Two Modes of 'Curing' Transmissible Bacterial Plasmids

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SUMMARY

F'*lac* was efficiently eliminated by acridine orange from *Escherichia coli* K12, whether sex pilus synthesis was constitutive or repressed by an fi^+ R factor. R factors, F-like or I-like, repressed or derepressed, were not eliminated by acridine orange. Sodium dodecyl sulphate (SDS), known to eliminate certain plasmids determining constitutive synthesis of F pili, had no effect on cultures with wild-type (repressed) R factors. Bacteria with derepressed synthesis of pili, either F-like or I-like, but particularly the latter, showed increased sensitivity to SDS. SDS treatment selected from such cultures non-piliated cells, with lost or mutant R factors. Thus acridine orange was a true 'curing' agent, specific for F, whilst SDS acted only by selection of spontaneous variants.

INTRODUCTION

Acridine dyes are very effective in curing *Escherichia coli* of the sex factor F (Hirota, 1960). This plasmid produces sex pili constitutively. Acridines have little, if any, effect in elimination of R factors (Mitsuhashi, Harada & Kameda, 1961; Watanabe & Ogata, 1966). We have found that F factors can be eliminated equally efficiently from cells in which pili are produced constitutively and from those in which pilus production is repressed by the presence of an fi^+ R factor. Elimination of F factors by acridines seems to be due to a selective interference in the replication of the plasmid (Hohn & Korn, 1969; Yamagata & Uchida, 1969) and is most clearly demonstrable in exponentially growing cultures (Hirota, 1960). Thus curing by acridines normally involves loss of the whole plasmid.

We also show that mutants of F-like and I-like R factors with derepressed (*drd*) pilus synthesis are as little influenced by acridine orange as are the wild-type (pilus repressed) plasmids.

Tomoeda and co-workers (Tomoeda, Inuzuka, Kubo & Nakamura, 1968; Inuzuka, Nakamura, Inuzuka & Tomoeda, 1969) showed that the detergent sodium dodecyl sulphate (SDS) was effective in eliminating F-*lac* and the R factor R100*drd*1. Both these plasmids produce F-like pili constitutively (Nishimura, Ishibashi, Meynell & Hirota, 1967). We have investigated the effect of SDS on *Escherichia coli* K12 carrying R factors, repressed or derepressed for the synthesis of F-like or I-like pili. Cultures of bacteria synthesizing pili constitutively were more sensitive to SDS than repressed cultures, in which pili are produced by only a minority of cells. Exposure to SDS led to selection of non-piliated cells; in some cases, but not all, the non-piliated cells had also lost resistance determinants.

METHODS

Bacteria. *Escherichia coli* K12 strains, J53 F⁻ *pro met lac*⁺ and J62 F⁻ *pro his try lac*⁻ (Clowes & Hayes, 1968).

Media. Nutrient broth was Oxoid no. 2 (CM67). Nutrient agar was the same, solidified with 1·2 % New Zealand agar (Oxoid L13). Acridine orange broth was as described by

Table 1. *Plasmids and phages used in these experiments*

Resistance markers: A = ampicillin, S = streptomycin, T = tetracycline,
C = chloramphenicol, K = kanamycin, Su = sulphonamide.

Plasmids		Resistance pattern	Reference
F-like	F'*lac*	—	Jacob & Adelberg, 1959
	R1	ASCKSu	Lawn *et al.* 1967
	R1*drd*19	ASCKSu	Lawn *et al.* 1967
	R1*drd*16	K	Frydman & Meynell, 1969
	R192	SCTSu	Lawn *et al.* 1967
	R192*drd*7	SCTSu	Lawn *et al.* 1967
	R538-1	SCSu	Romero & Meynell, 1969
	R538-1*drd*	SCSu	Romero & Meynell, 1969
I-like	R144	TK	
	R144*drd*3	TK	Lawn *et al.* 1967
	R64	ST	
	R64*drd*11	ST	

Phages	Receptor	
MS2	F pili	Davis *et al.* 1961
If1	I pili	Meynell & Lawn, 1968

Hirota (1960). MacConkey agar was Oxoid (CM7*b*). SDS was from Koch–Light Laboratories, Colnbrook, Buckinghamshire.

