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Mechanism of O-Antigen Distribution in Lipopolysaccharide

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O-antigen units are nonuniformly distributed among lipid A-core molecules in lipopolysaccharide (LPS) from gram-negative bacteria, as revealed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate; the actual distribution patterns are complex, multimodal, and strain specific. Although the basic biochemical steps involved in synthesis and polymerization of O-antigen monomers and their subsequent attachment to lipid A-core are known, the mechanism by which specific multimodal distribution patterns are attained in mature LPS has not been previously considered theoretically or experimentally. We have developed probability equations which completely describe O-antigen distribution among lipid A-core molecules in terms of the probability of finding a nascent polymer (O antigen linked to carrier lipid) of length k (T_k) and the probability that a nascent polymer of length k will be extended to $k + 1$ by polymerase (p_k) or transferred to lipid A-core by ligase (q_k). These equations were used to show that multimodal distribution patterns in mature LPS cannot be produced if all p_k are equal to p and all q_k are equal to q, conditions which indicate a lack of selectivity of polymerase and ligase, respectively, for nascent O-antigen chain lengths. A completely stochastic model ($p_k = p$, $q_k = q$) of O-antigen polymerization and transfer to lipid A-core was also inconsistent with observed effects of mutations which resulted in partial inhibition of O-antigen monomer synthesis, lipid A-core synthesis, or ligase activity. The simplest explanation compatible with experimental observations is that polymerase or ligase, or perhaps both, have specificity for certain O-antigen chain lengths during biosynthesis of LPS. Our mathematical model indicates selectivity probably was associated with the polymerase reaction. Although one may argue for a multimodal distribution pattern based on a kinetic mechanism, i.e., varying reaction parameters in space or in time during cell growth, such a model requires complex sensory and regulatory mechanisms to explain the mutant data and mechanisms for sequestering specific components of LPS biosynthesis to explain the distribution pattern in normal cells. We favor the simple alternative of enzyme specificity and present generalized equations which should be useful in analysis of other analogous biochemical systems.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria and consists of three biosynthetically distinct chemical structures: (i) lipid A, consisting of a β1-6 linked diglucosamine backbone containing phosphate, fatty acids, and other substituents; (ii) core oligosaccharide, consisting of specific carbohydrate residues, including ketodeoxyoctonate, in defined linkages; and (iii) O antigen, consisting of serologically and chemically distinct repeating carbohydrate structures which are strain specific (for reviews, see references 17, 19, and 21).

The biosynthetic pathways of lipid A (19), core oligosaccharide, and O antigen (17) have been extensively investigated and complemented by genetic studies. Lipid A precursor is assembled in the inner membrane, and core sugars are sequentially added to form a complete lipid A-core unit. O antigen monomers are assembled on a C55 polyisoprenoid carrier lipid and translocated to the outer surface of the inner membrane where transfer to lipid A-core occurs (16). Monomers, as well as polymers of various lengths, are then transferred to complete lipid A-core, and mature LPS is then translocated to the outer membrane.

The basic structure of LPS from *Salmonella typhimurium* is known (Fig. 1). The monomeric unit of O antigen is a tetrasaccharide consisting of galactose, rhamnose, mannose, and abequose. Once assembled on a lipid carrier, monomers of O antigen are available for polymerization. The polymerization reaction is analogous to protein synthesis in that a monomer is utilized in each reaction of chain extension, and the growing polymer is added to the nonreducing end of a monomer (22). Furthermore, initiation of a new polymer requires reaction of two monomeric units, yielding a dimer which can be extended in length as above; monomeric units are thus central to each polymer extension reaction as well as to the initiation of new polymers. At some time during polymerization, a polymer or a monomer is withdrawn from the polymerizing pool as a result of enzymatic transfer from the lipid carrier to a complete lipid A-core molecule. The completed LPS molecule is then translocated to the outer membrane. The activity responsible for polymerization was identified genetically (the *rfc* locus) and is referred to as polymerase, while the activity responsible for transfer of O antigen from carrier lipid to complete lipid A-core is apparently dependent on two genetic loci (*rfbT* and *rfaL*) and is referred to as ligase.

