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Effect of Some R Factors on the Sensitivity of Rough *Enterobacteriaceae* to Human Serum

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Eight plasmids out of 26 tested confer partial serum resistance to *Escherichia coli K-12* strains (Bhs character). Four Bhs$^+$ plasmids were tested in a rough *Salmonella typhimurium* host, and three conferred partial serum resistance to this host as well. Plasmids representative of 19 different incompatibility groups were examined; the Bhs$^+$ property was found in some (but not all) representatives of groups N, O, S, T, W, and F'*lac* (FI), and there was no apparent association between other plasmid markers and Bhs. It is likely that more than one mechanism can promote the Bhs$^+$ phenotype and that Bhs genes are responsible for modifications of the surface structures involved in serum sensitivity.

Serum kills many gram-negative organisms by a reaction requiring antibodies, at least some components of the complement system, and possibly other serum factors (3, 9, 17). Among *Enterobacteriaceae*, most rough strains are sensitive and many smooth strains are resistant to serum. *Escherichia coli* cells killed by serum show evidence of both cell wall and cell membrane damage (20, 23).

Feingold et al. (7, 8) have shown that the primary complement-mediated effect of serum is on the outer lipopolysaccharide-containing membrane, followed by peptidoglycan degradation by lysozyme and complement-mediated damage to the inner cell membrane; bacterial killing should be due primarily to this second effect, since serum retains most of its killing activity even if depleted of lysozyme (6, 22).

Roantree and Rantz (16) found a much higher proportion of serum-resistant strains among enteric bacteria isolated from blood than among those isolated from stools or urine. A possible correlation between serum resistance and virulence has long been recognized (12, 18). The nature of the terminal sugars of lipopolysaccharides and the total amount of lipopolysaccharide (11), as well as capsular structures such as Vi and K antigens (13), seem to be relevant for both serum sensitivity and virulence.

Some plasmids are known to modify the bacterial surface as evidenced by the appearance of new antigenic determinants (2, 10, 21) or an alteration of the lipopolysaccharide layer (5), and there is evidence that some contribute to bacterial pathogenicity (19). It is then plausible that plasmids exist that alter the serum sensitivity of sensitive bacteria. Reynard and Beck (15) have recently reported that two R factors (R1 and R100) increase the resistance of *E. coli* K-12 strains to the bactericidal effects of normal rabbit serum.

The present paper reports a survey on the effects of 26 plasmids (24 R factors and 2 F'*lac*) on the sensitivity of *E. coli* K-12 and rough *Salmonella typhimurium* strains to human serum.

MATERIALS AND METHODS

Plasmids. R factors harbored in a number of diffent hosts were obtained through the courtesy of N. Datta, Y. A. Chabbert, and C. Monti-Bragadin; F'*lacW* was from N. Willets, and F'*lacC* was from Y. A. Chabbert. All plasmids were transferred into the tester strains described below.

Organisms, cultural conditions, and bactericidal assay. *S. typhimurium* TA1535 *hisG46 uvrB bio rfa* (1) and the *E. coli* K-12 derivatives J6-2 *pro his trp lac*, J5-3 *met pro*, and C600 *lac thr leu thi* (4) were used; spontaneous mutants of the K-12 strains resistant to streptomycin, nalidixic acid, or rifampin, respectively, i.e., J6-2 *str*, J5-3 *nal*, and C600 *rfm*, were isolated and used as recipients of R factors from various sources in order to facilitate the counterselection of the donors.

The bacteria were grown in antibiotic medium no. 3 (Difco) at 37°C with agitation overnight and then diluted 1:40 in fresh broth and grown to 0.6 absorbancy at 560 nm in the same medium. The doubling time and the exponential nature of the growth were regularly checked spectrophotometrically. The bacteria were harvested by centrifugation, washed once in phosphate-buffered saline (Oxoid), and diluted to a final concentration of 5×10^6 cells/ml for the bactericidal assay. The bacterial suspensions were mixed with

serum dilutions (in phosphate-buffered saline; see below) and incubated at 37°C with agitation. Viable counts were obtained by plating suitable dilutions (in phosphate-buffered saline) on nutrient agar plates.

Serum source and treatments. The blood of five healthy donors was allowed to clot for 1 h at room temperature; the sera were separated by centrifugation, pooled, divided into 0.2-ml samples, and stored in liquid nitrogen. A single preparation lasted throughout the experiments. Where indicated, heat-labile components were inactivated by incubation at 56°C for 30 min; adsorption was carried out by incubating 10^{10} bacteria in 1.0 ml of 20% serum at 0°C for 60 min with occasional stirring. The adsorbed serum was separated by centrifugation and sterilized by membrane (0.45-μm pore size) filtration (Millipore Corp.).

