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Dedicated to Professor David Shugar on his 80th birthday

Mutagenesis and ultraviolet inactivation of transforming DNA of *Haemophilus influenzae* complexed with a *Bacillus subtilis* protein that alters DNA conformation*

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The wild-type *Bacillus subtilis* spore protein, $SspC^{wt}$, binds to DNA *in vitro* and *in vivo* and changes the conformation of DNA from B to A. Synthesis of the cloned $SspC^{wt}$ gene in *Escherichia coli* also causes large increases in mutation frequency. Binding of $SspC^{wt}$ to transforming DNA from *Haemophilus influenzae* made the DNA resistant to ultraviolet (UV) radiation. The mutant protein, $SspC^{ala}$, which does not bind DNA, did not change the UV resistance. The UV sensitivity of the DNA/$SspC^{wt}$ complex was not increased when the recipients of the DNA were defective in excision of pyrimidine dimers. These data indicate that the *H. influenzae* excision mechanism does not operate on the spore photoproduct formed by UV irradiation of the complex. Selection for the streptomycin- or erythromycin-resistance markers on the transforming DNA evidenced significant mutations at loci closely linked to these, but not at other loci. $SspC^{wt}$ apparently entered the cell attached to the transforming DNA, and caused mutations in adjacent loci. The amount of such mutations decreased when the transforming DNA was UV irradiated, because UV unlinks linked markers.

The protein $SspC^{wt}$ from *Bacillus subtilis* is one of a group of closely related α/β-type small, acid-soluble proteins (SASP) that bind DNA both *in vivo* and *in vitro* and change the DNA conformation from a B-like to an A-like form [1, 2]. Analysis of the UV photochemistry of DNA complexed with $SspC^{wt}$ or other α/β-type SASP has shown that cyclobutane dimers are not produced by UV irradiation of the complexes, but rather a thyminyl-thymine adduct termed spore photoproduct [3, 4]. When $SspC^{wt}$ is synthesized from a plasmid in *Escherichia coli*, the protein binds to the DNA of the cell [5], causes numerous mutations [6], and also alters the DNA's UV photochemistry as described above [3]. There are two reasons why it seemed desirable to study the effects of $SspC^{wt}$ on UV inactivation of transforming DNA from *Haemophilus influenzae*. Since the transformation is very efficient in this bacterium [7], the repair of the special UV lesions formed when $SspC^{wt}$ was bound to the DNA could be readily assessed with the use of repair-defective mutants [8–10]. The second reason for the present work was to determine whether $SspC^{wt}$ bound to the transforming DNA could produce mutations, as it does when synthesized in *E. coli* cells [6].

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Abbreviations: SASP, small acid-soluble proteins; $SspC^{wt}$, wild-type *Bacillus subtilis* spore protein.

MATERIALS AND METHODS

Bacteria, DNA transformation and protein purification. The wild-type *H. influenzae* and its *uvr2* and *uvr1* mutants have been described previously [8–10], and were grown in Difco brain heart infusion supplemented with hemin and NAD. *H. influenzae* transforming DNA was purified according to Marmur [11]. In the transformation assay, cells made competent in MIV medium [7] were exposed to DNA with or without $SspC^{wt}$ for 30 min at 37°C, and after appropriate dilution plated in 10 ml of nutrient agar medium. After hardening, the plates were incubated for 1.5 h before addition of antibiotic in 10 ml of top agar. For assay of total viable cells (about 10^9/ml) a second 10 ml of nutrient agar medium without antibiotic was added.

The transformation frequency (number of transformants/ml divided by the number of viable cells/ml) was 10^{-2}–10^{-3} a value not appreciably affected by the presence of $SspC^{wt}$ or its mutant derivative $SspC^{ala}$. $SspC^{wt}$ and $SspC^{ala}$ were purified as described [3, 12]. ^{14}C-Labeled $SspC^{wt}$ was prepared and purified as previously [3, 12], from *E. coli* expressing $SspC^{wt}$ grown in 50 ml of culture containing 120 µCi of a U-^{14}C-L-amino-acid mixture (ICN). The final purified [^{14}C]$SspC^{wt}$ contained about 5×10^2 c.p.m./µg protein.