We (7) and others (18) have developed high-resolution gel electrophoretic techniques for precise analysis of O-antigen distribution in mature LPS from the outer membrane. We now know that (i) monomers of O antigen are not distributed uniformly among lipid A-core molecules; (ii) O-antigen distribution is organism specific, with different patterns occurring in different strains and species; and (iii) slight alterations in O-antigen distribution can drastically alter resistance of bacteria to host immunodefense systems. However, little is known regarding the biosynthetic mechanism by which specific O-antigen distribution patterns are presented on the bacterial surface.

In this report we will (i) present a mathematical model of

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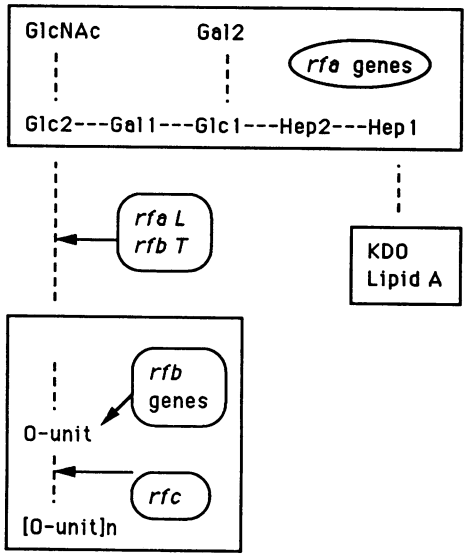

FIG. 1. Structure of LPS from *S. typhimurium* LT2. The basic structure of LPS from *S. typhimurium* LT2 is shown along with the genes involved in synthesis and assembly. KDO, 3-Deoxy-D-*manno*-octulosonate; Hep, heptose; Gal, galactose; Glc, glucose; GlcNac, *N*-acetylglucosamine; *rfa*, genes involved in core synthesis; *rfa L* and *rfb T*, genes involved in transfer of O antigen to lipid A-core; *rfb*, genes involved in O-antigen synthesis; *rfc*, genes involved in polymerization of O-antigen units.

O-antigen polymer synthesis and distribution in LPS, (ii) present a mathematical argument indicating that most likely polymerase or perhaps ligase or both have a predisposition to react with O-antigen polymers of specific lengths in order to produce distributions of O antigen observed in LPS, and (iii) use this model to evaluate experimentally observed effects of limiting production of O antigen, complete lipid A-core, or activity of ligase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. typhimurium* LT2 strains used in this study are listed in Table 1. These strains represent the following classes with respect to LPS biosynthesis: (i) parental strains (smooth) without lesions in LPS biosynthetic genes (SL1027); (ii) parental strains (*pmi*, smooth when grown with mannose; SL1644 and SL1647); (iii) mutants with temperature-sensitive (TS) lesions in *rfa* genes (rough mutants TS in core biosynthesis; SA117 and SA120 [12], and SL1373 and SL1377 [14]); (iv) mutants with partial defects in *rfb* genes (leaky mutants in O-antigen biosynthesis; SL1112, SL1113, SL1114, and SL1115); (v) *rfc* mutants (defective in polymerization of O antigen monomeric units SL901, SL1034, SL4919, and SL4920); and (vi) *rfb*T mutants (defective in transfer of O antigen to the lipid A-core; SL1196 and SL1197).

Cells were grown in defined medium as previously described (7). Mannose was added to 0.1% for *pmi* mutants in order to supplement this genetic lesion and allow synthesis of O-antigen units.

Isolation and analysis of LPS. Cells were labeled with [^{33}P]orthophosphoric acid, and LPS was isolated and prepared for gel electrophoresis (6, 7) as previously described. The distribution of O antigen among lipid A-core molecules (as revealed by gel electrophoresis) was quantitated following radioautography and microdensitometry with a Perkin-Elmer 1010 G densitometer. Data were digitized and quantitated by image analysis programs written for the PDP-11/70 computer as previously described (6).