Plasmids and phages are listed in Table 1.

Acridine orange (AO) treatment. A small inoculum (200 to 500 bacteria) was added to acridine orange broth (Hirota, 1960) with varying concentrations of acridine orange and incubated at 37° overnight. Cultures containing the highest concentration of acridine orange in which growth was clearly visible were diluted and spread on MacConkey agar (when cultures contained F-*lac*⁺) and nutrient agar plates. Where cultures contained R factors, colonies from the nutrient agar plates were replicated with velvet pads to nutrient agar plates incorporating appropriate antibiotic.

SDS treatment. To 100 ml of nutrient broth 1 g SDS was added. The solution was auto-claved, the pH adjusted to 7·6 and the solution steamed for 1 h. Control broth without SDS was also adjusted to pH 7·6. Overnight stationary broth cultures were diluted 100-fold and 0·5 ml added to each 100 ml of broth. Cultures were incubated with aeration at 37°. Where SDS was required 8 h after bacterial inoculation a volume of sterile 10 % (w/v) SDS solution in nutrient broth pH 7·6 was added, sufficient to bring the concentration to 1 % (w/v). When chloramphenicol was added, 2·5 ml of a solution containing 1 mg/ml was added to 100 ml broth to give a final concentration of 25 μg/ml. Viable counts were made after spreading 0·1 ml of dilutions of culture on nutrient agar plates and incubating at 37°. Extinction (*E*) was measured at 675 nm with a spectrophotometer (Unicam SP600). Colonies from the viable count plates were tested for phage- and drug-sensitivity.

Testing for phage- and drug-sensitivity. Ditch plates containing appropriate concentrations of drug in nutrient agar ditches were used to test for drug resistance. Colonies were streaked across the ditches and also across streaks of phage (approximately 10¹⁰ particles per streak on 9 cm nutrient agar plates) to test for phage sensitivity. Phage- or drug-sensitive and phage- or drug-resistant cultures were streaked as controls on each plate. Visible lysis by phages MS2 or If1 was our criterion for the constitutive production of F-like or I-like pili.

Matings. Procedure was as described by Datta *et al.* (1971).

Table 2.

Acridine orange curing of F'*lac* from cultures repressed and derepressed for pilus production

Culture	Exposure to acridine orange	*lac*+ clones	*lac*− clones	Total	Loss of *lac*+ (%)
J62(F'*lac*+)	−	320	21	341	6·1
	+	52	372	424	88
J62(F'*lac*+)(R1)	−	490	27	517	5·2
	+	4	324	328	99

Acridine orange curing of R factors from cultures repressed and derepressed for pilus production

Culture	Exposure	K-resistant clones	K-sensitive clones	Total	Loss of K resistance (%)
J62(R1)	−	1069	3	1072	< 1
	+	499	0	499	< 1
J62(R1*drd*19)	−	1381	2	1383	< 1
	+	554	0	554	< 1
J62(F'*lac*+)(R1)	−	496	1	497	< 1
	+	402	1	403	< 1
J62(R144)	−	682	4	686	< 1
	+	976	1	977	< 1
J62(R144*drd*3)	−	1288	3	1291	< 1
	+	560	0	560	< 1

RESULTS

Acridine orange treatment

F'*lac* was eliminated with high efficiency from J62(F'*lac*) and from J62(F'*lac*)(R1) by 25 μg acridine orange/ml. No elimination of R factors, F-like or I-like, repressed or derepressed for pilus synthesis, was observed (Table 2).

SDS treatment

(a) *Control cultures.* SDS had no effect on exponential growth of cultures without plasmids but in stationary phase the viable count fell and the extinction decreased disproportionately.

(b) *F-like R factors.* Cultures with wild-type (repressed) R factors behaved like R⁻ controls. Cultures with the derepressed R factors, R1*drd*19 or R538-1*drd*, were also unaffected by SDS during exponential phase; the decline in viable count and reduction in E brought about by SDS during stationary phase were accelerated. The effect of SDS on J53(R538-1*drd*) was less than on J53(R1*drd*19) (Fig. 1).

