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Cloning and characterization of a DNA repair gene in *Haemophilus influenzae*

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Abstract. Using a high-efficiency DNA cloning vector pJ1-8, a DNA repair gene *uvr*1 has been self-cloned in bacterium *Haemophilus influenzae.* Chimeric plasmid pKuvrl, carrying wild type allele of *uvr*1 gene and flanking DNA sequences, specifically complements a *uvr*l gene mutation in the bacterial chromosome. A *uvr*1^{-} mutation could be transferred from chromosome by *in vivo* recombination to pKuvr1 and isolated and designated as plasmid pKuvrl $^{-}$. Plasmid pKuvrl carries a 11·3 kb chromosomal DNA insert which was scanned for the presence of any other DNA repair genes by a novel method of directed mutagenesis. Preliminary analysis of the 3 new mutants isolated by this method supports the notion that the insert contains more than one gene concerned with ultraviolet radiation-sensitivity.

Keywords. *Haemophilus influenzae*; self-cloning; *uvr*1 gene; directed mutagenesis.

Introduction

DNA repair has been extensively studied in the naturally-transformable species of bacterium *Haemophilus influenzae* (reviewed in Notani and Kanade, 1985). At least two genes, *uvr*1 and *uvr*2*,* have been implicated in the repair of ultraviolet radiation (UV)-damage caused to DNA of *H. influenzae* (LeClerc and Setlow, 1973). These genes also condition Psoralen plus near-UV radiation-sensitivity of *H. influenzae* (George and Notani, 1981).

In *Escherichia coli,* products of *uvr*A*, uvr*B and *uvr*C genes act in concert to remove the DNA damage caused by agents like UV (Howard-Flanders *et al.* 1966). Cloning of these genes helped to reconstitute *in vitro* a novel UvrABC excision nuclease which cuts phosphodiester bonds on either side of the damaged segment and releases the latter (Sancar and Rupp, 1983). A human DNA repair gene has also been cloned by Westerveld *et al.* (1984). We have cloned *uvr*1 gene of *H. influenzae* and describe some of its characteristics.

Materials and methods

Bacterial strains

H. influenzae strains and plasmids used in the study are listed in table 1.

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Abbreviations used: UV, Ultraviolet radiation; BHI, brain heart infusion; NAD, nicotinamide adenine dinucleotide; Amp^{R}, ampicillin resistant; CFU, colony forming units; OD, optical density; Rd, rough strain, serotype d.

Table 1. *H. influenzae* strains and plasmids used in the study.

Strain	Phenotype/Genotype	Source or reference
Rd	Wild type	Goodgal and Herriott (1961)
Uvr1⁻	UV-sensitive mutant of *uvr*1 gene	LeClerc and Setlow (1973)
Uvr2⁻	UV-sensitive mutant of *uvr*2 gene	LeClerc and Setlow (1973)
Rec1⁻	Recombination-deficient	Setlow *et al.* (1972)
HIU04	UV-sensitive mutant of uvr^{sens1} gene	Present communication
HIU21	UV-sensitive mutant of uvr^{sens2} gene	Present communication
HIU36	UV-resistant mutant of uvr^{res} gene	Present communication
MC1	Minicell-producing strain	Sedgwick *et al.* (1975)
Plasmid pJ1-8	Ampicillin resistant	Joshi and Notani (1983)
pKuvr1	Recombinant plasmid containing pJ1-8 and *uvr*1 gene	Present communication
pKuvr1⁻	Recombinant plasmid containing pJ1-8 and *uvr1*⁻ mutation	Present communication

Media, growth and storage conditions

H. influenzae strains were grown in 2·5% Difco Brain Heart Infusion (BHI) broth supplemented with 2 μg/ml nicotinamide adenine dinucleotide (NAD), 10 μg/ml hemin and 1 μg/ml L-histidine at 37°C. BHI agar contained 1·5% Difco agar. Cells were made competent by aerobic-anaerobic-aerobic method of Goodgal and Herriott (1961). Transformation for plasmid marker (ampicillin resistance) was detected by resistance to 5 μg/ml of ampicillin. UV-sensitivities of the cultures were tested at different UV exposures as described earlier (Kanade and Notani, 1986). Cultures grown from single colony isolates were stored in 15% glycerol at –73°C.