TABLE 1. *S. typhimurium* strains

Strain	Genotype	Source
SL1027	*metA22 trpB2 H1-b H2-e, n,x* (fels 2) *flaA66 rpsL120 xyl-104 metE551*	B. Stocker
SA117	As SL1027, *rfa-3004*	K. Sanderson
SA120	As SL1027, *rfa-3006*	K. Sanderson
SL1644	*pmi-410*	B. Stocker
SL1647	*pmi-409*	B. Stocker
SL1373	As SL1644, *rfa-973*	B. Stocker
SL1377	As SL1647, *rfa-977*	B. Stocker
SL4917	*metA22 trpB2 H1-b nml H2-e,n,x rpsL120 flaA66 xyl-104 metE551 hspLT6 hspS29 ilv-452 his-7073*	B. Stocker
SL4919	As SL4917, *rfc-985*	B. Stocker
SL4920	As SL4917, *rfc-986*	B. Stocker
SL901	*metA22 trpB2 H1-6 H2e,n,x flaA66 rpsL120 rfc-497*	B. Stocker
SL1034	As SL901, *rfc-465* not *rfc-497*	B. Stocker
SL1196	*hisD27 trpA8 colE-30 H1-a H2-e,n,x gal-437 xyl-412 rfb-580*	B. Stocker
SL1197	As SL1196, *rfb-604* not *rfb-580*	B. Stocker
SL1112	As SL1196, *rfb-548* not *rfb-580*	B. Stocker
SL1113	As SL1196, *rfb-598* not *rfb-580*	B. Stocker
SL1114	As SL1196, *rfb-599* not *rfb-580*	B. Stocker
SL1115	As SL1196, *rfb-600* not *rfb-580*	B. Stocker

Description of the model. We will describe a mathematical model of the synthesis of lipopolysaccharide that is based on the following assumptions. (i) The addition of an O-antigen unit to a nascent polymer (consisting of an O-antigen chain attached to a carrier lipid) is an irreversible event. It occurs independently of any past or future additions with a probability that may depend on the length of the O chain at the time of polymerization. (ii) The ligase reaction is an irreversible event that occurs with a probability that may depend on the length of the O chain. (iii) Chains of all lengths, k, where $0 \leq k \leq N$ and N is the largest possible chain length, are found in the outer membrane, although the frequencies of some lengths may be quite low.

We can now introduce the variables and parameters used in the model. If k is the length of an O chain, then T_k is the probability a nascent polymer has an O chain of length k at a given time, p_k is the probability that a nascent polymer of length k undergoes a polymerization reaction that results in a polymer of length $k + 1$, q_k is the probability that a nascent polymer of length k undergoes a ligase reaction that results in the incorporation into the outer membrane of a complete lipopolysaccharide molecule with O-antigen chain of length k, r_k is the probability that a nascent polymer of length k remains unchanged, and χ is the probability that a lipid A-core unit (without an O chain) is incorporated into the outer membrane.

A few remarks can be made here. First, since N is the longest possible chain length, $p_N = 0$. We will also set $q_0 = 0$, and because of assumptions i and ii, the reaction probabilities (p_k, q_k, and r_k) may vary with k. The equations that constitute the model are based on the idea that in a single time step, a nascent polymer either remains unchanged or undergoes a polymerization or ligase reaction. If $\{T_k'\}$ is the nascent polymer distribution at the next time step (braces denote that all T_k are being considered as a class)

$$T_k' = p_{k-1}T_{k-1} + r_kT_k \text{ where } k = 1, 2, \ldots N \quad (1)$$

Indeed, polymer chains of length k at the next time step arise from chains of length k at the present time step with probability r_kT_k or from nascent polymers with chains of length $k-1$ that undergo a polymerization reaction, an event with probability $p_{k-1}T_{k-1}$.

