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Properties of a *Salmonella typhimurium* Mutant with an Incomplete Deficiency of Uridinediphosphogalactose-4-Epimerase

V. KRISHNAPILLAI,[1] D. G. MACPHEE,[2] AND B. A. D. STOCKER
Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305

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A galactose-negative mutant, nonleaky in respect to fermentation and utilization, isolated from a smooth *Salmonella typhimurium* strain by phage selection and inferred deficient of uridine diphosphate (UDP)-galactose-epimerase, was used for experiments on relation of somatic lipopolysaccharide (LPS) character to virulence. Extracts of induced mutant cells retained ca. 1% of wild-type epimerase activity and had only ca. 5% of wild-type kinase and uridyl transferase activities; also, some cultural properties of the mutant differed from those of mutants with complete defects of epimerase only. The mutant was not galactose sensitive, presumably because of its kinase defect. Although the mutant had the phage pattern (including C21-sensitivity) of an epimerase mutant, it was susceptible to transduction by phage P22 and was O-agglutinable, even when grown on defined medium; its LPS must therefore contain some O polymer, including endogenous galactose, resulting from residual epimerase activity. Growth on galactose-supplemented medium restored smooth phage sensitivity; since the mutant was partly inducible this may result, at least in part, from increased endogenous production of UDP-galactose. The mutant was made galactose positive by introduction of an F'-*gal*$^+$ plasmid. Base-change and frame-shift mutagens did not increase the frequency of reversion above the spontaneous rate. An insertion into the operator-promoter region of the *gal* operon seems the most likely mechanism of the mutation.

Uridinediphosphogalactose-4-epimerase, one of the three enzymes used for metabolism of exogenous galactose (Fig. 1), is used also for the synthesis from endogenous sources of uridine diphosphate (UDP)-galactose, the precursor of the galactose in the somatic lipopolysaccharide (LPS) of *Salmonella typhimurium*, which contains two molecules of galactose in each core chain and one galactose in each repeat unit of the O-specific side chains (Fig. 2). Mutants completely deficient of epimerase, through mutation at gene *galE*, have been intensively studied (1-4, 7, 13-15, 18). Such mutants do not ferment galactose nor utilize it as an energy source. If grown on medium lacking galactose, they make LPS of type Rc (galactose-deficient, Fig. 2) and are phenotypically rough, lacking the parental O antigens and being resistant to smooth-specific phages such as P22 but sensitive to various rough-specific phages including C21. If the other two galactose enzymes are retained, such mutants convert galactose provided in the medium to UDP-galactose and then make normal LPS, thus recovering smooth phenotype in respect of O antigenic character and sensitivity to phages. However, mutants deficient only of epimerase are sensitive to galactose, which causes them to lyse unless glucose also is present in the medium; they can become galactose-resistant by loss of galactokinase activity, by a second mutation affecting gene *galK*. For experiments on the relation of LPS character to mouse virulence, we had to obtain a mutant with a clearly defined LPS defect from a genetically mapped smooth *S. typhimurium* strain of high virulence. We chose to use a galactose-negative mutant, *gal-456*, the cultural properties of which indicated deficiency of UDP-galactose epimerase. Subsequent enzyme assays confirmed the inferred epimerase deficiency but showed it to be incomplete (residual activity ca. 1%) and showed also that galactokinase and uridyl transferase activities were reduced to ca. 5% of the parental levels. The ef-

[1] Present address: Department of Genetics, Faculty of Science, Monash University, Clayton, Victoria 3168, Australia.
[2] Present address: Department of Genetics and Human Variation, La Trobe University, Bundoora, Victoria 3083, Australia.

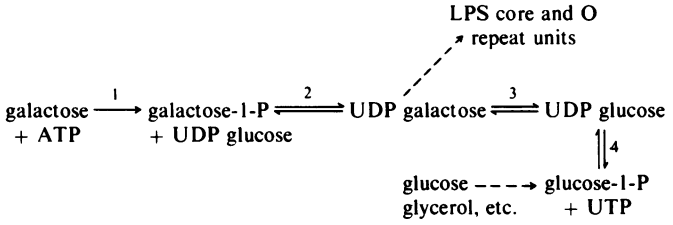

FIG. 1. *Galactose metabolism in S. typhimurium. 1 = galactokinase, 2 = galactose-1-P uridyl transferase, 3 = uridinediphosphogalactose-4-epimerase, 4 = UDPG pyrophosphorylase. Abbreviations: ATP, adenosine triphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; UDPG, uridinediphosphoglucose; P, phosphate; LPS, lipopolysaccharide.*