Recombinant DNA techniques

Chromosomal DNA of *H. influenzae* Rd was extracted as described by Marmur (1961). Plasmid DNA was isolated by the method of Hirt (1967) with minor modifications (Notani, 1981). *Eco*RI genomic library was prepared as described by Joshi and Notani (1983), using pJ1-8 as vector. The DNAs were partially (chromosomal) or fully (vector plasmid) digested with restriction endonucleases as per manufacturers' instructions (Bethesda Research Laboratories, Gaithersburg, Maryland, USA).

Agarose gel electrophoresis

Covalently closed circular DNA and linear (restriction endonuclease digested) forms of DNA preparations were electrophoresed through 0·7% and 1% agarose gels, respectively. Electrophoresis was carried out using Tris EDTA acetate buffer (pH 8). Both the gel and the buffer contained ethidium bromide (0·1 μg/ml). Gels were observed under UV illumination of 350 nm.

Directed mutagenesis

Exponentially growing cells of *H. influenzae uvr*1 (pKuvrl) were mutagenised with 0·1 mM N-methyl-N'-nitro-N-nitrosoguanidine for 5 min, chilled, washed and grown in fresh BHI broth with supplements for about 3 h. Plasmid DNA was isolated from these cells and used to transform wild type cells. The transformants were screened for UV-sensitivity.

Results

Cloning of uvr1 gene

*uvr*1$^-$ competent cells were transformed with an *Eco*RI genomic library prepared using pJ1-8 as a vector and chromosomal DNA of *H. influenzae* Rd cells. About 2000 ampicillin-resistant transformants thus obtained were pooled and tested for UV-resistance in 3 successive screenings by exposures to UV doses of 24, 48 and 72 J/m^2. One of the UV-resistant clones, designated pKuvrl, was found to confer on the UV-sensitive *uvr*1$^-$ strain an extent of UV-resistance that brought it on par with a wild type strain (figure 1A).