The next equation accounts for the frequency of polymers of zero length, that is, the free carrier lipids. With probability r_0T_0, a carrier lipid will remain free in the next time step. Free carrier lipids also result from the polymerization of nascent polymers of length k, an event with probability p_kT_k, or from a ligase reaction that occurs with probability q_kT_k. Adding all of these probabilities gives

$$T_0' = r_0T_0 + (p_1 + q_1)T_1 + (p_2 + q_2)T_2 + \ldots (p_k + q_k)T_k + \ldots (p_{N-1} + q_{N-1})T_{N-1} + q_NT_N \quad (2)$$

The reaction probabilities are subject to certain constraints:

$$p_k + q_k + r_k = 1 \text{ where } k \neq 1 \quad (3)$$

$$(1 - p_1 - q_1 - r_1)T_1 = p_1T_1 + p_2T_2 + \ldots p_{N-1}T_{N-1} \quad (4)$$

Equation 4 is a consequence of the special role of monomers (case $k = 1$) in chain synthesis. The left side of equation 4 is the probability that a monomer is involved in a polymerization reaction, and this must be equal to the probability that such a reaction occurs for a chain of some length 1, 2, ... $N-1$. The probability of the latter, however, is just the right side of equation 4.

Equations 1 through 4 define the model. $\{T_k\}$ as the frequency distribution of nascent polymers at a given time step means that

$$\sum_{k=0}^{N} T_k = 1$$

This implies that

$$\sum_{k=0}^{N} T_k' = 1$$

(as it should). In all subsequent discussions we will assume that frequency distributions for nascent polymers and lipopolysaccharide have reached steady state. This means that $T_k' = T_k$ for $k = 0, 1, 2, \ldots N$, even though the numbers of these molecules will increase during the growth phase of the cell. Let Γ_k be the lipopolysaccharide frequency distribution so that Γ_k is equal to the frequency of molecules of length k. Then

$$\Gamma_0 = \chi/\tau \text{ and}$$

$$\Gamma_k = q_kT_{k/\tau} \text{ if } k = 1,2, \ldots N$$

where

$$\tau = \chi + \sum_{J=1}^{N} q_jT_j$$

Note that τ is proportional to the total number of core units and polymer chains that are incorporated into the outer membrane. It can be shown (8) that when $k \geq 2$, $\{\Gamma_k\}$ can be expressed directly in terms of the reaction probabilities as