RESULTS

Definition of the serum bactericidal assay. Bacterial cultures display maximal serum sensitivity in log phase (8). Exponentially growing bacteria were washed in phosphate-buffered saline, diluted to approximately 5×10^6 cells/ml, and exposed to 1% serum at 37°C with agitation. The death curve was roughly exponential with respect to the time of incubation (Fig. 1). Heating the serum at 56°C for 30 min, or absorbing the serum with an excess of the same bacteria at 0°C for 30 min, completely suppressed the bactericidal activity, but this could be totally restored by mixing the treated sera in equal amounts (Fig. 1). The bactericidal system seems therefore to be dependent on both antibodies and complement (9).

Figure 2 shows the dose-response curves with different serum dilutions on the four tester strains used.

The derivation of the K-12 strains used is reported by Clowes and Hayes (4); no selection for serum resistance has ever been applied, but

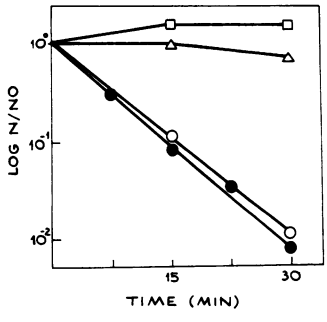

FIG. 1. *Bactericidal effect of human serum. Log-phase E. coli J6-2 str cells were exposed to 1% serum either untreated (●), heat-treated (□), or "adsorbed" (△). Curve (○) shows the effect of the treatment with both heat-treated and adsorbed sera (both at 1%).*

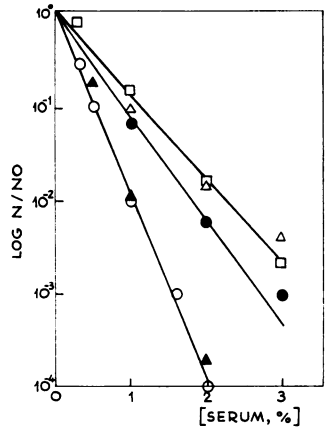

FIG. 2. *Serum sensitivity of the four tester strains used. Bacteria were exposed for 30 min to the indicated serum concentrations. Symbols: J6-2 str (○, ▲); J5-3 nal (●); C600 rfm (△); TA1535 (□).*

the rather strong mutagenic treatments might have led to the accumulation of unselected mutations, which make the various derivatives significantly different in this respect, e.g., strains J6-2 *str* and C600 *rfm* (see Fig. 2), the slopes of whose survival curves differ by a factor of 2. Replicated experiments with the same strain (and the same serum) gave only minimal variations, and the difference between the various strains was reproducible.

Both kinds of assay (i.e., the first with respect to time of incubation and the second with respect to serum concentration) were independent of the bacterial titer between 10^3 and 10^7 cells/ml.

Effect of plasmids on serum sensitivity. R^+ derivatives of the tester strain J6-2 *str* were studied for their serum sensitivity as compared with that characteristic of the R^- parent. None tested so far conferred total resistance (i.e., to undiluted serum), and most had no effect, whereas some conferred a significant relative increase in resistance.

Most R factors were tested also in C600 *rfm*, and those conferring serum resistance were also tested in J5-3 *nal*. No discrepancy in the effect of a particular R factor was found in any of the *E. coli* derivatives tested.

To exclude the possibility that the transfer system itself might result in the selection of recipients that are more resistant to serum, spontaneous R^- segregants of R^+ strains were tested for their serum sensitivity; in all cases, a return to a "parental" level of sensitivity was obtained.

Four R factors conferring resistance in *E. coli* strains were tested in the rough *S. typhimurium*

strain TA1535; three of them (RN3, Rtsl, and RS-a) conferred resistance to this host as well, but one (R-Ecl) did not show any effect.

Figure 3 shows the effect of two R factors (RS-a and Rtsl) on three tester strains, and Fig. 4 shows the time course of the killing by 1% serum on J6-2 *str* R^- and its (RS-a) derivative.

Plasmids represent a vary large and complex class of genetic elements (see Novick [14] for a review). One classification is based on "incompatibility groups," defined as the group of plasmids that cannot coexist in the same bacterium. We have tested at least one representative of all the incompatibility groups available to us. The results are reported in Table 1. We propose the symbol Bhs for the plasmidic character, resistance to the *b*actericidal effects of *h*uman *s*erum.

No evidence of association between Bhs and other plasmid markers, including the incompatibility group, was observed.

The extent of resistance conferred by the plasmids has been evaluated as the ratio of the serum dose necessary to reduce the viable count to 1% in R^+ and R^- strains (1% DR/D); these values (Table 2) seem to be very similar in all R^+ derivatives containing the same plasmid, independently of the base level sensitivity of the tester strain, with the exception of R-Ec1, which is ineffective in the *Salmonella* host.