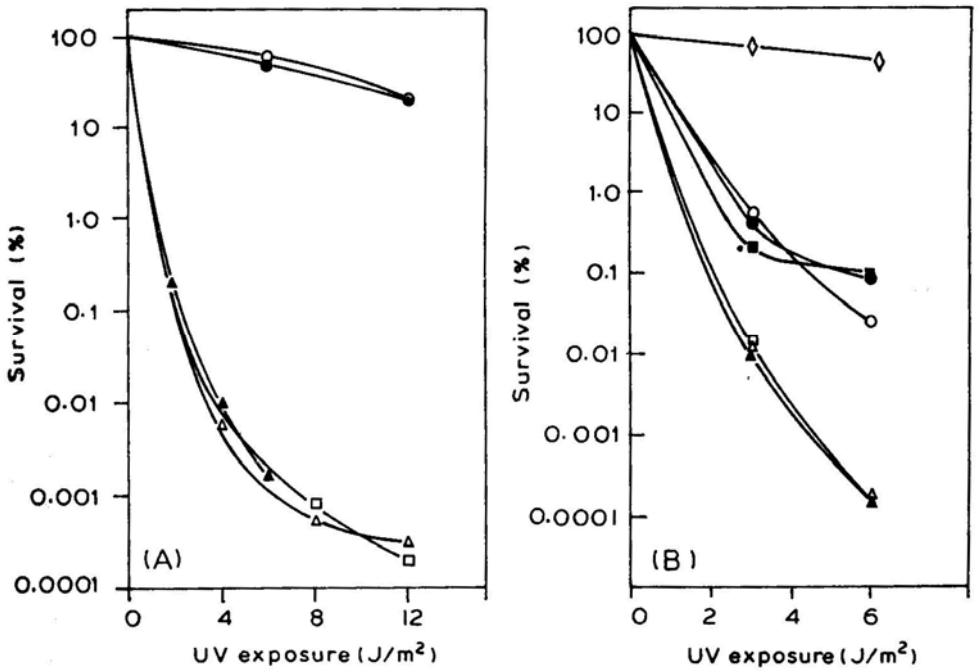

Figure 1. A. UV-inactivation of colony formation of *H. influenzae* strains. (O), Rd (wild type); (▲), *uvr*1 mutant strain; (●), *uvr*1 (pKuvrl); (Δ) *uvr*1 (pKuvr1$^-$). **B.** UV-inactivation of colony formation of *H. influenzae uvr*1 cells transformed by pKuvrl or pKuvrl$^-$ DNAs. (◊), Rd; (□) *uvr*1; (■), 2% Rd + 98% *uvr*1 cells; (●), pKuvr1 transformants plated without ampicillin agar; (O), pKuvrl transformants plated with ampicillin agar; (▲), pKuvr1$^-$ transformantsplated without ampicillin agar, (Δ), pKuvr1$^-$ transformants plated with ampicillin agar.

Chimeric plasmids in *H. influenzae* recombination-proficient strains undergo genetic recombination between the chromosomal splice of the plasmid and the corresponding homologous chromosomal DNA segment (Setlow *et al.,* 1981; Joshi and Notani, 1984) yielding chimeric plasmids that now carry the resident-DNA allele. Thus, as expected *uvr*1^{-} mutation could be isolated on the plasmid by transformation of *uvr*1^{-} cells with pKuvrl DNA carrying wild type allele of *uvr*1 gene. Out of 78 ampicillin resistant (Amp^{R}) transformants, 11 were found to be as UV-sensitive as *uvr*1^{-} strain (figure 1A, two isolates of pKuvrl^{-} are shown) while the rest were UV-resistant. The UV-sensitive Amp^{R} transformants contained plasmid of the same size as pKuvrl and presumably carried *uvr*1^{-} allele in both plasmid as well as the chromosome. The plasmid in these UV-sensitive transformants was designated as pKuvr1-.

As shown in figure 1B, when pKuvr1 and pKuvr1^{-} plasmid DNAs were used to transform *uvr*1 competent cells only pKuvrl DNA yielded a fraction of 2% cells that acquired both ampicillin as well as UV-resistance. UV-resistant fraction was not observed amongst transformants of pKuvrl^{-} DNA confirming the location of UV-resistant (wild type) or UV-sensitive (mutant) alleles of *uvr*1 gene on respective plasmids.

*Physical characterization of the plasmids pKuvrl and pKuvr*l^{-}

The size of the plasmid pKuvrl was estimated to be 15·05 kb by comparing mobility of linear DNA fragments (McDonell *et al.,* 1977). Both the plasmids (pKuvrl and pKuvrl^{-}) exhibited identical restriction digestion patterns with the enzymes *Eco*RI, *Pvu*II and *Pst*I. Figure 2 shows restriction map of pKuvrl derived by using enzymes *Eco*RI, *Puv*II, *Bam*HI and *Pst*I. *Eco*RI and *Bam*HI did not cut in the insert, while *Pvu*II and *Pst*I cut the insert only once and inactivated UV-resistance property of the plasmid as judged by transformation. In pKuvrl the insert size was estimated at 11·3 kb and the vector at 3·75 kb. *Sal*I, *Bcl*I and *Mbo*I did not cut the plasmid DNA.