$$\Gamma_2 = \frac{q_2}{p_2 + q_2} \cdot \frac{p_1T_1}{\tau} \text{ and}$$

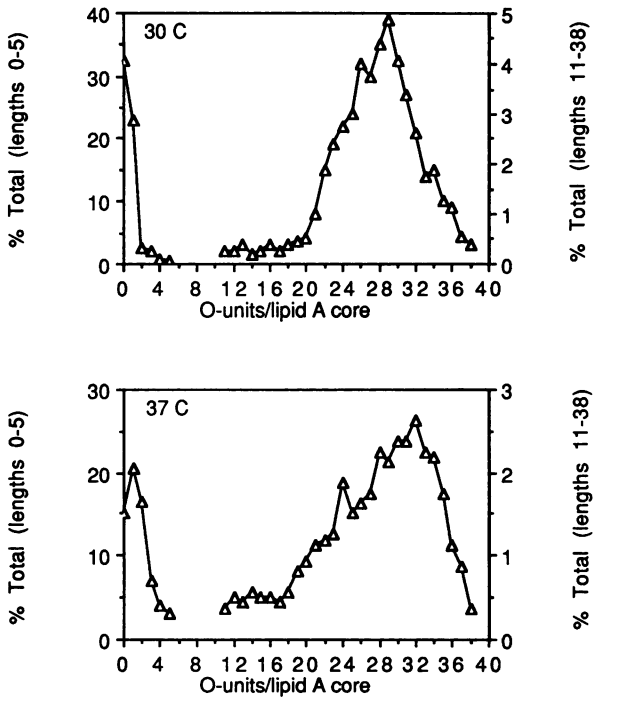

FIG. 2. Analysis of O-antigen distribution in wild-type *S. typhimurium* SL1027. Cells were grown at 30 or 37°C and labeled with [^{33}P]orthophosphoric acid. LPS was prepared and separated by gel electrophoresis, and the gel was exposed to X-ray film. Individual bands of LPS were quantitated by densitometry, and data shown were calculated from these data. Strain SL4917 and strains SL1644 and SL1647 grown with mannose were identical within experimental error. The zero value on the x axis represents lipid A-core molecules lacking O antigen. Missing data points are due to low levels of specific size classes resulting in poor accuracy of quantitation.

$$\Gamma_k = \frac{q_k}{p_k + q_k}\left(\prod_{J=2}^{k-1} \frac{p_{j-1}}{p_{j-1} + q_{j-1}}\right) \cdot \frac{p_1T_1}{\tau} \text{ if } k = 3, \ldots N$$

RESULTS

The distribution of O antigen in LPS isolated from the outer membrane can be accurately determined by gel electrophoresis in sodium dodecyl sulfate (7, 18). LPS molecules are completely dissociated in this detergent, and migration is dependent upon size, which is dependent on the amount of carbohydrate (core sugars and O antigen) attached to lipid A. LPS species containing from 1 to greater than 40 O-antigen units can be resolved by using gradients of acrylamide in 20-cm gels (7). The amount of each species can be directly determined by quantitating the amount of some component, such as phosphate, which is present only in the lipid A-core region of LPS.

We determined the distribution of O-antigen units in LPS from a smooth strain of *S. typhimurium* grown at 30 or 37°C (Fig. 2; Table 2). The most significant difference was the percentage of lipid A-core molecules lacking O antigen: 13% in cells grown at 37°C compared with 33% in cells grown at 30°C. Other parameters which differed were (i) the peak

TABLE 2. Analysis of LPS from *S. typhimuirum* strains[a]

Strain	Growth temp (°C)	Avg no. of O-units/lipid A-core	% of lipid A-core lacking O antigen	% of incomplete lipid A-core	Amt[b] of O antigen (nmol of abequose/mg of protein)	Length (O-units/lipid A-core) of peak frequency of lipid A-core with greater than 10 O-units[c]
SL1027[d]	30	10.7	35	<1	152	28–29
	37	11.4	13	<1	161	31–33
	42	11.9	15	<1	183	31–33
SA117 *rfa*	30	11.9	39	11.3	148	31–33
	37	2.0	80	35	81	26–28
	42	1.5	92	40	37	18–22
SA120 *rfa*	30	11.9	47	15.5	130	31–33
	37	1.4	95	76	67	26–28
	42	ND[f]	ND	ND	34	ND
SL1373[e] *rfa*	30	11.5	52	34	148	31–35
	37	3.7	85	48	130	27–30
	42	ND	ND	ND	112	ND
SL1113 *rfb*	37	1.5	64	<1	ND	18–20
SL1115 *rfb*	37	3.1	56	<1	ND	21–24
SL4917 *wt*	37	11.6	14	<1	ND	31–33
SL4919 *rfc*	37	0.80–0.85	15–20	<1	ND	NA[g]
SL4920 *rfc*	37	0.80–0.85	15–20	<1	ND	NA
SL901 *rfc*	37	0.80–0.85	15–20	<1	ND	NA
SL1034 *rfc*	37	0.80–0.85	15–20	<1	ND	NA
SL1196 *rfb*[h]	37	>0.05	>95	<1	ND	NA
SL1197 *rfb*[h]	37	>1	>95	<1	ND	20–22

[a] Data were collected following gel electrophoresis of ^{33}P-labeled LPS and densitometry of subsequent autoradiograms. The average number of O-units per lipid A-core was calculated from these data.

[b] These data represent quantitation of abequose by the thiobarbituric acid method (6) and are given as nanomoles of abequose per milligram of cell protein.

