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Salmonella typhimurium Tn*phoA* mutants with increased sensitivity to biological and chemical detergents

F.J.-C. Lacroix [1], C. Avoyne [1], C. Pinault [1], M.Y. Popoff [2] and P. Pardon [1] (*)

[1] *INRA, Centre de Tours-Nouzilly, Pathologie Infectieuse et Immunologie, 37380 Nouzilly (France), and*
[2] *Institut Pasteur, Unité des Entérobactéries, Unité INSERM 389, 75724 Paris Cedex 15*

SUMMARY

***Salmonella typhimurium* is a ubiquitous pathogenic bacterium able to sustain the environmental conditions of the gastrointestinal tract, including biliary salts. To understand the mechanisms involved in bile salt resistance and, more generally, detergent resistance, we investigated *S. typhimurium* mutants produced with the random mutagenic Tn*phoA* transposon. A total of 3,000 transpositional mutants were isolated. Three strains among the 1,432 first mutants lost the ability to grow in the presence of biological and chemical detergents. They were prototrophic and exhibited normal lipopolysaccharide and outer membrane protein profiles after SDS-PAGE. They did not show sensitivity to dyes but showed very different sensitivities to antibiotics. For each mutant strain, Southern blotting analysis revealed a unique Tn*phoA* insertion at different chromosomal locations. These observations were confirmed by transduction experiments.**

Key-words: Bile salt, Detergent, *Salmonella typhimurium;* Mutants, Transpositional mutagenesis, Detergent and antibiotic resistance, OMP, LPS, Tn*phoA*.

INTRODUCTION

Ubiquitous pathogenic *Salmonella* are able to colonize the host intestinal tract and tissues and can survive or multiply in the external environment. During their propagation, the *Salmonella* are exposed to radical changes while retaining their pathogenic potential. In the gastrointestinal tract, orally ingested *Salmonella* are notably resistant to many conditions generally hostile to other non-indigenous bacteria. Low pH, intestinal microflora, anaerobiosis and host-produced substances such as mucus, antimicrobial peptides, proteases, antibodies and biliary salts are the main factors which *Salmonella* are able to resist. In bacteriology, biliary salts such as sodium deoxycholate are used in selective media for the isolation of Gram-negative enteric pathogens. Such resistance to biliary salts could give a selective advantage to *Salmonella* in the intestinal tract and the bile-secreting apparatus (Hentges, 1983; Rolfe, 1991; Savage, 1977), but relatively little is known about how they survive (Nickerson and Aspedon, 1992).

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(*) Corresponding author.

Wild-type *Enterobacteriaceae* and particularly *S. typhimurium* are known to be resistant to detergents, while Gram-positive bacteria are sensitive and are killed, even at low concentrations. This differing behaviour of Gram-negative and Gram-positive bacteria can be explained by the presence of an outer membrane in the former which constitutes a selective barrier limiting the intracellular entry of many drugs (antibiotics, dyes, detergents, etc), and allowing only the permeation of hydrophilic molecules (Vaara and Nikaido, 1984).

The isolation of mutants altered in resistance to biliary salts constitutes an approach to the study of the intrinsic resistance of *Salmonella* to biological detergents. Many mutants were isolated by chemical mutagenesis or were found as byproducts in studies searching for other kinds of mutations. Mainly *Escherichia coli* and a few *S. typhimurium* mutants sensitive to hydrophobic antibiotics or detergents have been described, but the mutations generally identified were those affecting the lipopolysaccharide (LPS) structure (Nikaido, 1976; Roantree *et al.*, 1977) or outer membrane protein (OMP) synthesis (Schweizer *et al.*, 1976; Sukupolvi and Vaara, 1989). Other mutations were primarily associated with alteration of cell division (Antón and Orce, 1976; Klein *et al.*, 1990; Normark, 1969), growth rate (Nikaido, 1976), cell shape (Antón, 1978; Antón and Orce, 1976), induction of autolysis (Antón, 1978; Antón, 1981; Antón and Orce, 1976), release of periplasmic enzymes (Normark, 1969), sensitivity to diverse agents like hydrophobic and hydrophilic antibiotics (Antón, 1978; Klein *et al.*, 1990), EDTA (Antón and Orce, 1976; De Zwaig and Luria, 1967), basic dyes (De Zwaig and Luria, 1967; Klein *et al.*, 1990), lysozyme (Klein *et al.*, 1990) and UV light (Antón, 1978; Antón, 1981) and have been described as also affecting resistance to detergents. However, pleiotropism of envelope mutations is not surprising when taking into account the complexity of the envelope of Gram-negative bacteria (Cronan *et al.*, 1987; Nikaido and Vaara, 1987).