SDS did not affect the properties of R1 wild-type or R1*drd*19 during exponential phase. During stationary phase R1 was still unaffected but the majority of surviving clones from the J53(R1*drd*19) culture had lost phage sensitivity and all drug-resistance markers (Table 3). R538-1 wild-type was completely stable throughout. R538-1*drd* was almost equally stable. No loss of phage sensitivity or drug resistance occurred in any clone tested up to 48h incubation. At 72 h 10 % of colonies from the SDS culture had lost all plasmid-determined characters.

The effect of SDS on cultures carrying R192 and R192*drd*7 was also tested. In one experiment, in which R192*drd*7 was completely stable in the control, half of the surviving colonies from the SDS culture retained phage sensitivity and all three drug resistances, 20 % had lost
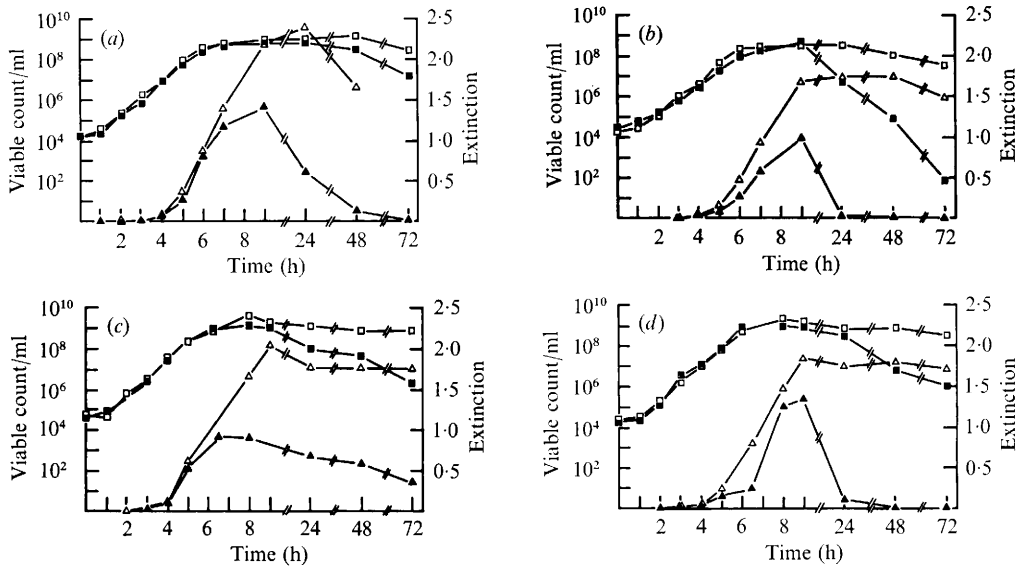
Fig. 1. Effect of SDS on *Escherichia coli* carrying F-like R factors. (*a*) J53(R1); (*b*) J53(R1*drd*19); (*c*) J53(R538-1); (*d*) J53(R538-1*drd*). □, Viable counts, control; ■, with SDS; △, extinction, control; ▲, with SDS.

Table 3. *Plasmid determined properties of clones from cultures containing repressed and derepressed F-like R factors, after growth with and without SDS*

		+SDS			Control		
Culture	Time of sample	Drug^r MS2^s	Drug^r MS2^r	Drug^s MS2^r	Drug^r MS2^s	Drug^r MS2^r	Drug^s MS2^r
J53(R1)	0	—	20/20	—	—	20/20	—
	4	—	20/20	—	—	20/20	—
	5	—	20/20	—	—	20/20	—
	9	—	20/20	—	—	20/20	—
	24	—	20/20	—	—	20/20	—
	48	—	20/20	—	—	20/20	—
	72	—	20/20	—	—	20/20	—
J53(R1*drd*19)	0	20/20	—	—	20/20	—	—
	4	20/20	—	—	20/20	—	—
	5	30/30	—	—	20/20	—	—
	9	20/20	—	—	20/20	—	—
	24	20/20	—	—	20/20	—	—
	48	4/20	—	15/20	20/20	—	—
	72	6/20	—	14/20	20/20	—	—
J53(R538-1)	0	—	20/20	—	—	20/20	—
	5	—	20/20	—	—	20/20	—
	9	—	20/20	—	—	20/20	—
	24	—	20/20	—	—	20/20	—
	48	—	20/20	—	—	20/20	—
	72	—	20/20	—	—	20/20	—
J53(R538-1*drd*)	0	20/20	—	—	20/20	—	—
	5	20/20	—	—	20/20	—	—
	9	20/20	—	—	20/20	—	—
	24	20/20	—	—	20/20	—	—
	48	20/20	—	—	20/20	—	—
	72	18/20	—	2/20	20/20	—	—