*Genetic characterization of pKuvr*l *and pKuvr*l^{-}

It was noted that pKuvrl specifically complemented a *uvr*1^{-} gene mutation but not a *uvr*2^{-} mutation. No UV-resistant transformants appeared in Uvr2^{-} competent cultures exposed to pKuvrl DNA. Similarly, UV-sensitivity of Uvr2^{-} (pKuvrl) was the same as that of Uvr2^{-} cells. Wild type strain carrying pKuvrl^{-} plasmid did not show increased UV-sensitivity. Also it was found that Recl^{-} cells carrying wild type allele of *uvr*1 gene in chromosome, but mutant *uvr*1^{-} gene in the plasmid pKuvrl^{-}, were almost as UV-sensitive as Recl^{-} cells. Recl^{-} cells are defective in homologous genetic recombination. This implies that wild type allele of *uvr*1 gene is dominant over the mutant allele.

Another characteristic of chimeric plasmid transformation in *H. influenzae* is that transformation for the chromosomal marker carried by the plasmid is atleast an order of magnitude higher than that for the plasmid marker (Setlow *et al.,* 1981; Joshi and Notani, 1984). Thus, it is quite easy to transfer mutations from specific DNA segments carried by the chimeric plasmid into the chromosome of the recipient cells. Making use of this property, an *in vivo* mutagenized pKuvr1 DNA was used to

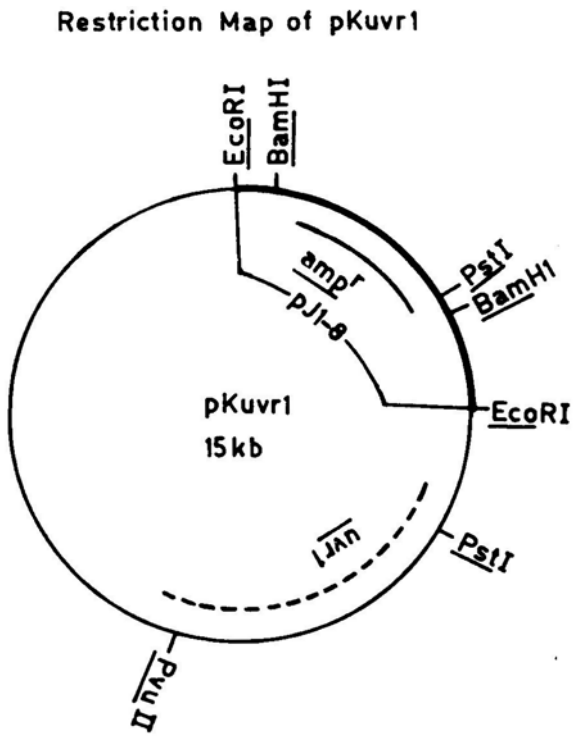

Figure 2. Restriction map of pKuvr1 showing probable location of *uvr*1 gene.

screen for any other DNA repair genes carried on the chromosomal DNA insert of the plasmid pKuvr1. The mutagenized plasmid DNA was used to transform wild type *H. influenzae* cells. From 250 transformants of this transformation, 3 new mutants with altered UV-sensitivity properties were isolated.

One of these was UV-resistant (HIU36) carrying a mutation designated *uvr*^{res}, while the other two were UV-sensitive-HIUO4 carrying a mutation *uvr*^{sens1} and HIU21 carrying a mutation *uvr*^{sens2}. Figure 3 shows UV-sensitivity of these isolates. The presence of pKuvr1 decreases UV resistance of HIU36 (figure 4A). However, both the plasmids pKuvrl and pKuvrl^{-}, increased UV resistance of HIUO4 giving only partial complementation of its UV sensitivity. The partial complementation may be due to instability of the plasmid while complementation even by pKuvrl^{-} can imply that the mutant carries wild type allele of *uvr*1 gene in the chromosome and is mutated in the neighbourhood of *uvr*1 which is present in wild type on the plasmid pKuvrl- .