Preparation of DNA/SspC complexes and UV irradiation. The transforming DNA (50 µg/ml in 25 mM Tris/acetate, pH 7.0, 50 mM KOAc, 1 mM EDTA), carrying either a streptomycin-resistance (to 250 µg/ml), a novobiocin-resistance (to 50 µg/ml) or an erythromycin-resistance (to 10 µg/ml) marker, was incubated with purified $SspC^{wt}$ (0.3–1 mg/ml) at 37°C for 2 h. These amounts of $SspC^{wt}$ are above the amount needed to saturate the DNA [1, 3]. The mixture was then exposed to ultraviolet radiation at 254 nm under various conditions: wet (in a 70 µl spot on a plastic petri dish), or dry (50 µl air-dried on either a glass microscope slide or on parafilm). The UV intensity was measured with a UVX meter (Ultraviolet Products, Inc.). Since $SspC^{wt}$ is a protein with a relative lack of UV-absorbing residues [13], the different amounts used did not affect the actual dose to the DNA. In some experiments with dried DNA the mixture of transforming DNA and $SspC^{wt}$ also contained a small amount of radioactive DNA (either ^3H-labeled *H. influenzae* DNA with the same genetic marker as in the unlabeled DNA or *Eco*RI linearized ^3H-labeled pUC19 DNA) in order to determine the recovery of DNA from the dried spots. After irradiation the dry samples were taken up in 0.2 ml sterile 0.15 M NaCl. Recovery from glass was poor (8 to 16%), but was much better from parafilm (45 to 90%). Adjustments were made in the concentration of DNA used for transformation, such that the amount of DNA used was constant throughout each irradiated series. With dry samples, control experiments without $SspC^{wt}$ proved difficult because there was great variability in the assays for transformation, due to variability in DNA recovery. Therefore we decided to use a protein that did not bind to DNA for the control experiments, to improve the recovery. The protein used was a variant of $SspC^{wt}$ termed $SspC^{ala,}$ in which an alanine has been substituted for a highly conserved glycine residue [12]. $SspC^{ala}$, does not bind to DNA *in vivo* or *in vitro*, and also fails to cause mutation in *E. coli* [6, 12].

In one experiment cells were transformed with DNA which had been complexed with $SspC^{wt}$, but the protein subsequently was removed prior to transformation. Transforming DNA carrying the streptomycin-resistance marker was complexed with $SspC^{wt}$ as described above. Then 25 µl containing 0.18 µg DNA and 2.1 µg $SspC^{wt}$ were added to 100 µl 1.25% SDS and 8 µl 1.5 M NaCl. The DNA was then precipitated with ethanol, and redissolved in 25 µl 0.15 M NaCl. Previous work has shown that this treatment removes $SspC^{wt}$ from DNA [14]. The protein-free DNA was then used to transform the *H. influenzae* Rd strain to streptomycin resistance in parallel with transformation by an equivalent amount of complexed DNA.

Selection for antibiotic resistant transformants. The method used previously for selecting transformants [15] was modified somewhat. DNA or an $SspC^{wt}$/DNA complex and competent cells were incubated together at 37°C for 30 min, diluted ten-fold in growth medium, incubated 1.5 h at the same temperature with aeration, and further diluted four-fold in growth medium with streptomycin (250 µg/ml) or erythromycin (10 µg/ml). Incubation was continued until an A_{675} of 0.8–0.85 was reached,

when the culture was frozen in 17% glycerol for subsequent assays of mutation.

Mutation assays. The frozen cultures were allowed to thaw, diluted for measurement of total cells, and also for analysis of mutations. Unlike assays for transformation, in which the cells were plated without antibiotic, incubated 1.5 h to allow expression of the antibiotic resistance and then overlaid with antibiotic-containing agar medium [7], for analysis of mutations the cells were plated with the desired concentration of antibiotic immediately after the culture was thawed, since the previous treatment had allowed mutations to be expressed. The number of total viable cells was approximately 4×10^9/ml, so that by plating 1 ml with an antibiotic a number of times, even mutation frequencies of 10^{-9} could be detected with reasonable statistical accuracy.