Drug^r = clones resistant to all antibiotics whose resistances are determined by the R factor.
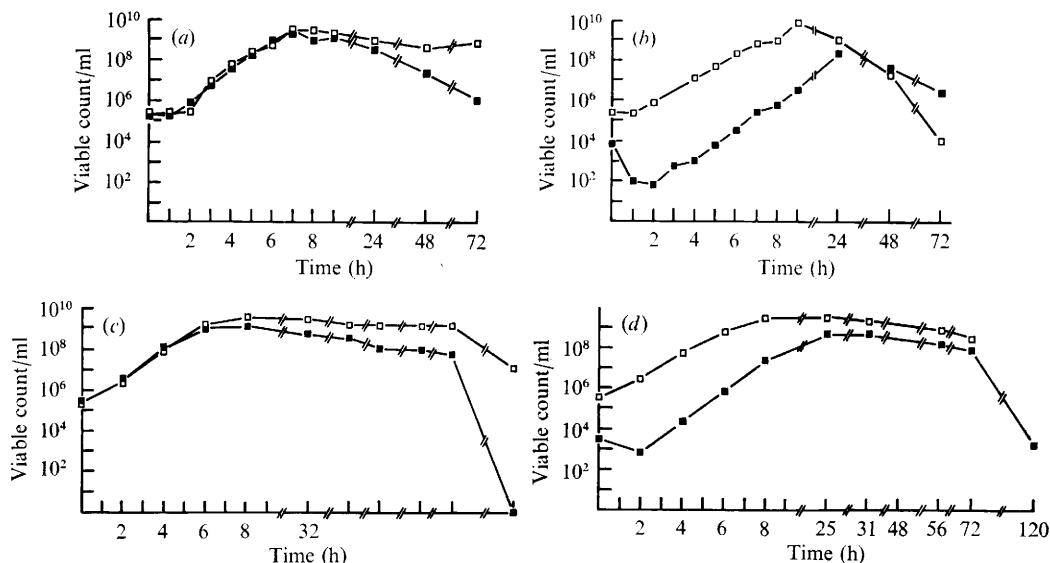Drug^s = clones sensitive to all antibiotics whose resistances are determined by the R factor.

Fig. 2. Effect of SDS on *Escherichia coli* carrying I-like R factors. (*a*) J53(R64); (*b*) J53(R64*drd*11); (*c*) J53(R144); (*d*) J53(R144*drd*3). Symbols as in Fig. 1.

Table 4. *Plasmid-determined properties of clones from cultures containing* R192*drd*7, *after growth with and without SDS*

	Time of sample (h)	+SDS			Control		
Culture		STC^r MS2^s	STC^r MS2^r	STC^s MS2^r	STC^r MS2^s	STC^r MS2^r	STC^s MS2^r
J53(R192*drd*7)	72	10/20	4/20	6/20	20/20	—	—

phage sensitivity but retained drug resistance and 30 % had lost all plasmid properties (Table 4). In other experiments, the tetracycline resistance and phage sensitivity of R192*drd*7 were unstable in the control culture, although resistance to streptomycin, chloramphenicol and sulphonamide was retained. When this was so, the loss was accelerated by SDS, but resistance to streptomycin, chloramphenicol and sulphonamide remained stable.