Mutant HIUO4 also shows some other phenotypes such as transformation defect, defect in prophage induction, defect in cell division, etc. Defect in cell division is reflected in altered cell size which can be observed microscopically or by measuring number of colony forming units (CFU) formed per unit optical density (OD). Table 2 shows stability and the number of CFU formed by cultures of comparable OD. Rd, Uvr1^{-} and Uvr1^{-} (pKuvrl) cultures show same number of CFU while MC1 — a mutant defective in cell division producing chromosomeless minicells and HIUO4 show a lower number CFU. Figure 5 showing light photomicrographs of these

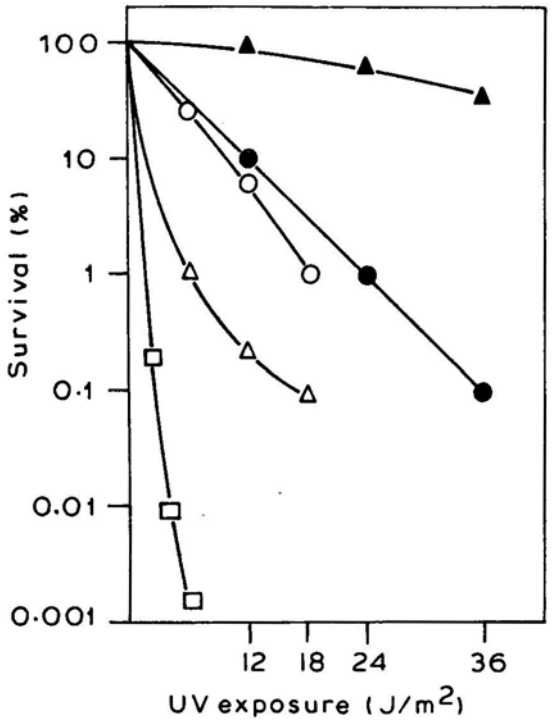

Figure 3. UV-inactivation of colony formation of mutant strains isolated by directed mutagenesis. (●), Rd; (O), *uvr*1; (Δ), HIUO4; (□), HIU21; (▲), HIU36.