Exposure of cells to ^{14}C-labeled SspC^{wt}. Competent cells of strain Rd were mixed with either purified [^{14}C]SspC^{wt} (about 10 µg with 0.5 ml cells) or the same amount of [^{14}C]-SspC^{wt} complexed with *H. influenzae* transforming DNA (1 µg). The mixtures were incubated 30 min at 37°C, and samples (0.05 ml) were removed from each for measurement of total radioactivity, and transformation frequencies (to assay for competence of the culture exposed to DNA). The cells were then centrifuged, with the supernatant retained (called supernatant 1), the cells resuspended in 0.5 ml MIV [7], recentrifuged, the pellet suspended in 0.5 ml water, and the supernatant 2 retained. Aliquots of both supernatant fluids and the resuspended pellet were counted with BioSafe II (Research Products International) as the scintillant. In some experiments proteinase K (Boehringer Mannheim) at 400 µg/ml was added after the 30 min at 37°C and there was an additional 30 min of incubation before the first centrifugation.

RESULTS AND DISCUSSION

Effect of SspC^{wt} on UV inactivation of transforming DNA

UV irradiation of uncomplexed transforming DNA in solution resulted in inactivation of the DNA (Fig. 1), with the DNA appearing much more UV-sensitive when assayed on excision-defective *H. influenzae* carrying either the *uvr2* (Fig. 1) or *uvr1* (not shown) mutations. However, the DNA in an SspC^{wt} complex was much more UV resistant than uncomplexed DNA, and there was no difference in its UV resistance when assayed in either wild-type or excision-defective hosts (Fig. 1, and data not shown). The transforming DNA used in these experiments carried a streptomycin-resistance marker; similar results were obtained with DNA carrying a novobiocin-resistance marker (not shown). DNA was also irradiated in the dry state in the presence of either SspC^{wt} or SspC^{ala}. SspC^{wt} had a strong protective effect against UV radiation as compared to SspC^{ala} (Fig. 2). Previous work has shown that SspC^{ala}, unlike SspC^{wt}, has no effect on the UV photochemistry of DNA, either in solution or dry [3, 4]. Consequently it is most likely that the data with SspC^{ala} are a good reflection of the results in the absence of any bound protein. DNA irradiated dry with either SspC^{wt} or SspC^{ala} showed similar UV inactivation on both wild-type and excision-defective hosts (Fig. 2). Similar results were obtained with DNA carrying either a novobiocin-resistance (Fig. 2) or streptomycin-resistance (not shown) marker. Recent work has shown that the photoproduct yield as a function of UV fluence is around fifteen-fold higher with protein-free DNA than with an SspC^{wt} DNA complex [16]. This is presumably the reason for the UV resistance provided to transforming DNA by SspC^{wt}. UV irradiation of an SspC^{wt}/DNA complex also generates little if any pyrimidine dimers [3, 4], which are major

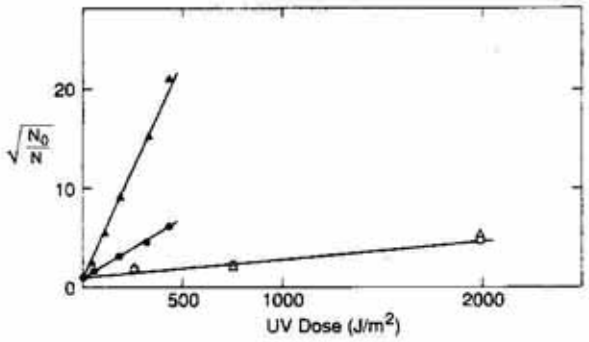

Fig. 1. UV irradiation of transforming DNA in solution with (open symbols) or without (closed symbols) SspC^{wt}.
The DNA was assayed on wild type (○, ●) or *uvr2* mutant (△, ▲) hosts. The transforming DNA carried the streptomycin resistance marker. N_0 is the number of streptomycin-resistant transformants obtained with no UV dose, and N the number after irradiation of the DNA.

targets of excision repair, but rather generates the spore photoproduct. Consequently, the lack of effect of the excision repair mutations (*uvr1* and *uvr2*) on the UV sensitivity of the $SspC^{wt}$ complex suggests that the spore photoproduct is not acted upon significantly by the excision repair pathway in *H. influenzae*.

The lack of effect of the excision mechanism on the survival of transforming DNA when the DNA was irradiated dry (Fig. 2) was in contrast with the results obtained with uncomplexed DNA in solution (Fig. 1). However, it is known that about one-tenth as many pyrimidine dimers are formed by UV irradiation of dry DNA as are formed in fully hydrated DNA [17]. Indeed, UV irradiation of dry films of DNA generates significant levels of a number of photoproducts, which are formed at only low levels, if at all, in fully hydrated DNA, with the ratios of these various photoproducts varying with relative humidity [18]. Presumably, the lack of significant effect of the excision mechanism on survival of transforming DNA irradiated dry without $SspC^{wt}$ (but with the mutant $SspC^{ala}$) is the result of the generation of few pyrimidine dimers under these conditions [17]. What is again implied is that the spore photoproduct, and probably other types of photoproducts formed by UV irradiation of dry DNA, are not subject to repair by the *H. influenzae* excision repair mechanism.