(*c*) *I-like R factors*. Wild-type I-like factors did not influence the growth or survival of their hosts in SDS medium. I-like factors with derepressed pilus production conferred marked sensitivity to SDS. Killing occurred as soon as detergent was added. Viable counts fell, but after 2 h rose at approximately the same rate as the control (Fig. 2). J53(R64*drd*11) but not J53(R144*drd*3) showed marked loss of viability in the control culture in stationary phase.

R64 was stable with and without SDS (Table 5). R64*drd*11 in the control cultures was unaltered during the first 48 h but clones isolated after the population decrease mentioned above were phage-resistant (Table 5). The effect of SDS on plasmid-determined characters of J53(R64*drd*11) was early loss of phage sensitivity.

R144 wild-type was unstable to the same extent in control and SDS cultures (Table 5). R144*drd*3 was stable in control cultures until well into stationary phase. After prolonged incubation phage-resistant mutants appeared. Two differences from the control were observed in the SDS cultures. Phage-resistant mutants appeared during log phase and many of the phage-sensitive clones (Table 5) were less clearly lysed than the parent J53(R144*drd*3).

Table 5. *Plasmid-determined properties of clones from cultures containing repressed and derepressed I-like R factors, after growth with and without SDS*

Culture	Time of sample (h)	+ SDS			Control		
		Drug^{r*} Ifi^s	Drug^r Ifi^r	Drug^s Ifi^r	Drug^r Ifi^s	Drug^r Ifi^r	Drug^s Ifi^r
J53(R64)	0	—	20/20	—	—	20/20	—
	4	—	20/20	—	—	20/20	—
	5	—	20/20	—	—	20/20	—
	9	—	20/20	—	—	15/15	—
	24	—	20/20	—	—	20/20	—
	48	—	20/20	—	—	20/20	—
	72	—	20/20	—	—	20/20	—
J53(R64*drd*11)	0	20/20	—	—	20/20	—	—
	4	18/20	2/20	—	20/20	—	—
	5	15/20	4/20	—	20/20	—	—
	9	3/20	17/20	—	19/20†	—	—
	24	—	20/20	—	20/20	—	—
	48	—	20/20	—	10/10	—	—
	72	—	20/20	—	—	20/20	—
J53(R144)	0	—	17/20	3/20	—	—	—
	2	—	20/20	—	—	17/20	3/20
	4	—	17/20	3/20	—	19/20	1/20
	6	—	18/20	2/20	—	17/20	3/20
	8	—	15/20	5/20	—	18/20	2/20
	25	—	17/20	3/20	—	16/20	4/20
	31	—	18/20	2/20	—	20/20	—
	48	—	18/20	2/20	—	18/20	2/20
	56	—	17/20	3/20	—	17/20	3/20
	72	—	17/20	3/20	—	17/20	3/20
J53(R144*drd*3)	0	7/8	1/8	—	20/20	—	—
	2	15/20	5/20	—	20/20	—	—
	4	15/20‡	5/20	—	20/20	—	—
	6	15/20‡	5/20	—	20/20	—	—
	8	14/20§	6/20	—	20/20	—	—
	25	12/20§	8/20	—	20/20	—	—
	31	9/20§	11/20	—	20/20	—	—
	56	9/20§	11/20	—	15/20	5/20	—
	72	7/10§	3/10	—	2/10	7/10	1/10

* Clones resistant to all antibiotics whose resistances are determined by the R factor.
† The exceptional clone (1/20) retained phage sensitivity and tetracycline resistance and had lost streptomycin resistance.
‡ Many of these clones showed a lesser degree of Ifi lysis than the control.
§ All these clones showed a lesser degree of Ifi lysis than the control.

Transfer from phage-resistant variants

From the SDS culture of J53(R192*drd*7), twenty phage-resistant mutant clones which had retained all drug-resistance markers were tested for ability to transfer drug resistance. Three of these gave no transfer (efficiency $< 10^{-7}$ per donor), eight transferred at rates between 10^{-6} and 10^{-7} per donor, nine transferred at wild-type frequency, which was 10^{-2} per donor. J53(R192*drd*7) control cultures transferred with an efficiency of 1 per donor cell.

