frequency per 10^7 viable cells. Table 1 presents a comparative picture of mutation induced by furazolidone when the challenging doses of streptomycin were 50 μg/ml and 100 μg/ml. Also, the mutation induced by a fixed dose of ultraviolet light is included in the same table for reference. It appears that the furazolidone-induced mutation frequency against the streptomycin level of 50 μg/ml was in general somewhat higher than that obtained against the antibiotic level of 100 μg/ml. Furazolidone appeared to be equally if not more effective than ultraviolet light in producing Str-r mutants of V. cholerae cells at the dose levels producing almost equal or comparable lethality.

Since most authors have experimented at the challenging dose level of 100 μg streptomycin/ml, the mutation of V. cholerae cells to Str-r with this challenging dose was studied with different doses of furazolidone. In this case the mutation index (MF/I/MF)s increased with increasing drug concentration up to about 7.0 μg/ml and thereafter exhibited a decline (Fig. 2). The maximum value of mutation index obtained was between 8 and 10. The viability of the cells decreased at a faster rate after an initial shoulder.

The question of significance of the mutation of furazolidine-treated cells to streptomycin resistance over the spontaneous mutation level of the same cells was then investigated statistically (t-test). For this purpose the drug-induced mutation frequency and the corresponding spontaneous mutation frequency obtained during each set of experiments were subjected to student’s t-test [24]. The t values were calculated and, taking account of the corresponding degrees of freedom, the probability values (P) were obtained from the relevant table [29]. The difference between the furazolidone-induced mutation frequency and the spontaneous mutation frequency was significant at a level better than 0.1% (P < 0.001) for all doses of furazolidone used, except that the significance was only better than 0.5% (0.005 > P > 0.001) when the drug concentration was 0.5 μg/ml. Variance analysis [24] was performed on induced mutation data vis-à-vis the data on spontaneous mutation to assess the significance of dose-dependent mutation. The F value in the variance ratio test was calculated to be 11.68, when the greater variance estimate had 7