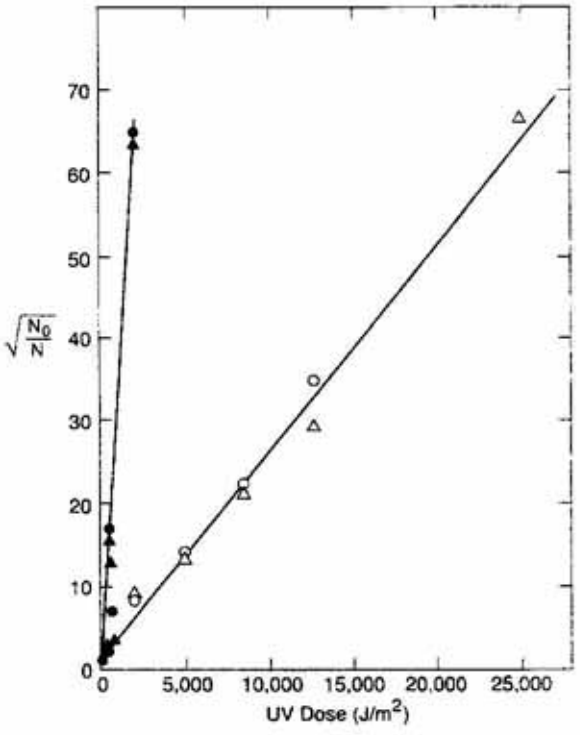

Fig. 2. UV irradiation of transforming DNA dried on parafilm with: $SspC^{wt}$ (open symbols) and the DNA assayed on wild type (○) or uvr2 mutant (△) hosts; or $SspC^{ala}$ (closed symbols) and the DNA assayed on wild type (●) or uvr2 mutant (▲) hosts. The transforming DNA carried the novobiocin resistance marker. N_0 and N are as in the legend to Fig. 1.

When the transforming DNA was irradiated either in solution or dry, with or without $SspC^{wt}$, the inactivation of the DNA followed the square root relationship, in that plots of $\sqrt{N_0/N}$ *versus* dose produced straight lines (Fig. 1 and 2). Earlier work showed that the square root relationship is caused by DNA damage repair [19]. Thus when protein-free DNA is irradiated in solution and assayed on an *H. influenzae* strain lacking both excision and post-replication repair (a double mutant, *uvr2recl1*), the dose survival curve is a simple exponential one [19]. With excision or post-replication repair alone, however, the square root relation holds [19]. Since the data with the $SspC^{wt}$ complex suggest that excision repair does not operate on the spore photoproduct as noted above, it thus seems likely that post-replication repair should act on the spore photoproduct in order for the square root relation to hold. We have attempted to analyze UV inactivation of $SspC^{wt}$/DNA complexes using the *H. influenzae rec1* mutant as a recipient. However, as noted previously [9], the transformation frequency in this strain is very low, too low for the amount of DNA that could be used in this experiment.

Mutation caused by $SspC^{wt}$ complexed with transforming DNA but not by uncomplexed DNA or streptomycin resistance

Analysis of spontaneous mutation in streptomycin resistant and sensitive wild-type and *uvr2* strains showed no significant effect of the streptomycin-resistance marker (Table 1). Use of uncomplexed DNA carrying the streptomycin-resistance marker to transform *H. influenzae* did not result in any significant increase in mutation of other antibiotic resistance markers (Table 2, Experiment 1). Similarly, $SspC^{wt}$ alone (Table 2, Experiment 3) or DNA which had been purified from an $SspC^{wt}$ complex (Table 2, Experiment 2), gave no increase in mutation of other markers. However, use of an $SspC^{wt}$/DNA complex for transformation resulted in significant mutation in the cells transformed to streptomycin resistance (Table

Table 1

Frequency of spontaneous mutations in streptomycin-resistant (R) and streptomycin-sensitive (S) H. influenzae[a]

