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SALMONELLA TYPHIMURIUM STRAINS CARRYING HAEMOLYSIN PLASMIDS AND CLONED HAEMOLYSIN GENES FROM *ESCHERICHIA COLI*

by **J. Hacker** (1) (*), **H. Hof** (2), **C. Hughes** (1) (**) and **W. Goebel** (1)

(1) *Institut für Genetik und Mikrobiologie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg and,*
(2) *Institut für Hygiene und Mikrobiologie, Universität Würzburg, J.-Schneider-Str. 2, D-8700 Würzburg (Federal Republic of Germany)*

SUMMARY

Like all other *Salmonella typhimurium* strains examined, the smooth variants SF1397 (LT2) and 1366 and also their semi-rough and rough derivatives are non-haemolytic. Nevertheless, two haemolysin (Hly) plasmids of *E. coli* belonging to the *inc* groups *inc*$F_{III,IV}$ (pSU316) and *inc*I_2 (pHly152) were able to be introduced into these strains by conjugation and stably maintained. A considerable percentage of the Hly+ transconjugants obtained had lost parts of their O-side chains, a result of selection for the better recipient capability of « semi-rough » variants rather than the direct influence of the Hly+ plasmids themselves. In contrast to the *inc*$F_{III,IV}$ plasmid pSU316, which exhibited higher conjugation rates with rough recipients, the *inc*I_2 plasmid pHly152 was accepted best by smooth strains. Transformation with cloned *E. coli* haemolysin *(hly)* determinant was inefficient ($<10^{-6}$) for smooth strains, but 10^2-10^3 times higher for rough recipients, and was increased by the use of *Salmonella*-modified DNA. The transformants and transconjugants were relatively stable and showed the same haemolytic activity as the *E. coli* donor strains.

The virulence of the Hly+ smooth, semi-rough and rough *S. typhimurium* strains was tested in two mouse models, and neither the mortality rate nor the ability to multiply within the mouse spleen was influenced by the *hly* determinants.

KEY-WORDS: *Salmonella typhimurium*, Plasmid, Haemolysin, *Escherichia coli*, Virulence.

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(*) To whom correspondence should be sent.
(**) Present address: Dept. of Pathology, University of Cambridge, Tennis Court Road, Cambridge (England).

INTRODUCTION

The *Escherichia coli* haemolysin (Hly) is an extracellular protein toxin which is produced by about 40% of *E. coli* strains causing extraintestinal infections [17], such as those of the urinary tract (UTI). Among such strains, the haemolysin determinant *(hly)* is generally found on the chromosome, in contrast to strains of animal origin which often carry *hly* genes on transmissible plasmids [5, 33]. Plasmid and chromosomal *hly* determinants, which share high sequence homology [25], have been cloned and genetically analysed [1, 8, 27], and all comprise four genes involved in the synthesis (*hly*A and *hly*C) and transport (*hly*B_a and *hly*B_b) of the active haemolysin [37].

Recent investigations using cloned *hly* genes provide evidence for a direct contribution of *E. coli* haemolysin to the virulence of *E. coli* strains in different animal models ([11, 39] reviewed in [10]).

In addition to *E. coli*, enterobacterial Hly^+ strains are found in the genera *Proteus* and *Serratia*, but *Salmonellae* with haemolytic activity have never been detected in routine laboratory analyses [21]. While Hly plasmids have been transferred from *E. coli* to strains of *Salmonella typhimurium* and other species of the *Enterobacteriaceae*, in the case of *Salmonella*, the Hly^+ transconjugants were very unstable [21, 33].

S. typhimurium is the cause of systemic and enteric infections in many hosts [14, 19, 35] and the O antigen is the most important contributory factor to virulence, acting as an endotoxin and protecting the strains against the host defence system [14, 22, 29]. The O antigen consists of lipopolysaccharides (LPS) and can be divided into three main parts. The outer part, the O-side chain, in an *rfb*-encoded polysaccharide of repeating units. The core region consists of a specific polysaccharide which is determined by the *rfa*-gene cluster, and the inner part, lipid A, is responsible for the endotoxin effects of the O antigen. Mutations in various parts of the *rfa* cluster give rough mutants, and a defect in the *rfc* locus, which directs the polymerization of the O-side chains, gives semi-rough (SR) mutants (fig. 1 and [34, 40]).

In this study, we examine the inheritance of conjugative Hly plasmids and cloned *hly* genes by smooth, SR and rough variants of *S. typhimurium*, and we assess the effect of such inheritance on mouse virulence.

MATERIALS AND METHODS

Media and chemicals. — Cultures were grown in Luria broth (LB; 10 g Difco Bacto peptone; 5 g Difco yeast extract, 5 g NaCl per litre H_2O, pH 7.2) washed

LPS = lipopolysaccharide.
PBS = phosphate-buffered saline.
RTD = routine test dilution.
SPF = specifically pathogen-free.
SR = semi-rough (strain).
UTI = urinary tract infection.

and resuspended in phosphate-buffered saline (PBS). Agarose was obtained from Seakem (Sweden), antibiotics from Bayer Leverkusen (FRG) and *S. typhimurium*-specific sera from Behring-Werke, Marburg/Lahn (FRG). Human erythrocytes were obtained locally and all other chemicals were bought from Merck, Darmstadt (FRG).