The work reported in this paper was initiated with the purpose of finding bacterial mutants that could be specifically altered in detergent resistance. We present a description of Tn*phoA* mutants of *S. typhimurium* which showed increased sensitivity to different biological and chemical detergents. We have characterized the phenotypic differences between parental and mutant strains and described the influence of the transpositional mutations upon viability, growth and both the detergent and antibiotic sensitivity of *S. typhimurium*.

MATERIALS AND METHODS

Media, chemicals and enzymes

The media used for cultivation of the bacteria were trypticase soy broth (TSB) (Bio Mérieux, Marcy l'Etoile, France) or trypticase soy agar (TSA) plates (Bio Mérieux) and Luria-Bertani medium (LB) containing: tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl (Merck) and distilled water, 1 l. *Salmonella-Shigella* medium (Diagnostics Pasteur) and minimal broth medium (M9) (Sambrook *et al.*, 1989) were used for selection of the transposon recipient strain and screening of the

Amp = ampicillin.
CDT = capacitance detection time.
CFU = colony-forming unit.
CHAPS = 3[(3-cholamidopropyl)-dimethylammonio]1-propanesulphate.
CHO = cholic acid.
CTAB = cetyltrimethyl ammonium bromide.
DHCHO = dehydrocholic acid.
DOC = deoxycholic acid.
Kan = kanamycin.
LB = Luria-Bertani (medium).
LPS = lipopolysaccharide.
MIC = minimal inhibitory concentration.
NP40 = Nonidet-P40.
OMP = outer membrane protein.
PAGE = polyacrylamide gel electrophoresis.
SDS = sodium dodecyl sulphate.
Sm = streptomycin.
TAU = taurocholic acid.
TBS = Tris-buffered saline.
TSA = trypticase soy agar.
TSB = trypticase soy broth.
TX100 = Triton-X100.
TX114 = Triton-X114.

mutant strains. The antibiotics (Sigma Chemical Co., St Louis, MO, USA) ampicillin (50 μg/ml; Amp), streptomycin (500 μg/ml; Sm) and kanamycin (50 μg/ml; Kan) were used for selection. Detergents were added to the nutrient agar for testing the sensitivity of the mutants: bovine bile and biliary acids used in sodium salts form [deoxycholic acid (DOC), taurocholic acid (TAU), cholic acid (CHO), dehydrocholic acid (DHCHO)] and chemical detergents represented by 3[(3-cholamidopropyl)-dimethylammonio]1-propanesulphate (CHAPS), Triton-X100 (TX100), Triton X114 (TX114), sodium dodecylsulphate (SDS), cetyltrimethyl ammonium bromide (CTAB) and Nonidet-P40 (NP40). All these detergents were purchased from Sigma, except NP40 (Fluka Bio Chemica, St Quentin Fallavier, France) and CHAPS (Serva; St Germain en Laye, France). Two-fold serial dilutions of the detergents were made with a concentration ranging from 16 to 0.5 g/l for the biological compounds and 16 to 3.9×10^{-3} g/l for the chemical detergents. To facilitate sterilization by filtration, the maximal detergent concentration did not exceed 16 g/l. Restriction enzymes (*Eco*RI, *Sac*I, *Hin*dIII) were purchased from Appligene (Illkirch, France).

Bacterial strains, bacteriophage and cultivation

S. typhimurium C5 (4511-2) strain (Furness and Rowley, 1956) was a gift from the Laboratory of *Enterobacteriaceae* of the Institut Pasteur, Paris. This prototrophic mouse virulent strain with a 90-kb plasmid (pIP1350) (Popoff *et al.*, 1984) was used as the parental strain to select a spontaneous Sm-resistant mutant called T39. For transduction experiments, we used *S. typhimurium* strain C5(3)s which is a spontaneous mutant resistant to Sm, obtained from the parental C5(3) strain (Pardon *et al.*, 1986). The latter is an isogenic variant of the C5 (4511-2) strain cured for the virulence-associated plasmid.

E. coli K12 strain SM10 (*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Kan λ *pir*) (Simon *et al.*, 1983) harbouring the suicide plasmid pRT733 (Miller and Mekalanos, 1988) carrying Tn*phoA* (*oriR6K* Tra^- Mob^+ Amp^r Kan^r) was used in random mutagenesis experiments (Manoil and Beckwith, 1985).

Bacteriophage P22 HT 105.1/int (P22) (Hoiseth and Stocker, 1985) used in transduction experiments was obtained from the Institut Pasteur, Paris.