Strain:	R (Rd)	S (Rd)	R (Rd*uvr2*)	S (Rd*uvr2*)
Antibiotic resistance	Mutation frequency			
Novobiocin	9.3×10^{-8}	3.6×10^{-8}	1.4×10^{-7}	5×10^{-8}
Kanamycin	2.7×10^{-7}	1.2×10^{-7}	1.8×10^{-7}	3.1×10^{-8}
Rifampin	1.4×10^{-8}	1.7×10^{-8}	9×10^{-7}	3×10^{-7}
Viomycin	1.2×10^{-6}	1.5×10^{-6}	3.7×10^{-7}	3.3×10^{-7}

[a]For each measurement of spontaneous mutations, nine different determinations were made with three different single colony isolates for novobiocin and kanamycin resistance, and three determinations with the same isolates for rifampin and viomycin resistance. The antibiotic concentrations used were: novobiocin — 0.43 μg/ml, kanamycin — 6 μg/ml, rifampin — 2 μg/ml, and viomycin — 100 μg/ml.

Table 2

Mutations in streptomycin-resistant transformants

Antibiotic resistance[a]	Mutation frequency	
	Experiment 1[b]	
	DNA alone	$SspC^{wt}$/DNA complex
Novobiocin (0.43)	1.4×10^{-7}	3×10^{-4}
Kanamycin (6)	1.8×10^{-7}	3×10^{-6}
Rifampin (2)	9×10^{-7}	3×10^{-7}
Viomycin (100)	4×10^{-7}	6×10^{-7}
	Experiment 2[c]	
	$SspC^{wt}$/DNA complex	DNA purified from an $SspC^{wt}$/DNA complex
Novobiocin (0.7)	5×10^{-6}	1.4×10^{-7}
Novobiocin (0.4)	1.6×10^{-3}	4.7×10^{-6}
Kanamycin (5.75)	2×10^{-4}	3×10^{-5}
Kanamycin (3)	3×10^{-4}	5×10^{-5}
	Experiment 3[d]	
	No $SspC^{wt}$	$SspC^{wt}$
Novobiocin (1)	3×10^{-7}	3×10^{-7}
Kanamycin (3)	2×10^{-5}	2×10^{-5}

[a]Values in parentheses are the antibiotic concentrations used in μg/ml.

[b]DNA carrying the streptomycin-resistance marker without and with $SspC^{wt}$ (a 15/1 w/w ratio) was used to transform the *uvr2* mutant of *H. influenzae* to streptomycin resistance, and transformants further tested for resistance to other antibiotics. These data were all obtained with the same sets of antibiotic containing agar.

[c]DNA carrying the streptomycin-resistance marker was complexed with $SspC^{wt}$ (an 11/1 w/w ratio), the complex used directly or after removal of $SspC^{wt}$ to transform the Rd strain to streptomycin resistance, and transformants further tested for resistance to other antibiotics. These data were all obtained with the same sets of antibiotic-containing agar.

[d]Competent Rd, exposed or unexposed to $SspC^{wt}$, was tested for resistance to novobiocin and kanamycin. The same antibiotic-containing agars were used to obtain these data.

2, Experiments 1 and 2). Mutation was most pronounced in genes very closely linked to the streptomycin marker, novobiocin and kanamycin resistances, and was not increased in markers more distant, those for viomycin and rifampin resistance (Table 2, Fig. 3). Note that the kanamycin- and novobiocin-resistance markers showed mutation frequencies which varied significantly from experiment to experiment (Tables 2 and 3). These differences appeared due to differences in the age of the antibiotic solutions in the different experiments, as well as in the different amounts of antibiotics used. When $SspC^{wt}$ complexed with DNA carrying an erythromycin-resistance marker was used for transformation, again there was a significant increase in mutation of a gene closely linked to erythromycin resistance, and specifying spectinomycin resistance, but not to more distant markers (Table 3, Fig. 3).