Kinetics of the SDS effect

SDS affected J53(R1*drd*16) in the same way whether it was added at the beginning of exponential phase or the beginning of stationary phase (Fig. 3). The addition of chlor-
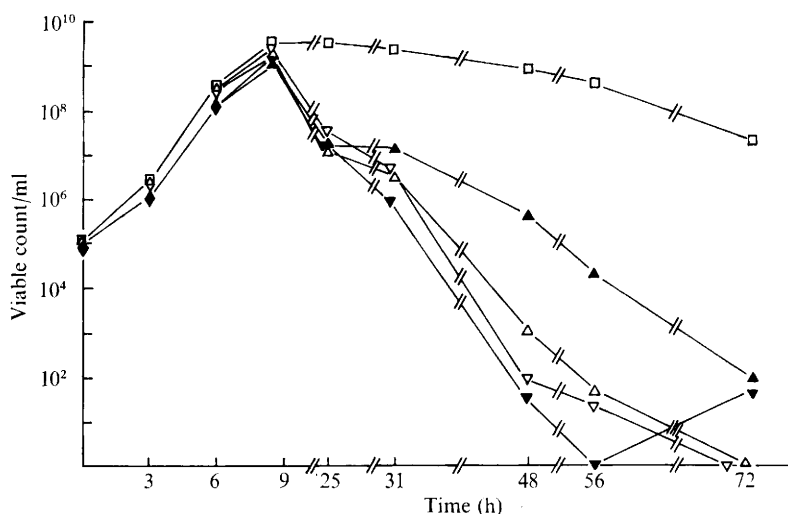
Fig. 3. Kinetics of SDS effect on *Escherichia coli* carrying an F-like R factor, with derepressed pilus synthesis. □, J53(R1*drd*16) control; ▽, SDS at 8 h; △, SDS at 8 h, chloramphenicol at 8 h; ▼, SDS at o h; ▲, SDS at o h, chloramphenicol at 8 h.

amphenicol at the end of exponential phase had little effect on the viable count, but prevented the appearance of phage-resistant mutants in the SDS cultures (Table 6).

DISCUSSION

Acridine orange efficiently cured bacteria of F'*lac*+, whether F pili were being synthesized constitutively or not. No acridine orange curing of R factors, F-like or I-like, with repressed or derepressed pilus synthesis was demonstrated. Acridine orange, which probably intercalates into DNA, appears to have a specific effect on replication of the F factor (Hohn & Korn, 1969; Yamagata & Uchida, 1969). The results with SDS were quite different.

Presence of wild-type (pilus repressed) F-like or I-like plasmids had no demonstrable effect upon the response of the host to SDS. The presence of derepressed mutant plasmids had a marked, though variable effect, making their hosts more sensitive. Their increased sensitivity may result from the presence of pili on all cells in the culture. Detergents, including SDS, are known to act on the bacterial membrane (Tremblay, Daniels & Schaechter, 1969) and sex pili may allow access of detergent to the membrane. This was suggested by Tomoeda *et al.* (1968), who postulated that the SDS might gain access to the membrane via the pili, that the plasmids are attached to membrane close to the pili and may thus be damaged. This implies a specific mutagenic action of the SDS on the plasmid. We consider it more probable that piliated cells themselves are especially susceptible to lysis by SDS and that the increase in the proportion of non-piliated cells is due, not to a direct effect on the plasmid, but to the selective advantage enjoyed by spontaneous non-piliated variants. Our results on the kinetics of the SDS effect (see Fig. 3 and Table 6) support this interpretation.

The presence of derepressed R-factors has other effects on the cell surface besides the constitutive synthesis of sex pili, for example that shown by a reduction in surface exclusion determined by the F factor (Willetts & Finnegan, 1970). Dowman & Meynell (1970) observed that the increased susceptibility to lysis by various agents, including SDS, conferred by derepressed I-like plasmids, was not due merely to the presence of sex pili. Dowman & Meynell studied R144*drd*3. We have consistently isolated clones from SDS cultures

Table 6. *Effect of SDS added at the beginning of log phase or in early stationary phase, with or without chloramphenicol in stationary phase on* J53(R1*drd*16)

Twenty colonies were tested for each sampling where available.