Generation of *S. typhimurium*::Tn*phoA mutants*

Tn*phoA* was introduced into *S. typhimurium* T39 on the suicide vector pRT733 by conjugation with the donor strain SM10 (Taylor *et al.*, 1989). Transconjugants were selected for antibiotic resistance, since the background phosphatase activity of parent colonies made selection of Tn*phoA* mutants virtually impossible. They were selected for Sm resistance carried by the recipient *S. typhimurium*, Kan resistance carried by the transposon and checked for Amp sensitivity, indicating loss of pRT733. No more than one colony was selected from an individual conjugation.

Screening of *S. typhimurium*::Tn*phoA* mutants

Mutant strains were selected for their sensitivity to biliary agents by use of the two following different culture conditions: TSA (Kan, Sm) containing bile or bile salts at different final concentrations and TSA (Kan, Sm) without detergent, incubated at 37°C. Minimal inhibitory concentrations (MIC) were determined, and growth of sensitive selected strains in TSB with either DOC, CHAPS or SDS (8 g/l) was measured (OD_{600}).

The bacterial sensitivities in liquid medium were further tested by monitoring microbial metabolic activities by the mediation of electrical capacitance (Firstenberg-Eden and Eden, 1984) using a "Bactometer" (BioMérieux) and sterile disposable 16-well modules. Two-fold serial dilutions of detergents were realized in distilled water and the detergents were added in Bactometer wells with TSB containing appropriate antibiotics to obtain the desired concentrations. The overnight cultures of mutants were adjusted in TSB (with appropriate antibiotics) and transferred to sterile wells to obtain 2×10^5 CFU/ml. The incubator was regulated at 37°C and capacitance readings were done every 6 min for 10 h. The electrical kinetics enabled estimation of the different inhibitions through capacitance detection times (CDT). CDT was the time interval between the beginning of culture at 37°C (start of the monitoring) and the beginning of the acceleration phase of the capacitance curve. Two parameters were used to approach the physiological behaviours of the *S. typhimurium* mutant strains. The MIC inducing total inhibition (MIC_t) was used when no CDT was measured by the Bactometer. When the CDT of the mutant was greater than the CDT of the parental T39 strain used as control, we characterized the mutant strain with the MIC inducing partial inhibition (MIC_p).

The ability to grow in M9 medium, motility and biochemical characteristics according to "API 50 CH" and "API 10 E" strips (Bio Mérieux) were tested as part of the characterization procedure. The morphology of the mutant strains was studied by electron microscopy on aliquots of exponentially growing cultures in LB medium at 45°C (Klein *et al.*, 1990).

Drug inhibition measurements

The agar dilution method (Sahm and Washington, 1991) was used to determine, in LX192, LX1054, LX1300 and their parental strain T39, the MICs of the following antibiotics: ampicillin, carbenicillin, nalidixic acid, spectinomycin, fosfomycin (Sigma), penicillin G, neomycin, tetracycline, rifampicin (Serva) and erythromycin (Aldrich Co.). The concentrations of inhibitors ranged from 0 to 512 µg/ml. The sensitivities to dyes and to EDTA of the four strains were investigated as described (De Zwaig and Luria, 1967). Methylene blue, acridine orange, acriflavine and cristal violet, purchased respectively from Prolabo, Serva, Sigma and Allied Chemical (Morriston Co., USA) and EDTA (Sigma) were used at concentrations ranging from 0 to 100 µg/ml.

LPS and OMP characterization

LPS composition was examined by use of O-specific agglutination sera and PAGE (Tsaï and Frasch, 1982). LPS samples were extracted by proteinase K treatment (Hitchcock and Brown, 1983).

OMPs were obtained as described (Leyer and Johnson, 1993) and analysed by SDS-PAGE using a discontinuous buffer system (Laemmli, 1970). The gels were stained with 0.2% (wt/vol) Coomassie brillant blue (R-250; Serva) in 50% (vol/vol) ethanol and 2% (vol/vol) phosphoric acid and destained in 20% (vol/vol) ethanol. Molecular weight standards (protein test mixture 4) were purchased from Serva.

Plasmid DNA analysis

Plasmid DNA obtained from the rapid alkaline procedure (Sambrook *et al.*, 1989) was analysed after digestion by *Hin*dIII in the appropriate reaction buffer (Appligene). Gel electrophoresis was performed with 0.7% agarose (Appligene) and the molecular weight standard (DNA ladder) used was obtained from GIBCO/BRL (Cergy-Pontoise, France).