Analysis of the effect of UV irradiation of an $SspC^{wt}$/DNA complex in which the DNA carried a streptomycin-resistance marker showed that mutations in linked markers decreased rapidly as a function of dose, with the mutation to novobiocin resistance declining faster than the kanamycin-resistance mutation (Fig. 4a). However, when the same streptomycin-selected cells were assayed for other mutations, these showed no change as a function of dose, and the mutation frequency remained at that of spontaneous mutation (Table 2, Fig. 4b). Given the order of these various markers on the *H. influenzae* chromosome (Fig. 3), these data suggest that mutations were generated only at linked loci in the transforming DNA which entered the cell with the streptomycin-resistance marker, and that UV irradiation unlinked the kanamycin and novobiocin markers from

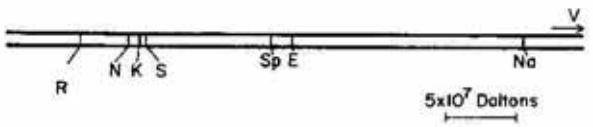

Fig 3. Genetic map of a part of the H. influenzae chromosome.
Data are from Herriott [20], S.H. Goodgal (personal communication) and J.K. Setlow (unpublished results). Antibiotic resistances: Sp, spectinomycin; E, erythromycin; Na, nalidixic acid; R, rifampin; N, novobiocin; S, streptomycin; V, viomycin; K, kanamycin.

streptomycin resistance. The novobiocin-resistance marker, which is farther from streptomycin resistance than is kanamycin resistance (Fig. 3), was unlinked more rapidly by UV than was the kanamycin-resistance marker (Fig. 4a).

The UV irradiation of *H. influenzae* transforming DNA containing linked markers has been previously shown to have a greater effect on co-transformation of the linked markers than on the transformed markers singly. It is clear that an increase in the number of photoproducts will cause the distance between a marker and the nearest UV lesion to decrease. Thus we postulate that increasing UV irradiation would decrease the probability that two linked markers would both become integrated into the host chromosome, although the single markers of the pair could survive.

The data further suggest that $SspC^{wt}$ itself might have entered the cells with the transforming DNA and thus generated the observed mutations. Therefore the uptake of $SspC^{wt}$ into competent cells of *H. influenzae* was measured.

Uptake of $SspC^{wt}$ into competent cells. There did appear to be some uptake of ^{14}C-labeled $SspC^{wt}$ into competent Rd cells (Table 4). However, there was no difference between the ap-

Table 3
Mutations in erythromycin resistant transformants[a]

Antibiotic resistance[b]	Mutation frequency	
	DNA alone	$Sspc^{wt}$/DNA complex
Spectinomycin (13)	1.4×10^{-9}	3×10^{-8}
Kanamycin (3)	7.7×10^{-6}	4.9×10^{-6}
Kanamycin (4)	4×10^{-7}	6.9×10^{-7}
Novobiocin (0.4)	2×10^{-8}	2×10^{-8}

[a]DNA carrying the erythromycin resistance marker without and with $SspC^{wt}$ (an 11/1 ratio) was used to transform the Rd strain to erythromycin resistance, and transformants further tested for resistance to other antibiotics.

[b]Values in parentheses are the antibiotic concentration in μg/ml.