Bacteria, plasmids and phages. — *S. typhimurium* strains used represent different O-antigen phenotypes. As shown in figure 1, smooth strains SF1397 (LT2) and 1366 exhibit the whole O antigen, repeating units, core and lipid A. Strain SF1512 is a semi-rough (SR) form, which can attach only one O-specific oligosaccharide unit to the R core because of a defect in the polymerase [34]; strains SF1592, SF1196 and SF1572, which exhibit different defects in the core polysaccharide, represent the Ra, Rc and Rd_1 chemotypes. All strains were resistant to streptomycin. The smooth wild-type strain 1366 was isolated in the Institut für Hygiene und Mikrobiologie, Würzburg, FRG ([14] and fig. 1); the other *Salmonella* strains were obtained from the Max-Planck Institut für Immunbiologie, Freiburg (FRG). As a control, *E. coli* K12 strain 33 (*nal*^{r}) was used [27]. All four haemolysin plasmids have been described in detail elsewhere [5] and the R plasmids TP114 and R124 are also listed in table I. The recombinant *hly*$^+$ plasmid pANN202-312 is a *Hin*dIII-*Sal*I fragment (9Kb) of pHly152, coding for the whole *hly* determinant, ligated into pACYC184 [8, 27]. Bacteriophages P22, FO, 6SR and C21 are specific for different O-antigen mutants and phage U3 for *E. coli* K12 strains.

Isolation of plasmid DNA. — Plasmid DNA from strains carrying recombinant DNA or conjugative plasmids was screened by the alkaline lysis procedure [2] and preparative DNA isolation was achieved as described previously [9].

Plasmid transfer. — Conjugations with Hly and R plasmids were performed on plates [27] and transformation of *S. typhimurium* and *E. coli* strains was achieved by a modified $CaCl_2$ cold-shock method [20]. Strains were checked for antibiotic resistance, Hly production and O antigen, the last by phage typing and agglutination with *S. typhimurium*-specific sera. Plasmid carriage was additionally controlled by electrophoresis of alkaline lysis extracts.

Phage propagation. — Phage were propagated on suitable hosts in broth and stored over chloroform at 4° C. The phages were used in routine test dilutions (RTD).

Assay for haemolysin activity. — Haemolysis was detected on blood agar plates (10 g Difco extract, 10 g Oxoid bacto peptone, 5 g NaCl, 60 ml washed erythrocytes per litre *aqua dest.*) and confirmed in a liquid assay [37].

Elimination of plasmids. — Hly plasmids were eliminated by treatment of cells with sodium dodecyl sulphate (0.02%; described in [15]).

Animal tests. — NMRI mice weighing 15-20 g (obtained from the Central Institute for Laboratory Animals, Hannover, FRG) were used in a specifically pathogen-free (SPF) state. In a mortality test, 10 mice per strain were injected intravenously with 2×10^6 bacteria and the number of mice dead between days 1 and 21 was recorded. In order to count the bacteria per spleen, mice were infected intravenously with 1×10^5 bacteria. At given intervals, animals were killed, the spleens removed aseptically and samples mixed with liquefied agar. The properties of bacterial strains reisolated from the spleens of mice were checked for the presence of covalently closed DNA, for plasmid-encoded markers (haemolysin production and/or chloramphenicol resistance) and for *Salmonella*-specific properties (LPS chemotype and streptomycin resistance).

RESULTS

1. — *Conjugal transfer of* E. coli *plasmids into* S. typhimurium *smooth strains.*

To evaluate the ability of Hly plasmids to infect *S. typhimurium* smooth strains, we performed conjugal tests with four plasmids of different size and *inc* group. As shown in table I, Hly$^+$ *S. typhimurium* LT2 transconjugants were obtained with 3 of the 4 plasmids. Only the *inc*F_{VI} Hly plasmid pSU105 was not able to infect the *S. typhimurium* strain. The *inc*F_{IV} plasmid pSU233 segregated: after 20 generations, only 10% of the transconjugants still produced haemolysin and the Hly$^-$ phenotype resulted from a loss of the plasmid, not from deletion. In contrast to pSU105 and pSU233, the plasmids pSU316 (*inc*$F_{III,IV}$) and pHly152 (*inc*I_2) gave rise to stable transconjugants following transfer to LT2. The transfer rates of the Hly plasmids were nearly the same as those obtained with the R-plasmids TP114 and R124, which belong to the same *inc* groups as pHly152 and pSU316. Similar results were obtained following transfer of the different plasmids into the *Salmonella* smooth strain 1366 (data not shown).