Transduction experiments

The Tn*phoA* insertions were isolated by P22-mediated transduction, using the high-transducing derivative of *S. typhimurium* bacteriophage: P22 HT 105.1/int (HT phage) as described (Davis *et al.*, 1980). HT phage lysates were prepared on the three mutant strains and transductions were performed by infecting *S. typhimurium* T39 and C53s. After 18 h of incubation at 37°C, 12 colonies of each transduction experiment were selected, cloned and transferred onto TCP96. The transductants were tested for detergent sensitivity by a plating procedure on TSA (with Sm and Kan) with detergent sensitivity at MIC determined for the mutant parental strains.

Southern blot analysis

Chromosomal DNA was isolated (Sambrook *et al.*, 1989) from 10 ml of bacterial culture grown overnight at 37°C in TSB with appropriate antibiotics. The DNA from Tn*phoA* mutants digested with the restriction enzymes *Hin*dIII, *Sac*I and *Eco*RI in the appropriate reaction buffers supplied by the manufacturer was transferred to nitrocellulose (Hybond-N Nylon; Amersham, Les Ulis, France) after separation on 0.7% agarose gel (molecular biology quality; Appligene). Southern blotting was performed as described (Sambrook *et al.*, 1989). Hybridization experiments were set up with a 3.4-kb-pair Tn*phoA-Hin*dIII internal fragment as a probe, labelled with α^{32}P-dCTP by a random priming method (Feinberg and Volgelstein, 1983) using a "Multiprime" kit (RPN 1601-Y; Amersham).

RESULTS

Selection of *S. typhimurium*::Tn*phoA* mutants

Spontaneous mutants resistant to Sm obtained from the C5 (4511-2) strain were selected and tested for agglutination with anti-O and anti-H diagnostic sera, growth rate in TSB, aspect of the colonies on plates, ability to grow on *Salmonella-Shigella* medium, ability to survive in distilled sterile water and ability to grow in M9. Only one strain (T39) presenting same characteristics of the parental wild type of *S. typhimurium* was retained after this screening.

The *S. typhimurium*::Tn*phoA* mutants were further obtained by conjugation between *S. typhimurium* T39 strain and *E. coli* SM10 strain. They were selected on the basis of resistance to both Kan and Sm and sensitivity to Amp. A total of 3,000 colonies were selected and cloned.

Screening of mutant strains defective in resistance to detergents

Among the bank of Tn*phoA* mutants, 1,432 strains were screened for loss of resistance to biliary salts and bile. Three mutants (LX192,

LX1054 and LX1300) were susceptible to various biological detergents. These mutant strains exhibited variable MIC for the tested detergents (table I).

In a second stage, the parental strain and the mutants were tested for their sensitivity to chemical detergents. According to MIC values, the Tn*phoA* mutants presented different phenotypes of sensitivity. The parental strain T39 was not sensitive to any detergent at the concentrations used in this study except to CTAB at 1 g/l and to CHAPS at 8 g/l.

LX1054 strain was the most sensitive, presenting an increased sensitivity to all biliary salts, to bile and chemical detergents except TX100 and NP40 (table I).

In a third stage, the growth of the mutant strains was tested in liquid media by capacitance measurements, with figure 1 illustrating one of the curves obtained in these experiments. MIC_t values were quite similar to those obtained with the plate procedures (table I), validating the capacitance measurements. Acording to MIC_t and MIC_p values, each mutant had a different spectrum of resistance to detergents in liquid medium (table II), as already observed with the plate procedure (table I). Taking into account both MIC_t and MIC_p, the ranking of the strains from the most to the least sensitive was as follows: LX1054, LX1300, LX192.

Growth, membrane composition and metabolism of screened mutants

The growth rate in TSB of the mutants LX1054 and LX1300 was similar to that of parental strain T39 (fig. 2). The LX192 curve indicated an alteration of the growth rate. Electron microscopy made on aliquots of overnight cultures in LB medium with respective antibiotics at 45°C (Klein *et al.*, 1990) indicated no alteration in the cell morphology of the mutants (data

Table I. Comparative susceptibilities of *S. typhimurium*::Tn*phoA* mutants and of the parental strain to detergents.