[c] Data obtained from final histograms of distribution of O-units per lipid A-core.

[d] Data from strain SL4917 and strains SL1644 and SL1647 grown in mannose were identical to data for SL1027 within experimental error.

[e] Strain SL1373 yielded several bands of incomplete core at the restrictive temperature, indicating that it may be an *rfa* H mutation (14). Furthermore, the amount of O antigen (nanomoles of abequose per milligram of cell protein) decreased only 12% at 37°C, while the average of O-units per lipid A-core dropped from 11.5 to 3.7. This may reflect increased LPS synthesis.

[f] ND, Not determined.

[g] NA, Not applicable.

[h] The lesions in SL1196 and SL1197 map in an *rfb* cluster but are known to affect ligase function rather than O-antigen synthesis.

frequency for LPS molecules containing greater than 10 O-antigen repeats per molecule of lipid A-core (31 to 32 for cells grown at 37°C, compared to 28 to 29 for cells grown at 30°C), (ii) the average number of O-antigen units per lipid A-core (11.9 at 37°C, compared with 10.1 at 30°C), and (iii) the relative shapes of the distribution patterns.

Palva and Makela had analyzed LPS from polymerase- and ligase-deficient mutants of *S. typhimurium* (18). Cells lacking polymerase (only monomers of O antigen are available for transfer to lipid A-core) contained one major LPS component which was slightly larger than the single component present in ligase-deficient cells in which only complete lipid A-core is accumulated in the outer membrane. We found strains SL4919, SL4920, SL901, and SL1034 (polymerase-deficient strains) and SL1196 (a ligase-deficient strain) to behave accordingly (Table 2).

We reasoned that examination of mutants partially defective in specific steps of LPS biosynthesis would yield information reflecting the mechanism by which the normal distribution of O antigen was attained in wild-type cells. LPS from partially defective *rfb* mutants (strains SL1112, SL1113, SL1114, and SL1115) was analyzed to determine the effect of restriction of O-antigen monomer availability on subsequent O-antigen distribution in LPS isolated from the outer membrane. On the basis of the model, we predicted that if the distribution pattern in wild-type cells was the result of purely stochastic interactions between polymerase and ligase and O-antigen polymers of varying lengths, then under conditions of O-antigen monomer limitation, O-antigen polymers linked to lipid A-core would be drastically shorter.

O-antigen synthesis was inhibited by 73% in strain SL1115, but the peak frequency for greater than 10 O-antigen units (O-units) per lipid A-core was reduced by only 9 units (32 in the wild type compared with 22 in SL1115 [Table 2; Fig. 3]). Furthermore, the percentage of lipid A-cores lacking O antigen increased 4.3-fold: from 13% in the wild type to 56% in strain SL1115. Similar results were obtained for strain SL1113 (Table 2), which showed 87% reduction in total O antigen, and for strains SL1112 and SL1114, which were inhibited to greater than 90% (data not shown). There is a strong preference for maintaining synthesis of LPS with long O-antigen polymers at the expense of leaving large amounts of lipid A-core devoid of O antigen; this is not consistent with a purely stochastic model (see Discussion).

In contrast, on the basis of a model of purely stochastic interactions, partial inhibition of synthesis of complete lipid A-core (the acceptor for O antigen) should lead to an increase in polymer length. This is not the case when TS *rfa* mutants are grown at elevated temperatures (Fig. 4; Table 2). Mutant strain SA117 has a near-normal LPS profile when grown at 30°C. Some incomplete lipid A-core molecules have accumulated, but the distribution of O-antigen units and the average number of O-antigen units per lipid A-core is similar to those of the wild type. The peak distribution of LPS with greater than 10 O-units per lipid A-core was only slightly greater than that of the wild-type strain SL1027 (Table 2). Growth at 37°C led to accumulation of incomplete lipid A-cores (35% compared with <1% in the wild type) and to only a slight decrease in polymer lengths, rather than the increase predicted by the stochastic model (Fig. 4). At 42°C,