TABLE I. — **Transfer of plasmids from « E. coli » K12 into « S. typhimurium » smooth strain SF1397 (LT2).**

	Plasmid					
	pHly152	pSU316	pSU105	pSU233	TP114	R124
inc-group of plasmid	*inc*I_2	*inc*$F_{III,IV}$	*inc*F_{VI}	*inc*F_{IV}	*inc*I_2	*inc*F_{IV}
Selective marker	Hly$^+$	Hly$^+$	Hly$^+$	Hly$^+$	Km^r	Tc^r
Transfer frequency (1)	40	8	—	12	35	50
Segregation of plasmid (2)	<1 %	<1 %	—	91 %	<1 %	10 %
Smooth recipients (%) (3)	65	85	—	100	100	100

(1) Transconjugants/donor cell $\times 1 \times 10^{-6}$.
(2) % segregation following 20 generations without selective presure, 1,000 colonies tested.
(3) Indicated by P22 phage lysis, 100 colonies tested; P22-resistant strains have been termed « SR ».

2. — S. typhimurium *O-antigen mutants as recipients for different conjugative plasmids.*

The *Salmonella* smooth strains 1397 (LT2) and 1366 and the 4 well-defined R mutants SF1512 (SR), SF1592 (Ra), SF1196 (Rc) and SF1572 (Rd_1; see fig. 1) were crossed with *E. coli* K12 strains bearing different Hly$^+$ and R$^+$ plasmids. As demonstrated in figure 2, the mutations in LPS have an influence on the transfer frequency of Hly$^+$ plasmids. Compared with the smooth strain SF1397, the SR strain SF1512 and the R mutants were

better recipients of the Hly+ plasmids which belong to the *inc*F family, the semi-rough strain being the best. In contrast, the transfer frequency of *inc*I_2 plasmid pHly152 decreased when SR or rough strains were used as recipients. The *inc*F and *inc*I_2 Hly+ plasmids gave the same transfer rates as the R factors used as controls.

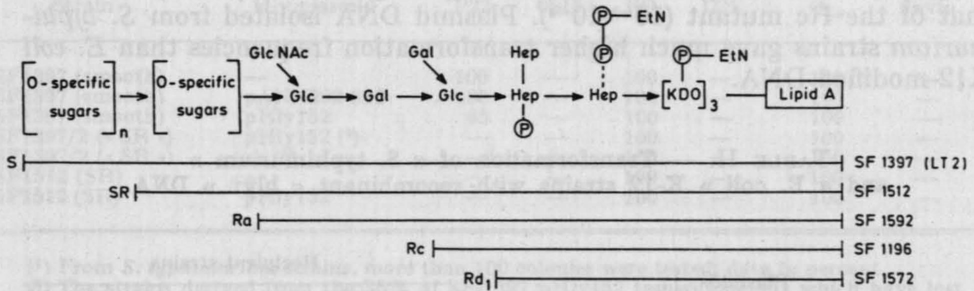

FIG. 1. — *Structure of the O antigen of* S. typhimurium *and mutant strains.*

The smooth strains SF1397 (LT2) and 1366 exhibit all surface sugar components; the semi-rough strain 1512 has only one repeating unit. The rough mutants SF1592 (Ra), SF1196 (Rc) and SF1572 (Rd_1) have lost these side chains and contain progressive defects in the structure of the core polysaccharide.

Abbreviations are as follows: GlcNAc = N-acetylglucosamine; Glc = glucose; Gal = galactose; Hep = heptose; P = phosphate residue; EtN = ethanolamine; KDO = 2-keto-3-deoxy-octane.

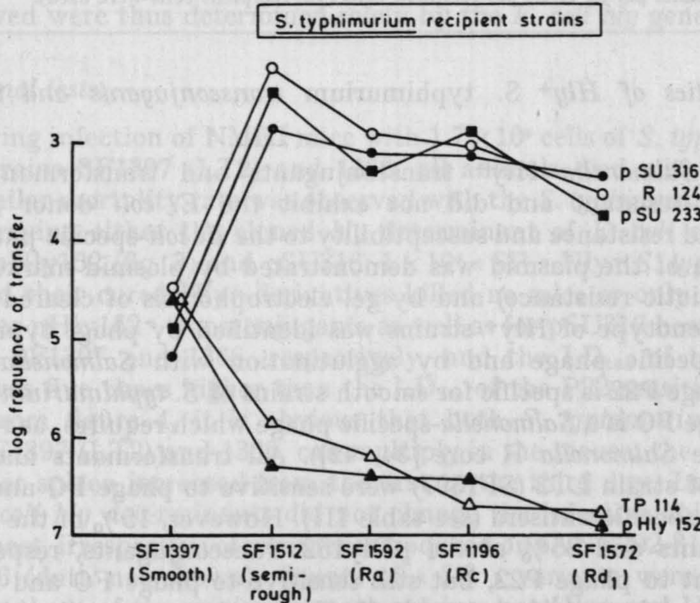

FIG. 2. — *Transfer frequencies of the Hly plasmids pHly152* (incI_2), *pSU316* (inc$F_{III,IV}$) *pSU233* (inc?) *and the R-plasmids TP114* (incI_2) *and R124* (inc$_{IV}$) *between* E. coli *K12 and* S. typhimurium *strains.*

The *S. typhimurium* recipients represent different O-antigen phenotypes.