	MIC (g/l)(*) for: mutant strains			parental strain
	LX1054	LX1300	LX192	T39
Biological detergents				
Anionic				
Sodium deoxycholate	4	1	4	>16
Sodium cholate	4	>16	>16	>16
Sodium dehydrocholate	6	>16	>16	>16
Sodium taurocholate	8	>16	>16	>16
Bile	8	>16	>16	>16
Chemical detergents				
Cationic				
CTAB(**)	0.015	0.25	1	1
Ampholytic				
CHAPS(***)	2	8	8	8
Anionic				
Sodium dodecyl sulphate	0.06	>16	>16	>16
Non-ionic				
TX114	0.5	>16	0.5	>16
TX100	>16	>16	>16	>16
NP40	>16	>16	>16	>16

(*) Determined on TSA plates supplemented with appropriate antibiotics, after overnight incubation at 37°C.
(**) Cetyltrimethyl ammonium bromide.
(***) 3[(3-Cholamidopropyl)-dimethylammonio]1-propanesulphate.

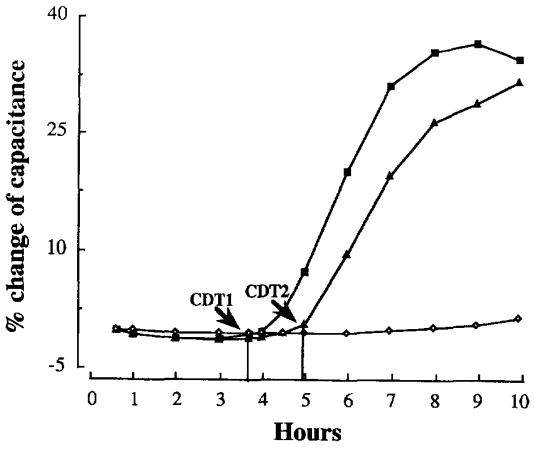

Fig. 1. Capacitance curves during the growth of *S. typhimurium*::Tn*phoA* mutant strain LX1054 in TSB (Kan) without (■) or with sodium deoxycholate [0.5 g/l (▲), 2 g/l (◇)].

CDT1 = capacitance detection time in TSB; CDT2 = capacitance detection time in TSB with sodium deoxycholate (0.5 g/l).

not shown), as in the parental strain. No defects in cell division, such as growth in chains, were observed.

Mutants which did not autoagglutinate in 2% NaCl were agglutinated with specific anti-O and anti-H antisera. Moreover, the Tn*phoA* mutants were tested for smooth LPS biosynthesis using P22 bacteriophage sensitivity. The three strains were sensitive to lysis by the P22 smooth specific phage. In electrophoresis, the characteristic doublet ladder was seen in sensitive mutants as well as in the wild-type parental strain, and changes in the migration pattern were not observed (data not shown).

The electrophoretic pattern of OMPs extracted from the mutants was qualitatively and quantitatively similar to that of parental strain T39 (not shown). Thus, Tn*phoA* inserts did not seem to affect expression of any of the detectable major *S. typhimurium* OMPs.

The Tn*phoA* mutants were able to grow in M9, indicating that auxotrophic requirements were not developed during construction of the strains. API strips did not detect differences in biochemical profiles between mutant strains and the parental strain.

Evidence of unique but distinct chromosomal insertions

*Hin*dIII restriction analysis of plasmid DNA of the mutants and of the parental strain T39 exhibited similar migration patterns, therefore exclud-

Table II. Comparative effects of detergents determined by capacitance.

	MIC (g/l) for: mutant strains LX1054		LX1300		LX192		parental strain T39	
Biological detergents								
Sodium deoxycholate	0.5(*)	(2)(**)	1	(2)	1	(2)	8	(>16)
Sodium cholate	0.5	(4)	4	(16)	16	(>16)	>16	(>16)
Sodium taurocholate	1	(8)	16	(16)	8	(>16)	>16	(>16)
Bile	0.5	(8)	8	(16)	8	(>16)	>16	(>16)
Chemical detergents								
CHAPS(***)	1	(2)	4	(4)	4	(8)	8	(8)
Sodium dodecyl sulphate	0.03	(0.125)	0.25	(>16)	>16	(>16)	>16	(>16)

Results of four independent measurements of capacitance detection time in TSB with appropriate antibiotics at 37°C for 10 h.
(*) Minimal concentration inducing partial inhibition (detection time enhanced comparatively to control).
(**) Minimal concentration inducing total inhibition (no acceleration of the capacitance curve during the 10-h culture).
(***) 3[(3-Cholamidopropyl)-dimethylammonio]1-propanesulphate.