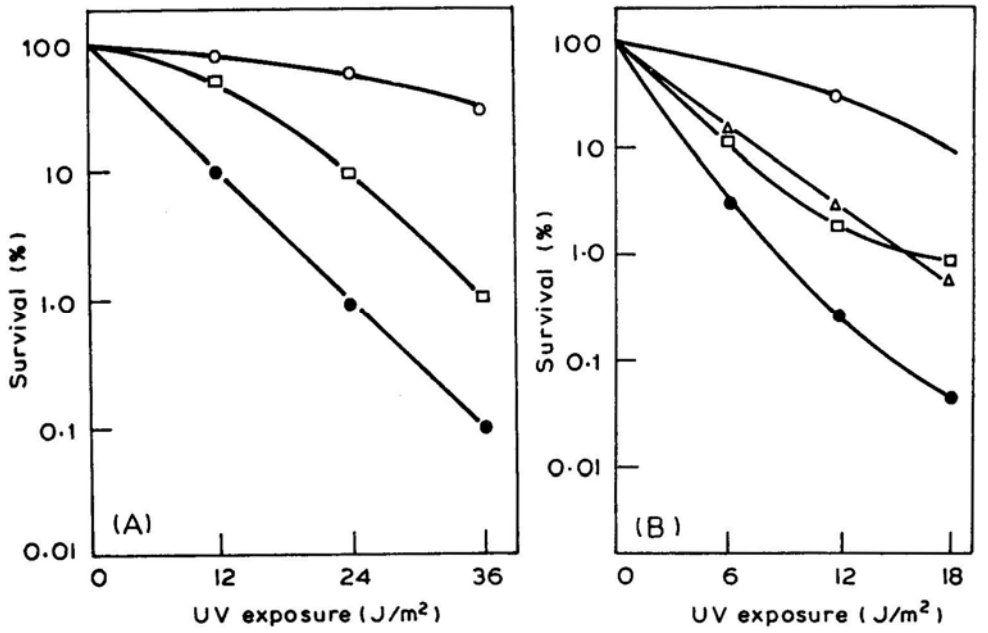

Figure 4. A. Complementation of HIU36 mutant by plasmid pKuvrl. (●) Rd; (O), HIU36; (□), HIU36(pKuvrl). **B.** Complementation of UV sensitivity of HIUO4. (O); Rd; (●), HIUO4; (▲), HIUO4 (pKuvr1); (■), HIU04 (pKuvrl⁻).

Table 2. Colony forming ability of *H. influenzae strains.*

H. influenzae strain	Amp^R colony forming units (%)	CFU (X 10^9) per unit absorbance of culture
Rd	—	5·0
*uvr*1	—	5·0
*uvr*1 (pKuvr1)	100·0	5·0
HIU04	—	3·7
HIU04 (pKuvr1)	0·1	3·5
MC1	—	2·3
MC1 (pKuvr1)	2·0	4·0

cultures supports these observations. It is seen that MC1 and HIUO4 strains produce longer cells than those of wild type. It is also seen from table 2 that pKuvrl is stable in $Uvr1^-$ cells but is unstable in MC1 and extremely so in HIUO4. Presence of pKuvrl abolished the ability of MC1 mutant to produce minicells as well as increased its number of CFU per unit OD. However, this complementation is partial which may be due to instability of the plasmid. Plasmid pKuvrl does not significantly affect CFU per unit OD of HIUO4 which may be due to its extreme instability in the mutant.

Discussion

We have self-cloned *uvr*1 gene of *H. influenzae.* At least two genes, namely *uvr*1 and *uvr*2 governing UV sensitivity are known in *H. influenzae.* From genetic and other experiments (Beattie and Setlow, 1970; Notani *et al.,* 1971) it was inferred that the action of *uvr*1 gene precedes that of *uvr*2 gene. The pKuvrl clone fully and specifically complements *uvr*1 gene but not *uvr*2 gene. It is concluded that *uvr*2 gene is not carried on the 11·3 kb insert of pKuvrl and is therefore not linked to *uvr*1 gene.

To scan for other genes concerned with DNA repair functions which might be carried on the 11·3 kb chromosomal insert of pKuvrl, a novel method of directed mutagenesis was successfully applied. Mutations induced in pKuvrl DNA were transferred to resident DNA by transformation. By this method many new phenotypes were found to be associated with chromosomal DNA insert of pKuvrl. These include UV resistance, UV sensitivity, filament formation, etc. These observations suggest that there is a cluster of more than one gene on *uvr*1 clone pKuvrl. Mutation designated uvr^{sensl} has phenotypes similar to that of *ssb* mutant of *E. coli.*

The *uvr*A clone of *E. coli* was found to carry *ssb* gene on a 9·4 kb chromosomal DNA insert of plasmid pDR2000 (Sancar and Rupp, 1979). *ssb* mutants are UV sensitive and exhibit number of phenotypes that include filamentation, defective induction of prophage among others (Vales *et al.,* 1980). Also *lon* or *cap*R mutants of *E. coli* are UV sensitive and form filaments. In addition *Ion* gene regulates a number of genes such as *sul*A and *sul*B which are involved in cell division (Gayada *et al.,* 1976). Ward and Lutkenhaus (1985) have shown that 2–7 fold increase in *sul*B protein induces minicell formation.