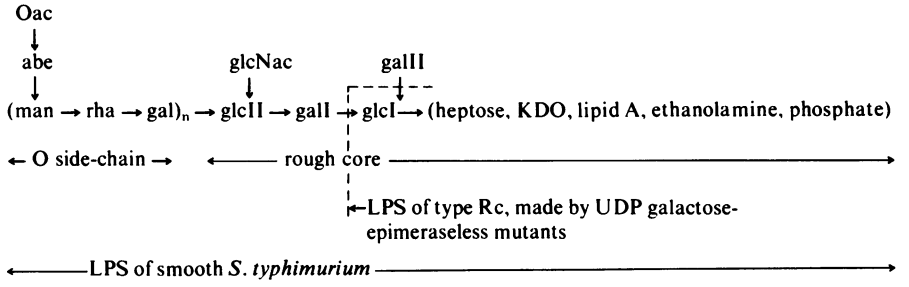

FIG. 2. *Lipopolysaccharide structure of S. typhimurium, smooth or galactose-epimerase-deficient. Abbreviations: abe, abequose; Oac, O-acetyl; man, mannose; rha, rhamnose; gal, galactose; glc, glucose; glcNac, N-acetyl glucosamine; KDO, 2-keto-3-deoxyoctonic acid; n, number of repeating units of O side-chain.*

fect of the mutation on various cultured properties differed in some respects from those produced by complete loss of epimerase function only. We here record the isolation, genetic analysis, enzyme activities, and various cultural properties of mutant *gal-456*. Our observations serve (i) to justify our inference as to the nature of the LPS defect responsible for the diminished virulence and increased susceptibility to phagocytosis of this mutant (Krishnapillai, *unpublished data*); (ii) to indicate the probable nature and site, within the *gal* operon, of the mutation; (iii) to illustrate some properties of *Salmonella* mutants with incomplete epimerase defects, especially phage sensitivities and serological character (and, by inference, LPS constitution), which are less well documented than those of mutants with complete epimerase defects.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* strain TV253 (T. V. Subbaiah, Ph.D. thesis, University of London, 1963) carries colicine factor *colEl-30* and is leucine- (*leu1051*), cystine- (*cysG1175*), and histidine- (*hisC1150*) requiring and maltose negative (*malB479*) as a result of successive ethyl methane sulfonate-induced mutations of a prototrophic wild-type strain, M7471, of the "FIRN" subtype (12)—i.e., non-fimbriate, rhamnose- and inositol-negative. TV253, like its parent M7471, is mouse virulent (LD_{50} by intraperitoneal route <20 bacteria).

Media. Oxoid nutrient broth #2 (CM67) and Oxoid blood agar base (CM55) were used. When appropriate, galactose (0.5%) or glucose and galactose (0.5% of each) were added. Galactose fermentation was tested in Difco purple broth base and on Difco EMB agar (which contains lactose and saccharose—0.5% of each) with 1% galactose added. The defined medium used was Davis minimal agar, without citrate but with 0.2% glycerol or galactose, supplemented when appropriate with amino acids at 20 μg/ml.

Serological tests. Rabbit anti-*Salmonella* O sera were from the center for Disease Control, Atlanta, Ga.; for slide agglutination tests, they were diluted 1:10 in 0.2% NaCl (to avoid salt agglutination of rough bacteria).

Phage pattern tests. See references 5, 18, and 20 for details of smooth-specific and rough-specific phages used. Plates of nutrient agar, or of sugar-supplemented nutrient agar, were inoculated by flooding with broth cultures which had been inoculated from single colonies and incubated, unshaken, at 37 C overnight—or shaken for 3.5 hr when "young" cells were desired. Excess broth was pipetted off; when the agar surface had dried, drops (0.01-ml volume) of phages at concentration of ca. 10^8 plaque-forming units (PFU)/ml were applied. The plates were read after overnight incubation at 37 C.