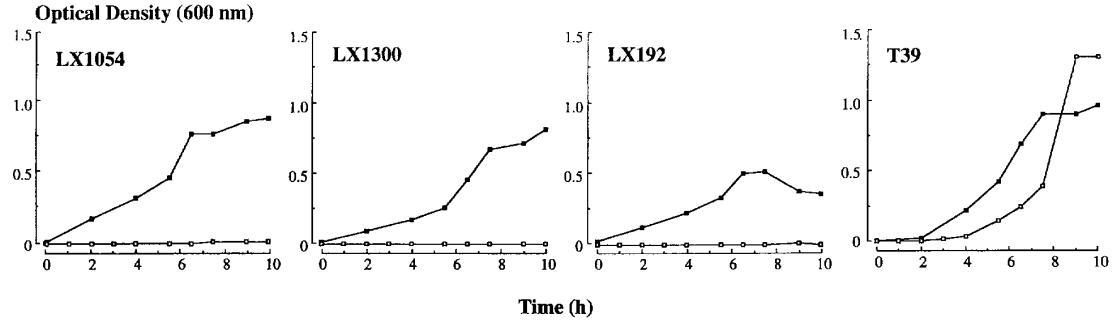

Fig. 2. Kinetics of growth in TSB (■) or in TSB with 8 g/l sodium deoxycholate (□) for parental (T39) and for *S. typhimurium* mutant strains (LX192, LX1054 and LX1300).

The experiments were carried out at 37°C in TSB with appropriate antibiotics.

ing the existence of Tn*phoA* insertions in the virulence-associated plasmid (Michiels *et al.*, 1987). The three mutants were reconstructed into both *S. typhimurium* strain T39 and its isogenic plasmid free variant (C53s) by P22-mediated transduction and tested for bile and biliary salt resistance. Transductants presented the same sensitivity pattern as their respective parental mutant strain (data not shown), clearly indicating that each of the transpositional mutants harboured, a single Tn*phoA* insertion in a chromosomal location.

To localize the Tn*phoA* insertions, chromosomal DNA was restricted by *Hin*dIII, *Eco*RI or *Sac*I (both *Hin*dIII and *Eco*RI, but not *Sac*I, cleave the Tn*phoA* internal sequence). DNA from the parent strain did not hybridize with the probe (^{32}P-labelled 3.4-kb-pair *Hin*dIII fragment, entirely contained within the transposon), whereas bands were detected in all chromosomal preparations from the three defective mutants (fig. 3). Bands detected with *Hin*dIII cleavage enabled visualization of the probe for every Tn*phoA* mutant. However, their Southern profiles could be distinguished by using either *Sac*I or *Eco*RI cleavage. In these profiles, LX192, LX1054 and LX1300 strains presented different hybridization patterns, suggesting that the transposon had inserted into three distinct chromosomal sites.

Transposon Tn*phoA* may insert into genes or regulatory sequences and consequently disrupt the function of genes. The existence of three mutations indicates that disruption of three different genes decreased the ability of these mutants to resist biological and chemical detergents, with a specific behaviour for each of them (tables I and II).

Drug sensitivity of detergent-sensitive mutants

To test the effects of mutations conferring detergent sensitivity by affecting drug susceptibility the MIC of various drugs was determined. None of the mutants were significantly different from their parental strain in terms of sensitivity to basic dyes and EDTA (data not shown). Mutant LX192 exhibited parental levels of sensitivity to antibiotics (table III). The antibiotic sensitivity of mutant LX1300 did not significantly differ from that of the wild type (table III). The most sensitive strain was LX1054. In particular, this strain was more susceptible to erythromycin (512-fold) and penicillin G (64-fold) that the T39 strain.

DISCUSSION

During the initial stages of infection, *Salmonella* are exposed to multiple harsh conditions in the gastrointestinal tract of mammals. The bile