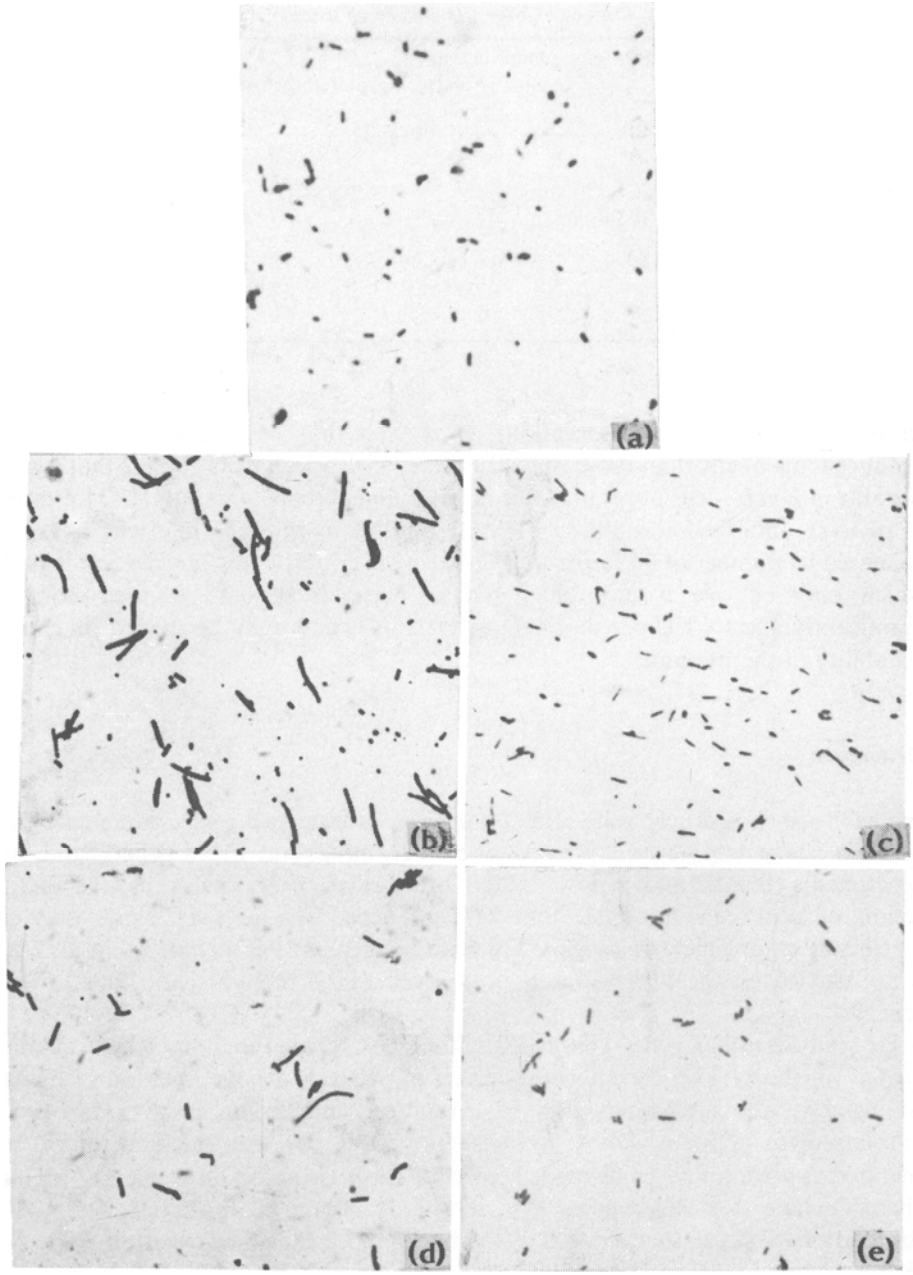

Figure 5. *H. influenzae* strains at a magnification of 1,000 X. (a), Rd; (b), MC1; (c), MC1 (pKuvrl); (d), HIUO4; (e), HIUO4 (pKuvrl).

With the cloning of a *uvr*1 gene from *H. influenzae* it should now be possible to see if any other DNA (*E. coli* or even human) contains sequences homologous to. *uvr*1 gene. Also it should be possible to isolate its product and study its role in DNA repair at the molecular level.

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