3. — *Transformation of the cloned* hly *determinant into smooth and rough strains of* S. typhimurium.

The recombinant *hly*+ plasmid pANN202-312 was transformed into *S. typhimurium* strains SF1397 (smooth), 1366 (smooth) and the Rc mutant SF1196. As can be seen in detail in table II, the transformation frequency of the smooth strains was very low (10^{-5}-10^{-6}) compared with that of the Rc mutant (10^{-2}-10^{-4}). Plasmid DNA isolated from *S. typhimurium* strains gave much higher transformation frequencies than *E. coli* K12-modified DNA.

TABLE II. — Transformation of « S. typhimurium » and « E. coli » K-12 strains with recombinant « hly+ » DNA.

Recombinant plasmid	Plasmid-borne genes	Source of plasmid DNA	Recipient strains: SF1397 (smooth)	1366 (smooth)	SF1196 (Rc)	*E. coli* K12
pANN202-312	*hly*C,A,B_a,B_b	*E. coli* K12 (5K)	—	—	5×10^{-5} (1)	5×10^{-3}
pANN202-312	*hly*C,A,B_a,B_b	*S. typhimurium* (SF1196, Rc)	8×10^{-6}	3×10^{-6}	6×10^{-3}	3×10^{-3}

(1) Transformants per µg DNA per recipient cells (10^8 recipient cells were used).

4. — *Properties of Hly*+ S. typhimurium *transconjugants and transformants.*

All the *Salmonella* Hly+ transconjugants and transformants were streptomycin-resistant and did not exhibit the *E. coli* donor markers (nalidixic acid resistance and susceptibility to the *E. coli*-specific phage U3). The presence of the plasmid was demonstrated by plasmid markers (Hly and/or antibiotic resistance) and by gel electrophoresis of cleared lysates. The LPS phenotype of Hly+ strains was identified by phage typing with *Salmonella*-specific phage and by agglutination with *Salmonella*-specific antisera (phage P22 is specific for smooth strains of *S. typhimurium* (04 and 12) and phage FO is a *Salmonella*-specific phage which requires, as receptor, the complete *Salmonella* R core [34, 42]). All transformants and transconjugants of strain LT2 (SF1397) were sensitive to phage FO and agglutinated in O-specific antisera (see table III). However, 15% of the pSU316 transconjugants and 35% of the pHly152 transconjugants, respectively, were resistant to phage P22, but still sensitive to phage FO and reactive in O-specific antisera (also see table I). The strains were still resistant to the rough specific phage 6SR, *i. e.*, they showed the same phage pattern as the SR strain SF1512. After curing the Hly plasmids, the « SR » strains retained resistance to P22 and the phage pattern of the various rough

TABLE III. — **Phage pattern and agglutination of Hly⁻ and Hly⁺ « S. typhimurium » strains.**

Strain	Hly plasmid	Phage lysis (1)				Agglutination	
		P22	6SR	FO	C21	$O_{4,5}$	Trypoflavin
SF1397 (smooth)	—	100	—	100	—	100	—
SF1397 (smooth)	pANN202-312	100	—	100	—	100	—
SF1397 (smooth)	pHly152	65	—	100	—	100	—
SF1397/2 (« SR »)	pHly152 (2)	—	—	100	—	100	—
SF1397/2 (« SR »)	— (3)	—	—	100	—	100	—
SF1512 (SR)	—	—	—	100	—	100	—
SF1512 (SR)	pHly152	—	—	100	—	100	—

(1) From *S. typhimurium* strains, more than 100 colonies were tested; data in percent.
(2) The strains derived from the 35 % of SF-1397 pHly152 transconjugants which have lost P22 sensitivity.
(3) Strains were cured from plasmid pHly152.

strains was indistinguishable for Hly^+ and Hly^- variants (table III). Southern hybridization (Hacker and Knapp, unpublished results) revealed that *Salmonella* strains used in virulence tests (see below) did not bear silent haemolysin sequences on their chromosome. The haemolytic activities observed were thus determined solely by the *E. coli hly* genes.