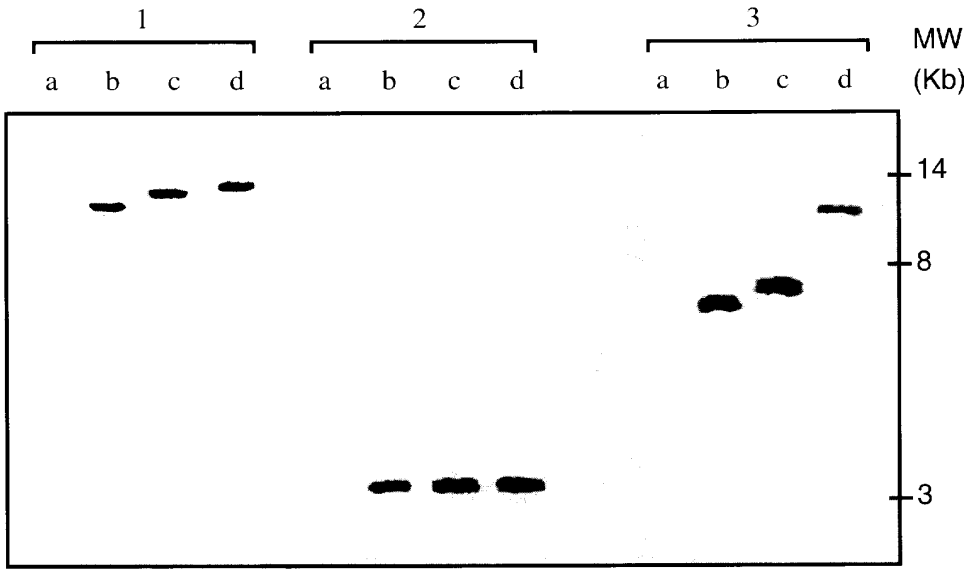

Fig. 3. Autoradiograph of a Southern blot from a gel of separated genomic DNA from *S. typhimurium* strain T39 and Tn*phoA* mutants digested with *Sac*I (1), *Hin*dIII (2) and *Eco*RI (3), probed with ^{32}P-labelled Tn*5phoA-Hin*dIII internal fragment.

Lane a=parent strain T39; lanes b, c and d=mutant strains, LX192, LX1054 and LX1300, respectively. Molecular weight markers (MW) are indicated on the right. Size in kb of fragments recognized by the probe are 11.6 for 1b, 12.7 for 1c, 13.3 for 1d, 3.4 for 2(b, c, d), 6.7 for 3b, 7.5 for 3c and 11.6 for 3d.

Table III. Comparative susceptibilities of *S. typhimurium*::Tn*phoA* mutants and of the parental strain to various antibiotics.

	MIC (μg/ml)(*) for:			
	mutant strains			parental strain
Antibiotic	LX1054	LX1300	LX192	T39
Ampicillin	0.5	4	2	2
Carbenicillin	2	8	8	8
Penicillin G	0.25	32	16	16
Erythromycin	0.25	64	128	128
Nalidixic acid	1	8	8	8
Neomycin	512	512	512	32
Spectinomycin	64	64	64	64
Tetracycline	1	2	2	2
Rifampicin	16	8	32	32
Fosfomycin	16	16	32	16

(*) Determined on TSA plates supplemented with appropriate antibiotics after overnight incubation at 37°C.

salts constitute one of the many regulating factors of the intestinal flora and counterselecting factors of some pathogens like *Salmonella* (Hentges, 1983; Rolfe, 1991; Savage, 1977).

Many mutations in the *E. coli* genome affecting detergent sensitivity have been described (Riley, 1993) as have a few in the *S. typhimurium* genome (Sanderson and Roth, 1988). These mutants, for which detergent sensitivity was observed as a phenotypic feature associated with pleiotropic effects, were obtained mostly by phage transductions or chemical treatments. Our approach was based on the expectation that some transpositional mutations could more specifically affect detergent resistance and provide mutants with new phenotypic patterns. We describe the isolation and characterization of *S. typhimurium* mutants defective in resistance to biliary salts and chemical detergents. In Gram-positive bacteria, most drugs appear to enter readily, and such organisms are generally more sensitive to these agents than are Gram-negative cells. Members of the *Enterobacteriaceae* are less sensitive than Gram-positive bacteria such as *Staphylococcus aureus*, but more sensitive than *Pseudomonas aeruginosa* (Russel and Chopra, 1990). Previous investigations on the detergent resistance of well-defined rough mutants of *S. typhimurium* have focused on the role played by the LPS (Nikaido and Vaara, 1987; Sukupolvi and Vaara, 1989; Wilkinson *et al.*, 1972). The LPS is involved in impermeability to hydrophobic compounds (Hancock, 1984; Sukupolvi and Vaara, 1989). Strains altered in LPS defined as "deep rough" mutants (*i.e.* in Rd1, Rd2 and Re mutants) are very sensitive to a wide range of hydrophobic compounds including antibiotics, mutagens, dyes, bile salts and other detergents (Nikaido, 1976). However, all the mutants recovered in our screening had a complete LPS with no observable differences as judged by SDS-PAGE. This implies that factors other than LPS contribute to the detergents' intrinsic resistance. This is in accordance with previous works suggesting that several other resistance mechanisms (chromosomal mutation or plasmid-mediated resistance) are operating in bacteria, such as expulsion of biocides from resistant cells (Russel and Chopra, 1990).