Transfer of F'-1-gal$^+$. TR112, an *Escherichia coli* K-12 *met* strain carrying the F'-1-*gal*$^+$ episome (10) was used as source of this factor. Recipients (*met*$^+$) acquiring the F'-*gal*$^+$ factor were obtained at a frequency of about one per 10^5 recipients plated.

Enzyme assays. Overnight broth cultures were di-

luted 100-fold into fresh broth and shaken at 37 C for 1.25 hr. D-Galactose (0.1% final concentration) was then added, and shaking was continued for a further hour. Bacterial extracts were made by the procedure of Nikaido (13), except that the cells were disintegrated in a sonic disintegrator. The enzymes were determined as described by Maxwell et al. (11) and protein content was determined by the Lowry method (9).

Effect of mutagens on reversion of gal-456 to gal$^+$. The method was similar to that of Whitfield et al. (19). Concentrated, unwashed bacterial suspensions were spread on defined medium with galactose as sole carbon source. Crystals of *N*-methyl-*N'*-nitro-nitrosoguanidine and of 2-aminopurine, and filter paper discs dipped in undiluted ethyl methane sulfonate or containing 10 μg of the nitrogen half-mustard compound ICR-191, were deposited on the agar surface; the plates were scored after 3 to 4 days of incubation.

Transduction procedures. The phage used was the *int* mutant L4 of P22 (17). Recipient bacteria were unshaken broth cultures grown for 18 hr at 37 C. To obtain *his*$^+$ transductants, 0.1-ml samples of unwashed bacteria mixed with phage, at a multiplicity of one, were spread on selective medium. To obtain *cys*$^+$ transductants, drops of phage (5×10^7 phage particles) were spotted onto selective medium surface-inoculated with unwashed bacteria.

RESULTS

Isolation and general properties of gal-456. Two of eight spontaneous mutants isolated from the smooth galactose-fermenting strain TV253 by selection with a mixture of the phages P22.*c2*, FO, and 6SR were galactose-negative and had the phage patterns typical of UDP-galactose-4-epimerase-deficient mutants (18, 20). One of them, SL4510 carrying *gal-456*, was studied in detail. The mutant was similar to the parent TV253 in colonial morphology and in not producing deposit when grown in broth; however, it was dissimilar in that its colonies crumbled when touched and that it was slightly agglutinable in 0.85% NaCl. It did not produce acid on galactose EMB or in galactose purple broth medium incubated for 2 days, nor utilize galactose as sole carbon source in defined medium; but late fermentation (>5 days) occurred in purple broth. Unlike typical epimerase-negative mutants, *gal-456* was not sensitive to galactose, for it grew equally well on minimal medium with glycerol (0.2%) or with glycerol plus galactose (0.2% of each) and on nutrient agar with or without galactose. Spontaneous galactose-utilizing revertants were obtainable on defined medium at a frequency of about 10^{-9} per cell plated; no increase over the spontaneous level was observed with the mutagens nitrosoguanidine, ethyl methane sulfonate, and 2-aminopurine nor with the nitrogen half-mustard ICR-191. Strain *gal-456* was "cured" of its galactose defect by transfer of the F'-1-*gal*$^+$ episome from *E. coli* K-12; the cured derivative was designated SL4521 with the genotype *gal-456* (F'-1-*gal*$^+$). Spontaneous loss of the episome was not very frequent, as indicated by the presence of only about 1% of galactose-negative colonies on EMB galactose plates inoculated from overnight broth cultures.

Galactose metabolic enzymes. The mutant *gal-456* and its parent TV253 were tested for the levels of enzymes of the galactose operon (Table 1). The mutant had low activity not only of epimerase but also of kinase and of transferase—between 1 and 5% enzyme activity for all three enzymes when compared to induced wild-type levels (Table 1). Whereas TV253 synthesized 11.6 times as much transferase after induction, the mutant after induction only synthesized about twice as much.