5. — *Animal tests.*

Following infection of NMRI mice with 1.7×10^6 cells of *S. typhimurium* smooth strains SF1397 (LT2) and 1366, all animals died within 6 days. A very similar mortality rate was observed with the *S. typhimurium* smooth strains carrying either the cloned *hly* determinant of *E. coli* or the Hly plasmids pHly152 (fig. 3) and pSU316: 1×10^6 « SR » Hly^+ *S. typhimurium* strains and their cured Hly^- derivatives killed no mice or only 10 %. This was true for pHly152⁺ transconjugants as well as for pSU316 bearing « SR » strains of SF1397 and 1366, respectively, and the LD_{100} of the « SR » variants was five times higher than the LD_{100} of the P22-sensitive smooth strains. From figure 4, it is obvious that both *S. typhimurium* smooth strains, SF1397 (LT2) and 1366, can multiply in the mouse; the number of bacteria per spleen increased from the first to the third day. Introduction of the *E. coli hly* determinants did not change the infective ability of the bacterial host, irrespective of whether introduced on pANN202-312, pHly152 or pSU316 (data not shown). Counts of « SR » variants were 100 times lower than those of the original smooth strains, but Hly^+ and Hly^- « SR » strains were both able to survive in the mouse, and both reached a titre of 10^5 bacteria per spleen on the sixth day.

Hly^+ and Hly^- variants of the SR strain SF1512 and the rough strains

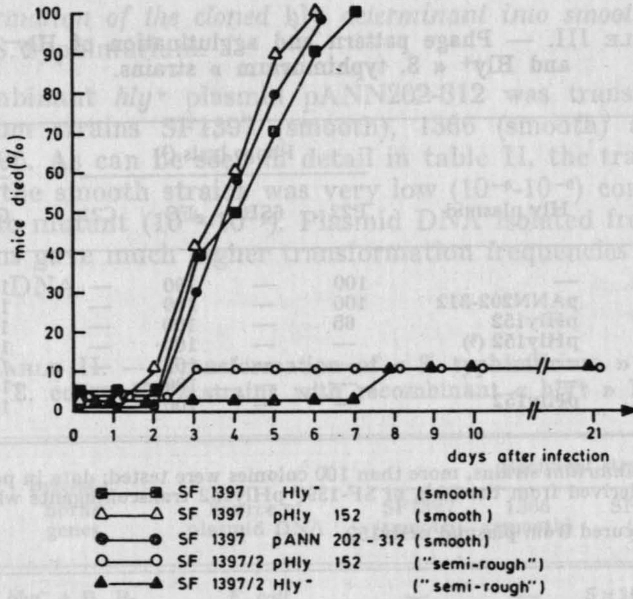

FIG. 3. — *Mortality rates of NMRI mice infected with 1.7×10^6 cells of the Hly⁻* S. typhimurium *smooth strain 1397 (LT2) and its Hly⁺ and « semi-rough » derivatives.*

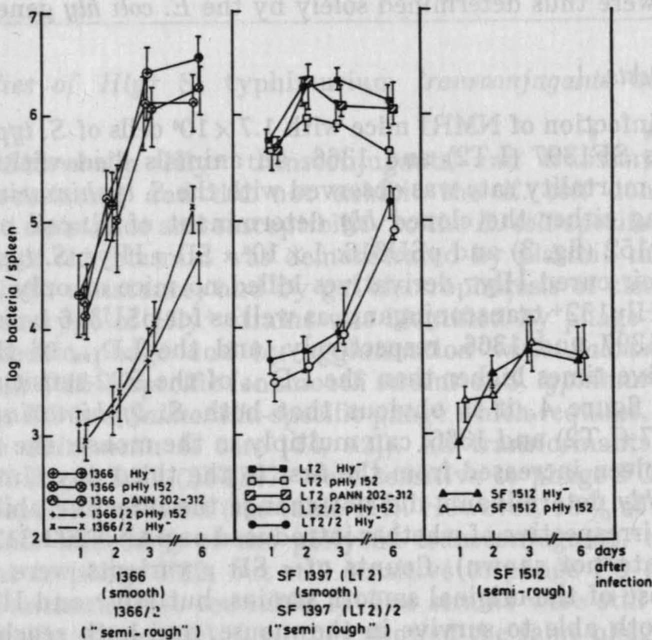

FIG. 4. — *Bacterial counts per spleen of Hly⁻ and Hly⁺ variants of* S. typhimurium *strains 1366 (smooth), 1397 (LT2, smooth) and SF1512 (semi-rough).*

SF1592 (Ra), SF1196 (Rc) and SF1572 (Rd_1) were also indistinguishable in both assays (data not shown). Thus, the *E. coli hly* determinant had no additional effect on the mouse virulence of *S. typhimurium* strains.