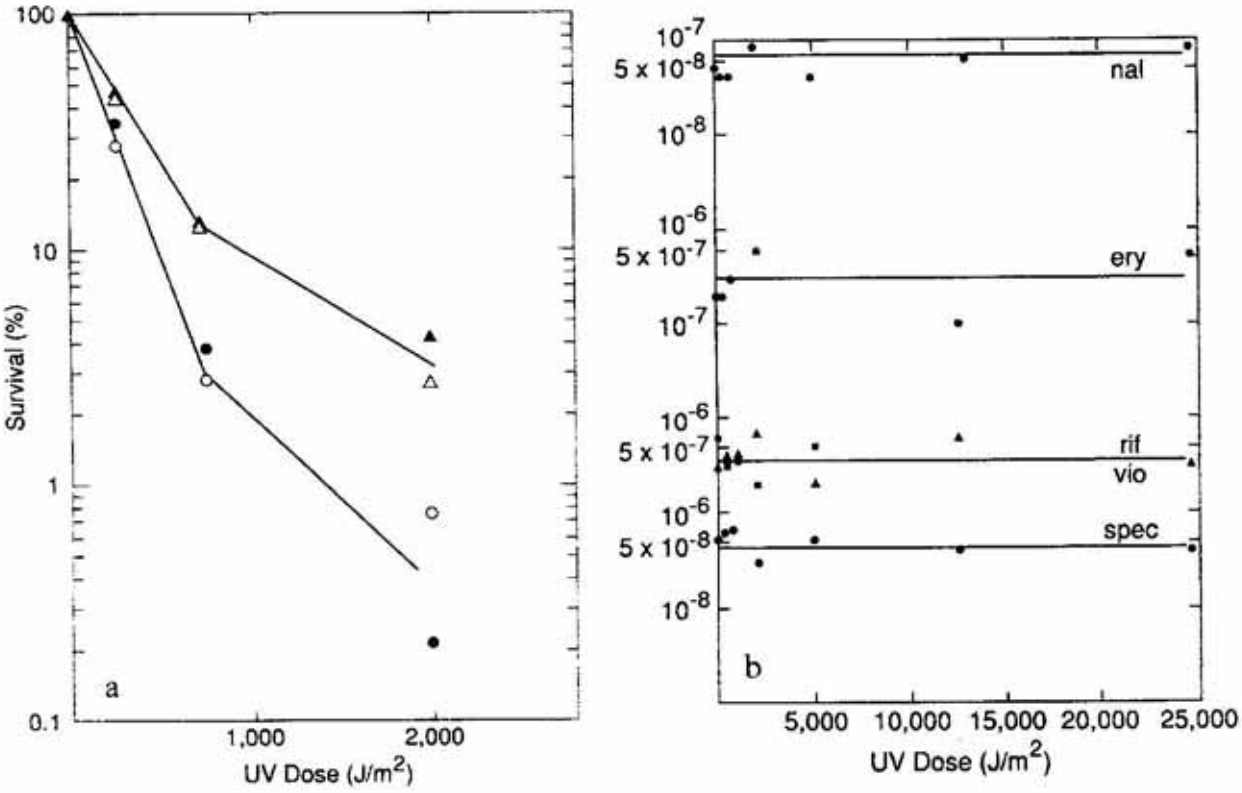

Fig. 4 a, b.
a, DNA carrying the streptomycin resistance marker was complexed with $SspC^{wt}$ and UV irradiated either in solution (open symbols) or dry (closed symbols) and used to transform Rd*uvr2* cells to streptomycin resistance. The selected cells were assayed for mutation to 20 μg/ml kanamycin (Δ, ▲) and 0.8 μg/ml novobiocin (○, ●). The frequencies of mutation at zero dose were 3×10^{-6} and 3×10^{-4}, respectively. b, The streptomycin-selected cells as described in a were assayed for mutation to 1 μg/ml nalidixic acid (nal), 6 μg/ml erythromycin (ery), 0.3 μg/ml rifampin (rif, ▲), 200 μg/ml viomycin (vio, ●) or 10 μg/ml spectinomycin (spec) resistance. Frequencies of mutation are shown.

parent uptake of protein, whether or not it was complexed with *H. influenzae* DNA. The $SspC^{wt}$ partitioning with the cells appeared to be intracellular, as most was not solubilized by extracellular proteinase K, and a control experiment showed that proteinase K at high concentration completely solubilized cell-free $SspC^{wt}$ bound to DNA (not shown). However, the data of Table 4 do not support the idea that only the protein complexed with transforming DNA entered the cells. If indeed there is $SspC^{wt}$ inside the cells, whether or not complexed with DNA, we might ask why only the complexed protein caused mutations. One possibility is that sole of the complexed protein went to the locus on the chromosome where the transforming DNA integrated, causing the cells selected for a particular marker to acquire mutations in adjoining loci. The uncomplexed protein which might also have entered cells did not cause observable

Table 4
Measurement of $SspC^{Wt}$ uptake by competent cells of H. influenzae[a]

Proteinase K	[^{14}C]$SspC^{wt}$/DNA complex		[^{14}C]$SspC^{wt}$ alone	
	no	yes	no	yes
	Percent of total ^{14}C			
Supernatant 1	82	91	83	89
Supernatant 2	3	0.4	3	0.6
Cell pellet	16	9	14	10

[a] [^{14}C]$SspC^{wt}$ with or without DNA was used to transform competent Rd cells. Competent cells were then fractionated by centrifugation with or without prior treatment with proteinase K, and radioactivity in various fractions quantitated.

mutations because it could have been broken down by cell proteases before it could act as a mutagen. It is known that binding of $SspC^{wt}$ to DNA to some extent protects the SspC protein against digestion by proteases [22]. Alternative explanation is that in the whole (non-selected) population the probability of detecting mutations caused by unbound $SspC^{wt}$ at any particular locus was too small.

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