Our study found that the LX1054 strain had the most sensitive phenotype and was found to be defective for resistance to all the biliary salts tested. The other two mutant strains were sensitive only to DOC. The secondary screening performed with chemical detergents showed different levels of sensitivity from those observed for bile salts. We have further studied the bacterial physiology of the mutants by using the capacitance technique, since it relies on the dynamic measurements of metabolic changes but not on the production of a visible biomass. Previous work indicated that bile acids and more generally detergents do not have the same power of bacterial inhibition (Binder *et al.*, 1975). Our work confirms these studies and provides new data on the behaviour of detergent-sensitive mutants with different gene disruptions. The three sensitive mutants exhibited different levels of MIC_p, while MIC_t values were identical. Among the strains and according to the detergent, two types of behaviour could be found. In the first, $MIC_t = MIC_p$, which expresses an "all or nothing" effect. There was total inhibition when the threshold value was attempted. Before the threshold concentration, the strain did not seem to be affected by the detergent. In the second type of behaviour, $MIC_t > MIC_p$ and, in this case, there was a "dose effect". We observed progressive enhancement of the CDT compared to the control, as the detergent concentration increased to a critical value for which there was total inhibition.

None of the mutants had alterations in the major OMPs as assessed by SDS-PAGE, but we cannot exclude minor alteration of components of the outer membrane. They were mobile and without metabolic requirement. Moreover, they showed no change in dye sensitivity as compared to the parental strain, in contrast to mutant strains identified in previous studies (Coleman and Leive, 1979; De Zwaig and Luria, 1967; Klein *et al.*, 1990; Nakamura, 1968; Vaara, 1993). No detectable morphological changes were found in isolated cells or filamentous growth at high temperatures (Klein *et al.*, 1991) (data not shown).

MIC of antibiotics showed that the levels of antibiotic sensitivity found for the LX1054 strain

in comparison with the T39 strain can be related to its hypersensitivity to detergents and bile. Since no apparent defect of the outer membrane was detected, a mutated gene encoding a drug efflux pump may explain the variable drug sensitivities observed. No detectable difference between the LX192 strain and the T39 strain was observed; likewise, the LX1300 mutants do not have antibiotic sensitivities significantly different from the wild type. Therefore, the hypothesis of an inactivated multidrug efflux pump can be applied only to mutant LX1054.

The screening method, including normal 18 h growth on TSA without bile salts, excluded any sensitive mutants with a weak growth rate. This method could explain why neither auxotrophic strains nor morphological changes were observed in isolated cells or during cell division.

In summary, the data obtained in these experiments identified three patterns of sensitivity to chemical and biological detergents in connection with three distinct and single chromosomal mutations. The results indicate that the LX1054 strain was much more sensitive to all detergents than the LX192 and LX1300 strains. However the three Tn*phoA* mutants remain very interesting because of their atypical and variable behaviour towards detergents and drugs. They constitute a tool in understanding a particular aspect of the host/pathogen relationship. They may be a model for the study of resistance mechanisms of *Salmonellae* to detergents and disinfectants, especially those having detergent activity. Moreover, they could be used to understand the potential effects of bile salts in gut colonization and systemic invasion. Analyses of the mutated genes and of their products are in progress to elucidate the molecular mechanisms governing detergent resistance.

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Mutants transpositionnels de *Salmonella typhimurium* sensibles aux détergents biologiques et chimiques

Pathogène ubiquitaire, *Salmonella typhimurium* est une bactérie capable de supporter les conditions environnementales du tractus digestif. Afin d'identifier des mécanismes spécifiques de la résistance aux sels biliaires et plus largement aux détergents, nous avons criblé des mutants de *S. typhimurium* créés par insertion aléatoire du transposon Tn*phoA*. Au total 3000 souches ont été isolées. Parmi les 1432 premiers mutants étudiés, trois souches ont perdu la capacité de se développer en présence de détergents chimiques ou biologiques. Ces trois mutants prototrophes possèdent un LPS complet et présentent, après électrophorèse en gel de polyacrylamide, un profil de protéines majeures de la membrane externe normal. Ils ne présentent aucune sensibilité aux colorants mais des sensibilités très différentes aux antibiotiques. L'analyse par hybridation après transfert d'ADN chromosomique montre une insertion unique du transposon, avec une position dans le génome différente pour chacune des trois souches. Ces observations ont été confirmées par transduction.

Mots-clés: Sel biliaire, Détergent, *Salmonella typhimurium;* Mutants, Mutagenèse transpositionnelle, Résistance aux antibiotiques et aux détergents, OMP, LPS, Tn*phoA*.

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