DISCUSSION

In this study, we have introduced the *E. coli* virulence determinant *hly* into *S. typhimurium* via conjugation of Hly plasmids and transformations of recombinant DNA. The efficiency of transformation of the *hly* determinant depended on the source of DNA and on the outer membrane composition of the recipient. Transformation of *E. coli hly* genes was nearly 100 times higher when DNA was isolated from an *S. typhimurium* host. In contrast to Lederberg and Cohen [20], we found rough *gal*E mutant (Rc) to be a better recipient for transformation than the smooth LT2 strain, and similar results have been reported for both plasmid [23, 31] and phage P22 [4] DNA, where rough strains were also better recipients than their smooth counterparts (with the exception of Re mutants). The composition of the outer membrane also drastically influences the conjugal transfer of plasmids between strains of *E. coli* belonging to different serogroups [16]. In the present study, the transfer frequencies of F plasmids from *E. coli* K12 to rough *S. typhimurium* during conjugation was increased compared with smooth recipients. Similar results were obtained by Watanabe *et al.* [38] and Sanderson *et al.* [30] in studies of *S. typhimurium*, and also from workers describing *S cholerae-suis* var. *kunzendorf*, *S. minnesota* and *E. coli* recipients [6, 18, 41].

In contrast to the *inc*F plasmids, transfer of the *inc*I_2 Hly plasmid pHly152 and the *inc*I_2 R factor TP114 was reduced when rough strains were used as recipients. The same observation has been made with the *inc*I R-plasmid R64drd11 [6] and, whatever the reason, it seems clear that *inc*I plasmids, unlike those of *inc*F, do not use the *omp*A protein as receptor in conjugation events [13]. The presence of the outer part of the LPS on the recipient cell may promote the transfer of *inc*I plasmids.

In this paper, we have shown that the *E. coli* haemolysin, stably expressed, has no influence on the virulence of *S. typhimurium* strains. Smith and Halls [33] also found that Hly plasmids from *E. coli* did not contribute to *Salmonella* virulence following subcutaneous application of strains into mice, but after nine days, more than 95% segregation was observed and this clearly influenced the outcome of the test. We were able to eliminate this problem by obtaining stable Hly^+ transformants and transconjugants.

We also found « SR » Hly^+ variants with a lower virulence than the original SF1397 and 1366 smooth strains. The fact that the strains do not regain their virulence following elimination of the Hly plasmids excludes the possibility that the Hly plasmid itself determines a reduction in virulence, *e. g.* via a direct influence on the biosynthesis of the LPS as described for the derepressed ColIb plasmid [15]. The phage and agglutination tests suggest, rather, that there is a selection for such « SR » forms, strains with

reduced O-side-chains [24] which are better recipients in conjugal transfer noted. Such selection of non-smooth forms has also been observed following R-plasmid transfer from *E. coli* into several strains of *Salmonella* [18, 32, 36]. In all cases, transconjugants had a much lower virulence, and the reason was not the carriage of the R factor *per se*, but rather a selection for the better recipient ability of rough forms. The R factors themselves had a negligible effect [3, 32, 36] on the virulence of *S. typhimurium*. This is also the case following introduction of *E. coli hly* genes, regardless of whether carried on transmissible plasmids or an multicopy cloning vectors.

Introduction of cloned virulence determinants into *Salmonella* is now of particular interest in view of possible multivalent protection afforded by live vaccine strains. Similar experiments have been performed in transferring the *Shigella sonnei* virulence plasmid [7] and the cloned *E. coli* S-fimbriae determinant [12] into an *S. typhimurium galE* mutant and introducing cloned *Klebsiella* common type I fimbriae [28] and *E. coli* LT enterotoxin plasmids [26] into *S. typhimurium* smooth strains. As with the *E. coli hly* determinants, all these foreign determinants were readily expressed in the *Salmonella* hosts.

RÉSUMÉ

SOUCHES DE « SALMONELLA TYPHIMURIUM » PORTEUSES DE PLASMIDES D'HÉMOLYSINE ET DE GÈNES D'HÉMOLYSINE CLONÉS PROVENANT DE « ESCHERICHIA COLI »

Comme toutes autres souches de *Salmonella typhimurium* examinées, les variantes « smooth » SF1397 (LT2) et 1366 ainsi que leurs dérivés « rough » se sont avérés non hémolytiques. Toutefois deux plasmides de *Escherichia coli* codant pour la production d'une hémolyse (Hly) et appartenant aux groupes d'incompatibilité *inc*$F_{III,IV}$ (pSU316) ou *inc*I_2 (pHly152) ont pu être introduits dans ces souches par conjugaison, ou se maintiennent stablement. La plupart des souches hémolytiques ont perdu une portion de la chaîne latérale de leur antigène O. Cela résulte plus d'une sélection des souches « semi-rough », qui apparemment acceptent mieux les plasmides, que d'une influence directe des plasmides Hly sur l'expression de l'antigène O. Alors que le plasmide pSU316 manifeste une meilleure affinité pour les souches « rough » que pour les souches « smooth », le plasmide pHly152 est accepté plus facilement par des souches « smooth ». La transformation des souches « smooth » de *Salmonella* avec les déterminants de l'hémolysine de *E. coli* clonés est assez inefficace, 10^{-6}; par contre, celles des souches « rough » est 10^2 à 10^3 fois plus efficace. Par ailleurs, ces expériences réalisées à partir d'ADN modifié, extrait de *Salmonella*, montrent une augmentation de la fréquence des souches hémolytiques. Le caractère hémolytique de *E. coli* s'exprime avec une activité identique chez les souches de *Salmonella* obtenues par transformation ou par conjugaison.