Serology of gal-456. Strain TV253 and its mutant *gal-456* were tested by slide agglutination tests with an anti-4,5,12 serum (known to have high anti-5 activity) and with an anti-4,5,12 plus anti-4,12,27 serum (with high anti-4 activity), and, as controls, with unrelated *Salmonella* O sera, anti-3,10 and anti-1,3,19. The anti-5 and anti-4 sera strongly agglutinated *gal-456*, regardless of whether it had been grown on nutrient agar with or without galactose or on defined medium with glycerol as sole carbon source. As expected, *gal-456* was not agglutinated by the control anti-O-sera. The agglutination of *gal-456* by anti-5 and anti-4 sera might have been due to antibodies against LPS of chemotype Rc (Fig. 2), perhaps present in the anti-O sera. These sera were therefore tested on an *S. typhimurium* LT2 mutant with an amber mutation in the LPS-galactosyl transferase I (*rfaH*) gene and consequently unable to add constituents distal to glucose I to its LPS (Fig. 2; 20; T. Kuo, Ph.D. thesis, Stanford University, 1969). This mutant, expected to be nonleaky (or nearly so) and to

TABLE 1. *Assay of galactose enzymes in extracts of mutant gal-456 and its parent strain*

Strain	Condition[a]	Specific activity[b]		
		Kinase (NADPH formed)	Transferase (NADPH formed)	Epimerase (NADH formed)
TV253	Induced	1.7	13.9	22.1
gal-456	Induced	0.09	0.57	0.2
TV253	Not induced		1.2	
gal-456	Not induced		0.32	

[a] For induced cultures, galactose (0.1% final concentration) was added 1 hr before harvesting.

[b] Expressed as micromoles per milligram of protein per hour at 30 C. Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide.

synthesize deficient LPS only (Fig. 2), was barely agglutinable by the anti-5 or anti-4 sera.

Transduction of TV253 and gal-456 with P22.L4. It is believed that the adsorption site for the smooth-specific transducing phage P22 is the O side-chain; thus nonleaky LT2 epimerase mutants are unable to act as recipients in P22 transduction (Fig. 2; reference 5). However, when the mutant *gal-456* and its smooth parent TV253 were tested as transductional recipients, *his*$^+$ transductants were obtained from each at a frequency of ca. one per 10^6 phage particles; *cys*$^+$ transductants were also easily obtained from *gal-456*.

Phage patterns of TV253, gal-456, and gal-456 (F'1-gal$^+$). Mutants of *S. typhimurium* unable to form either the galactose I or glucose I units in LPS, owing to deficiency of UDP-galactose-4-epimerase or of the galactosyl I or glucosyl I LPS-transferases, needed for attachment of the units (Fig. 2), are specifically sensitive to the phage C21; those with galactose-deficient LPS, but not those with glucose-deficient LPS, are also sensitive to phage Br2 (18, 20). The mutant *gal-456*, its *gal*$^+$ parent TV253, and its "cured" derivative *gal-456* (F'-1-*gal*$^+$) were therefore tested for sensitivity to a battery of phages (Table 2). When tested by the standard procedure (i.e., using nutrient agar plates flooded with 18-hr broth cultures), *gal-456* exhibited the pattern typical of epimerase-deficient mutants in that it was resistant to the smooth-specific phages P22.*c2*, P22h.*c2*, and 9NA and to the smooth-rough phage FO but sensitive to rough phages including C21 and Br2 (Table 2). As expected, its smooth parent, TV253, and cured derivative, *gal-456*(F'1-*gal*$^+$), showed the phage pattern typical of smooth strains. Epimerase-deficient mutants when tested on nutrient agar supplemented with galactose (plus glucose, added to relieve possible galactose toxicity) are found to have become phenotypically smooth, for they show the phage pattern typical of smooth strains (2, 10). Mutant *gal-456* behaved in this way, except that it remained sensitive to the rough-specific phage P221.*c2* (Table 2). An observation of interest was that, when the nutrient agar plates were inoculated from young broth cultures of *gal-456* (instead of from 18-hr cultures), the phage pattern obtained was that characteristic of smooth cells, except for sensitivity to phage P221.*c2*.

DISCUSSION

The galactose-negative mutant *gal-456* of the smooth, mouse-virulent *S. typhimurium* strain TV253 was chosen for use in mouse experiments (V. Krishnapillai, *manuscript in preparation*), because its phage resistance pattern on medium lacking galactose and the restoration of the smooth pattern by growth in the presence of galactose suggested that it was completely deficient of UDP-galactose-epimerase activity. However, enzyme assays showed that the mutant retained about 1% of the wild-type (induced) epimerase activity and had only about 5% of the wild-type (induced) levels for the other two galactose enzymes. [Typical *galE* mutants grow very poorly on galactose-containing media and can become galactose-resistant by further mutation causing loss of galactose kinase activity (2, 16). The low kinase activity of *gal-456* may account for its galactose resistance, despite very low epimerase activity.]