Treatment	Time (h)	K^r MS2^s	K^r MS2^r	K^s MS2^s	K^s MS2^r
Control	8½	20	0	0	0
	24	20	0	0	0
	32	20	0	0	0
	49	20	0	0	0
	57	20	0	0	0
	72	20	0	0	0
SDS 0 h	8½	19	0	1	0
	24	19	0	1	0
	32	14	6	0	0
	49	0	0	0	6
	72	0	10	0	0
SDS 0 h, CHL 8½ h	8½	20	0	0	0
	24	20	0	0	0
	32	20	0	0	0
	49	20	0	0	0
	72	13	0	0	0
SDS 8½ h	8½	20	0	0	0
	24	20	0	0	0
	32	19	0	0	1
	49	16	2	0	0
	57	0	5	0	0
SDS 8½ h, CHL 8½ h	8½	20	0	0	0
	24	20	0	0	0
	32	20	0	0	0
	49	17	0	0	3
	57	10	1	0	0

of J53(R144*drd*3), whose sensitivity to phage If1 was decreased (Table 5) although they were still visibly lysed. However, with many of the other derepressed R-factors studied, we obtained rather different results. Exposure to SDS led to selection of clones completely resistant to pilus specific phages. We believe therefore that non-susceptibility to SDS is strongly correlated with failure to produce pili. In one case, R192*drd*7, we have investigated the nature of the phage-resistant mutants. Some seemed to be revertants to the repressed condition. Others were apparently point (revertible) mutants analogous to the *tra* mutants of F′*lac* (Achtman, Willetts & Clark, 1971).

Derepressed I-like and F-like factors had strikingly different effects on susceptibility of bacteria to SDS. During exponential phase growth in the presence of SDS, phage-resistant variants occurred in strains with derepressed I-like but not with derepressed F-like factors. Immediately on exposure to SDS there was a sharp fall in viability where the cells carried derepressed I-like factors. Resumption of growth, at normal rates, after this fall could not be explained exclusively as multiplication of pre-existing phage-resistant mutants (Fig. 2, Table 5). With cultures carrying derepressed F-like factors, phage-resistant clones were only detected in stationary phase. Where the derepressed F-like R factor markedly increased the lethal effect of SDS in the stationary phase, there was strong selection for phage-resistant variants for example, with J53(R1*drd*19). Where the R factor had less influence on viability, there was less selection for phage resistance, as with J53(R538-1*drd*).

Phage-resistance, encountered in these experiments, was of two kinds – loss of phage

sensitivity alone, or loss of all plasmid-determined characteristics, presumably loss of the whole plasmid. Each R factor tested tended to produce one or other of these variants on exposure to SDS. For example, R1*drd*19, an *i⁻ drd* mutant, according to the terminology of Frydman & Meynell (1969), produced plasmidless variants but R1*drd*16, an o^e mutant (Frydman & Meynell, 1969; but see Willetts & Finnegan, 1971) of the same R factor, R1, produced exclusively drug-resistant, phage-resistant variants (i.e. retained the plasmid). This may reflect the reversion rates of the two *drd* mutants of R1. Both derepressed I-like factors lost phage resistance whilst retaining drug-resistance markers.

In cultures carrying F-like derepressed R factors the increase of phage-resistant mutants occurred in the stationary phase. The action of SDS, manifested in stationary phase, was actually exerted in this phase (Fig. 3). This could be due either to a direct effect of SDS on nondividing bacteria or to a replacement by turnover of phage-sensitive types by resistant variants. Treatment of the SDS stationary phase cultures with the bacteriostatic antibiotic, chloramphenicol, prevented the emergence of phage-resistant mutants (Fig. 3, Table 6). The simplest interpretation of these results is that the antibiotic prevented multiplication of phage-resistant mutants. Thus, according to the definition of Novick (1969), the effect of SDS on *drd* R factor carrying cultures is not true curing but rather a selection of plasmidless clones or of clones carrying mutant plasmids.

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