La virulence de *Salmonella* hémolytique (smooth, semi-rough et rough) a été examinée chez la souris. Ni la mortalité, ni la multiplication dans la rate ne sont influencées par le caractère hémolytique.

MOTS-CLÉS : *Salmonella- typhimurium*, *Escherichia coli*, Plasmide, Hémolysine, Virulence.

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REFERENCES

[1] BERGER, H., HACKER, J., JUAREZ, A., HUGHES, C. & GOEBEL, W., Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in *Escherichia coli*. *J. Bact.*, 1982, 152, 1241-1247.

[2] BIRNBOIM, H. C. & DOLY, J., A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.*, 1979, 7, 1513-1523.

[3] BRANA, H. & HUBACEK, J., Virulence of *Salmonella typhimurium* infected with the R-plasmid and relation with the *rec*-mutation. *Folia microbiol.*, 1977, 22, 451.

[4] BURSZTYN, H., SGARAMELLA, V., CITERRI, O. & LEDERBERG, J., Transfectability of rough strains of *Salmonella typhimurium*. *J. Bact.*, 1975, 124, 1630-1634.

[5] DE LA CRUZ, F., MÜLLER, D., ORTIZ, J. M. & GOEBEL, W., Hemolysis determinant common to *Escherichia coli* hemolytic plasmids of different incompatibility groups. *J. Bact.*, 1980, 143, 825-833.

[6] DUKE, J. & GUINEY, D. G. Jr, The role of lipopolysaccharide structure in the recipient cell during plasmid-mediated bacterial conjugation. *Plasmid*, 1983, 99, 222-226.

[7] FORMAL, S. B., BABON, L. S., KOPECKO, D. J., WASHINGTON, O., POWELL, C. & LIFE, C. A., Construction of a potential bivalent vaccine strain: introduction of *Shigella sonnei* form I antigen genes into the *gal*E *Salmonella typhi* Ty2a typhoid vaccine strain. *Infect. Immun.*, 1981, 34, 746-750.

[8] GOEBEL, W. & HEDGPETH, J., Cloning and characterization of the entire plasmid-encoded hemolysin determinant of *Escherichia coli*. *J. Bact.*, 1982, 151, 1290-1298.

[9] GRINSTEDT, J., BENNETT, P. M., HIGGINSON, S. & RICHMOND, M. H., Regional preference of insertion of Tn501 and Tn802 into RP1 and its derivatives. *Mol. gen. Genetics*, 1978, 166, 313-320.

[10] HACKER, J. & HUGHES, C., Genetics of *Escherichia coli* hemolysin. *Curr. Top. Microbiol. Immunol.*, 1985 (in press).

[11] HACKER, J., HUGHES, C., HOF, H. & GOEBEL, W., Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. *Infect. Immun.*, 1983, 42, 57-63.

[12] Hacker, J., Schmidt, G., Hughes, C., Knapp, S., Marget, M. & Goebel, W., Cloning and characterization of genes involved in the production of mannose-resistant, neuraminidase-susceptible (X) fimbriae from an uropathogenic O6:K15:H31 *E. coli* strain. *Infect. Immun.*, 1985, **47**, 434-440.

[13] Havekes, L., Tommassen, J., Hoekstra, W. & Lugtenberg, B., Isolation and characterization of *Escherichia coli* K-12 F-mutants defective in conjugation with an I-type donor. *J. Bact.*, 1977, **129**, 1-8.

[14] Hof, H., Emmerling, P., Hacker, J. & Hughes, C., The role of macrophage in primary and secondary infection of mice with *Salmonella typhimurium*. *Ann. Immunol. (Inst. Pasteur)*, 1982, **133 C**, 21-32.

[15] Hoffmann, J., Lindberg, B., Glowacka, M., Derylo, M. & Lorkiewicz, Z., Structural studies of the lipopolysaccharide from *Salmonella typhimurium* 902 (ColIb drd 2). *Europ. J. Biochem.*, 1980, **105**, 103-107.

[16] Hughes, C., Bauer, E. & Roberts, A. P., Spread of R-plasmids among *Escherichia coli* causing urinary tract infections. *Antimicrob. Agents a. Chemother.*, 1981, **20**, 496-502.

[17] Hughes, C., Hacker, J., Roberts, A. & Goebel, W., Hemolysin production as a virulence marker in urinary tract infections caused by *Escherichia coli*. *Infect. Immun.*, 1983, **39**, 546-551.

[18] Jarolman, H. & Kemp, G., Association of increased recipient ability for R-factors and reduced virulence among variants of *Salmonella choleraesuis* var. *kunzendorf*. *J. Bact.*, 1969, **97**, 962-963.

[19] Kühn, H., Tschäpe, H. & Rische, H., Enterotoxigenicity among *Salmonella* — a prospective analysis for surveillance programme. *Zbl. Bakt.*, I. Abt. Orig., 1978, **A 240**, 171-183.