Several explanations were considered for the reduction in activity of all the three enzymes determined by genes of the *gal* operon which was caused by the spontaneous, presumably single-

TABLE 2. *Phage sensitivity patterns of gal-456 and its parent and derivative strains*

Strain	Condition[a]	Phages[b]					
		O-specific	OR	R-specific			
		P22.*c2*, P22h.*c2* and 9NA	Felix O	6SR	Br2, Ffm and Br60	P221.*c2*	C21
TV253	Standard	+	+	−	−	−	−
gal-456	Standard	−	−	−	+	+	+
gal-456	Galactose agar	+	+	−	−	+	−
gal-456	Young cells	+	+	−	−	+	−
gal-456(F'-1-*gal*$^+$)	Standard	+	+	−	−	−	−

[a] Standard, nutrient agar plates inoculated from unshaken, overnight cultures; galactose agar, similar to standard, but nutrient agar was supplemented with glucose + galactose; young cells, similar to standard, but plates were inoculated from shaken, 3.5-hr cultures.

[b] Used at a concentration of ca. 10^8 plaque-forming units/ml. Clear plaque variants were used in case of phages P22, P22h, and P221. Symbols: +, lysis; −, no lysis.

step, *gal-456* mutation. This mutant cannot have arisen by "super-repressor" mutation at the *gal* repressor locus, because in such a mutant the *E. coli* wild-type *gal* genes in the F′ factor would also have been repressed. This, evidently, was not the case since *gal-456* (F′-1-*gal*$^+$) fermented galactose strongly. The residual activities of all three enzymes and the isolation of galactose-positive revertants show that the mutation cannot be an extensive deletion involving any of the three structural genes of the operon, *galK*, *galT*, and *galE*. Mutation at the operator end of the operon, affecting the promoter region [inferred binding site for ribonucleic acid (RNA) polymerase] might reduce activity for all three enzymes. Although spontaneous revertants were easily isolated, the frequency of reversion was not increased by base-substitution or frame-shift mutagens; this indicates that the *gal-456* mutation arose neither by change of a single base nor by insertion or deletion of a single base. In *E. coli*, galactose-negative mutants which revert spontaneously but do not respond to mutagens have been attributed to chromosomal aberrations (insertions, duplications, or inversions); in some of them, insertions comprising several hundred base-pairs within *galT* have been inferred (6). An insertion would account for the reversion properties of *gal-456*. A long insertion into the promoter region would be expected to prevent any synthesis of *gal* messenger molecules and thus to cause complete absence of all three enzymes. However, the residual activities in *gal-456* might be accounted for if the insertion was near one end of the promoter region, so that it retained some ability to bind RNA-polymerase, or if the inserted material included a base sequence with weak promoter activity. The ratio of induced to noninduced activities of galactose uridyl-transferase was only ca. 2:1 in the mutant, compared with ca. 12:1 in the wild-type parent. This partly constitutive character suggests that the postulated insertion (or other chromosomal aberration) affects not only the promoter but also the operator, which is probably immediately adjacent to it.

Although the assays showed that *gal-456* had between 1 and 5% normal (induced) activities for all three galactose enzymes, it neither fermented nor utilized galactose to a detectable extent—perhaps because these reactions involve the sequential action of all three affected enzymes. Thus, apparently complete loss of ability to metabolize galactose, as judged by tests of fermentation and utilization, does not necessarily mean that the mutation concerned has caused complete loss of any of the galactose enzymes.