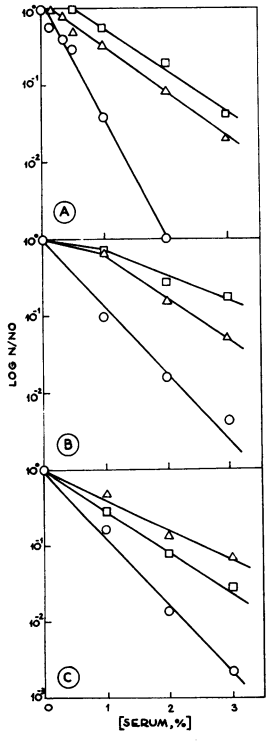

FIG. 3. *Effect of two R factors in three tester strains. Bacteria were exposed for 30 min to the indicated serum concentrations. (A) J6-2 str; (B) C600 rfm; (C) TA1535. Symbols: R^- strains (○); $(RS\text{-}a)^+$ (□); $(Rts1)^+$ (△).*

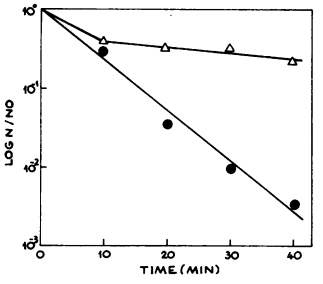

FIG. 4. *Time course of bacterial killing by 1% human serum. Symbols: J6-2 str R^- (●); J6-2 str (RS-a)* (△).

DISCUSSION

A plasmid-directed alteration of the serum sensitivity of serum-sensitive *Enterobacteriaceae* might have consequences on the rate of spread (both of the plasmid itself and of its host) in environments where serum is a possible selective force, and thus on the composition of the intestinal flora and possibly on bacterial virulence itself, e.g., by rendering capable of bacteremic invasion strains normally devoid of such capacity.

We have tested the serum sensitivity of a large number of plasmid-bearing derivatives of serum-sensitive *E. coli* K-12 and *Salmonella* strains. Only 8 out of 26 plasmids tested conferred relative serum resistance to the *E. coli* hosts in our bactericidal system (human serum), i.e., carried the Bhs trait. The plasmid R1, reported by Reynard and Beck (15) as conferring resistance to the effect of rabbit serum, is inactive in our system (see Table 1). The discrepancy is likely due to qualitative or quantitative differences in the "natural" antibodies of rabbit and human sera.

The finding that one plasmid active in *E. coli* is inactive in the *S. typhimurium* host suggests that more than one mechanism can promote serum resistance. A possible interpretation of our findings is that some R factors code for enzymes that can modify the bacterial surface, using different structures as substrates; one of these substrates might be lacking in *S. typhimurium* TA1535.

TABLE 1. *Characteristics of the plasmids studied for their effect on the bactericidal activity of human serum*

Plasmid	Characteristic[a]									
	IG	fi	T	A	S	C	Su	K	Tm	Bhs
R386	FI	+	+	−	−	−	−	−	−	−
R1	FII	+	−	+	+	+	+	+	−	−
R124	FIV	+	+	−	−	−	−	−	−	−
RA1	A	−	+	−	−	−	+	−	−	−
R40a	C	−	−	+	−	−	+	+	−	−
R27	H	−	+	−	−	−	−	−	−	−
R144	Iα	−	+	−	−	−	−	+	−	−
R621a	Iγ	−	+	−	−	−	−	−	−	−
R391	J	−	−	−	−	−	−	+	−	−
R1066	K	−	+	−	+	+	+	−	−	−
R387	K	−	−	−	+	+	−	−	−	−
R471a	L	−	−	+	−	−	−	−	−	−
R446b	M	−	+	−	+	−	−	−	−	−
R67	N	−	−	−	−	−	−	+	+	−
RN3	N	−	+	−	+	−	+	−	−	+
pKM101	N	−	−	+	−	−	−	−	−	−
R16	O	−	+	+	+	−	+	−	−	+
RP4	P	−	+	+	−	−	−	+	−	−
R478	S	−	+	−	−	+	−	+	−	+
R-Ec1	S	−	−	−	+	+	+	−	+	+
Rts1	T	−	−	−	−	−	−	+	−	+
R401	T	−	−	+	+	−	−	−	−	−
RS-a	W	−	−	−	+	+	+	+	−	+
R7K	W	−	−	+	+	−	−	−	−	−
F′*lacW*										+
F′*lacC*										+

[a] IG, Incompatibility group; fi, fertility inhibition; T,A,S,C,Su,K,Tm, resistance to tetracyclin, ampicillin, streptomycin, chloramphenicol, sulfonamide, kanamicin, trimethoprim, respectively; Bhs, resistance to the bactericidal activity of human serum.

TABLE 2. *Quantitation of the increase in serum resistance conferred by plasmids in different hosts*

Plasmid	1% DR/D ratio[a] in host:		
	J6-2 *str*	C600 *rfm*	TA1535
RN3	1.57	1.48	1.40
R478	1.40	1.46	NA
R-Ec1	1.69	1.80	1.00
Rts1	2.37	2.19	2.18
RS-a	2.80	3.12	2.00

[a] 1% DR/D is the ratio of the serum doses necessary to reduce the viable count to 1% in R^+ and R^- strains; 1.00 = no difference. NA, Derivative not available.

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