[20] Lederberg, E. M. & Cohen, S. N., Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. *J. Bact.*, 1974, **119**, 1072-1074.

[21] Le Minor, S. & Le Coueffic, E., Studies of haemolysins of *Enterobacteriaceae*. *Ann. Microbiol. (Inst. Pasteur)*, 1975, **126 B**, 131-332.

[22] Lyman, M.-B., Steward, J. P. & Roantree, R. J., Characterization of virulence and antigenic structure of *Salmonella typhimurium* strains with lipopolysaccharide core defects. *Infect. Immun.*, 1976, **13**, 1539-1545.

[23] MacLachlan, P. R. & Sanderson, K. E., Transformation of *Salmonella typhimurium* with plasmid DNA: differences between rough and smooth strains. *J. Bact.*, 1985, **161**, 442-445.

[24] McConnell, M. R. & Schoelz, J. E., Evidence for shorter average O-polysaccharide chain length in the lipopolysaccharide of a bacteriophage felix 01-sensitive variant of *Salmonella anatum* A1. *J. gen. Microbiol.*, 1983, **129**, 3177-3184.

[25] Müller, D., Hughes, C. & Goebel, W., Relationship between plasmid and chromosomal hemolysin determinants of *Escherichia coli*. *J. Bact.*, 1983, **153**, 846-851.

[26] Neill, R. J., Twiddy, E. M. & Holmes, R. K., Synthesis of plasmid-coded heat-labile enterotoxin in wild-type and hypertoxigenic strains of *Escherichia coli* and other genera of *Enterobacteriaceae*. *Infect. Immun.*, 1983, **41**, 1056-1061.

[27] Noegel, A., Rdest, U. & Goebel, W., Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. *J. Bact.*, 1981, **145**, 233-247.

[28] Purcell, B. K. & Clegg, S., Construction and expression of recombinant plasmids encoding type I fimbriae of a urinary *Klebsiella pneumoniae* isolate. *Infect. Immun.*, 1983, **39**, 1122-1127.

[29] Roantree, R. J., The relationship of lipopolysaccharide structure to bacterial virulence, *in* « Bacterial endotoxins » (S. Kadis, G. Weinbaum & S. J. Ajil), vol. *V* (p. 1-32). Academic Press, New York, London, 1971.

[30] Sanderson, K. E., Janzer, J. & Heat, J., Influence of lipopolysaccharide and protein in the cell envelope on recipient capacity in conjugation of *Salmonella typhimurium. J. Bact.*, 1981, **148**, 282-293.

[31] Sanderson, K. E. & Roth, J. R., Linkage map of *Salmonella typhimurium*, edition VI. *Microbiol. Rev.*, 1983, **47**, 410-453.

[32] Smith, H. W., The effect on virulence of transferring R-factors to *Salmonella typhimurium in vivo. J. med. Microbiol.*, 1972, **5**, 451-458.

[33] Smith, H. W. & Halls, S., The transmissible nature of the genetic factor in *Escherichia coli* that controls haemolysin production. *J. gen. Microbiol.*, 1967, **47**, 153-167.

[34] Stocker, B. A. D. & Mäkelä, H., Genetic aspects of biosynthesis and structure of *Salmonella* lipopolysaccharide, « Bacterial endotoxins » (S. Kadis, C. Weinbaum & S. J. Ajil), vol. *IV* (p. 369-439). Academic Press, New York, London, 1971.

[35] Takeuchi, A., Electron microscope observations on penetration of the gut epithelial barrier by *Salmonella typhimurium*, *in* « Microbiology 1979 » (D. Schlessinger) (p. 174-181).ASM, Washington, 1979.

[36] Thiele, E. H., Virulence of R-factor-bearing *Salmonella typhimurium. Infect. Immun.*, 1970, **2**, 516-518.

[37] Wagner, W., Vogel, M. & Goebel, W., Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. *J. Bact.*, 1983, **154**, 200-210.

[38] Watanabe, T., Arai, T. & Hattori, T., Effects of cell wall polysaccharide on the mating ability of *Salmonella typhimurium. Nature* (Lond.), 1970, **225**, 70-71.

[39] Welch, R. A., Dellinger, E. P., Mishew, B. & Falkow, S., Hemolysin contributes to virulence of extraintestinal *E. coli* infections. *Nature* (Lond.), 1981, **294**, 665-667.

[40] Westphal, O., Jann, K. & Himmelspach, K., Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. *Progr. Allergy*, 1983, **33**, 9-39.

[41] Wiedemann, B. & Schmidt, G., Structure and recipient ability in *E. coli mutants. Ann. N. Y. Acad. Sci.*, 1971, **182**, 123-125.

[42] Wilkinson, R. G., Gemski, P. & Stocker, B. A. D., Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. *J. gen. Microbiol.*, 1972, **70**, 527-554.