Slide-agglutination tests showed the presence of O-antigenic factors 4 and 5 in *gal-456* cells, even when grown on defined medium without galactose. These factors reflect the presence in LPS of O side-chains of the sort found in *Salmonella* of group B; these are made up of repeating units containing galactose and cannot be attached to uncompleted LPS core of type Rc, i.e, lacking the core galactose units (Fig. 2). O repeating units, in chains of length not less than two (5), are believed to constitute the adsorption site for phage P22. The susceptibility of *gal-456* to transduction by phage P22 is therefore evidence for presence of some polymeric O side-chains in its LPS. Since the galactose of LPS core and O repeating units is derived from UDP-galactose (which in the absence of exogenous galactose can only be made by the epimerization of UDP-glucose), the O agglutinability and susceptibility to P22 transduction of *gal-456* must reflect its residual epimerase activity, although this amounts, in extracts of induced cells, to only about 1% of the corresponding activity of wild-type cells (Table 1). However, the induced level of epimerase activity of wild-type cells is presumably that needed for the rapid catabolism of exogenous galactose, and calculation (cf. 8) shows that a small fraction of the wild-type enzymatic activity should suffice to generate UDP-galactose at the rate needed for synthesis of smooth LPS. Thus, 1 mg (dry weight) of smooth *S. typhimurium* cells, growing with a doubling time of 30 min, contain about 500 μg of protein and about 20 μg of LPS, of which about 14 μg is polysaccharide containing about 20% (i.e., 2.8 μg) galactose. Galactose for incorporation into LPS must therefore be made at a rate of about 2.8 μg per 30 min per 0.5 mg of protein, which is 2.8 per 180 μM galactose per 0.5 hr per 0.5 mg of protein, equaling 0.06 μM per hr per mg of protein. This corresponds to less than 1% of the enzymatic activity of extracts of induced wild-type cells and is of the same order of magnitude as the activity found in extracts of induced *gal-456* cells (Table 1). In *E. coli* K-12, some mutants with almost complete lack of epimerase make LPS containing considerable amounts of galactose; in extracts of some such mutants, epimerase activity was undetectable (less than 0.02% of the wild-type uninduced activity) and was far less than the activity calculated to be needed for synthesis of the galactose found in the LPS (8). In one such mutant, the explanation of the anomaly seems to be that whole cells have more epimerase activity than can be measured in their extracts (16); if this is so, then there is no need to postulate that some of the galactose of LPS comes from a precursor other than UDP-galactose, as previously surmised.

Growth of mutant *gal-456* on galactose-supplemented nutrient agar changed its phage sensitivity pattern from that typical of *Salmonella* making LPS of type Rc (galactose-deficient) to that characteristic of smooth strains, except that sensitivity to the rough-specific phage P221 persisted (Table 2). In the case of mutants with complete epimerase defects and normal kinase and uridyl transferase activities this "phenotypic curing" by galactose results from uptake of galactose from the medium, and its phosphorylation and conversion to UDP-galactose (Fig. 1); growth in the presence of galactose does not alter the phage sensitivity of mutants which are in addition completely deficient of galactokinase. The mechanism may be somewhat different in mutants such as *gal-456*. Exposure to galactose caused about a twofold induction of transferase activity (Table 1); the epimerase activity of extracts of induced cells, as calculated above, was about sufficient for synthesis of the galactose content of smooth LPS. In such mutants, therefore, phenotypic curing by exposure to galactose may result, at least in part, from induced increases in epimerase activity, rather than from conversion of exogenous galactose to UDP-galactose, a process which in *gal-456* would be hindered by the partial kinase and transferase defects. Similarly, the smooth phage pattern of cells from young broth cultures may reflect increased epimerase activity in such cells, perhaps induced by uptake of the traces of galactose commonly present in nutrient broth (16).

Cells from fully grown broth cultures of *gal-456* were resistant to the smooth-specific phages and to Felix O phage, and sensitive to rough-specific phages including C21 (Table 2), the pattern characteristic of mutants making LPS of type Rc (galactose-deficient). Thus, the epimerase defect in such cells, although incomplete, severely interferes with synthesis of smooth LPS. However, the susceptibility to transduction by P22 and the O agglutinability of such cells show that their LPS contains some O-specific material, presumably O polymeric chains attached to completed core chains (since the absorption site for P22 consists of more than one O repeat unit). The ability of *gal-456* cells to adsorb both P22 and rough-specific phages suggests that their LPS, like that of leaky *rfa* mutants (5), has core chains, some of which are completed and capped by O polymer and others (presumably the majority) which are uncompleted (in this instance, presumably lacking one or both galactose units). The resistance of *gal-456* cells to phages Felix O and 6SR, both of which attack mutants making "complete core" LPS, suggests that all or nearly all of such core chains as are completed in *gal-456* are covered by O chains. If this is so, it suggests that under conditions of UDP-galactose limitation the galactosyl transferase system involved in synthesis of O repeat units on antigen carrier lipid competes effectively for UDP-galactose with the transferase systems which form the two galactose units of the LPS core chains.

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