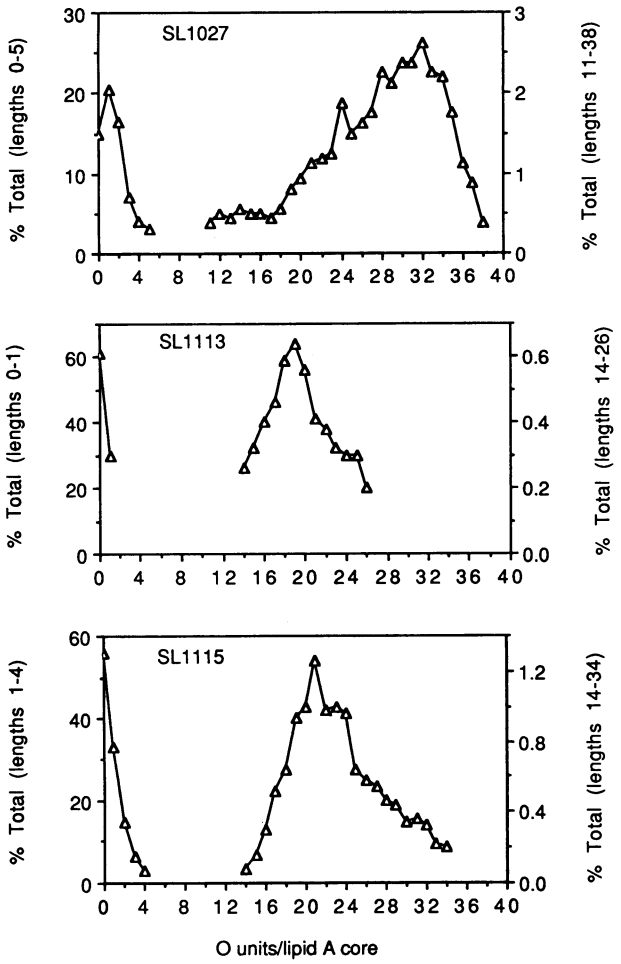

FIG. 3. Comparison of LPS from strain SL1027 and the partially defective *rfb* mutants SL1113 and SL1115. Cells were grown at 37°C and data were obtained as described in the Fig. 2 legend.

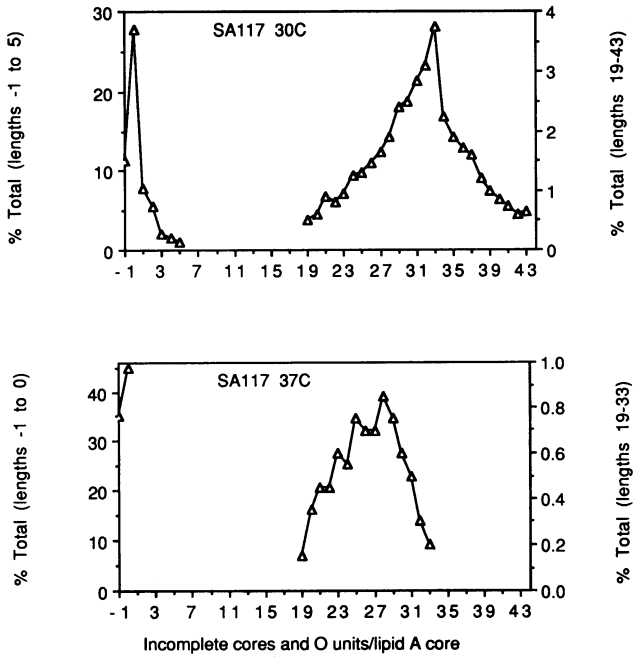

FIG. 4. LPS analysis of TS *rfa* strain SA117 grown at 30 or 37°C. Data were obtained as described in the legend to Fig. 2. The −1 value on the *x* axis represents incomplete core molecules.

incomplete lipid A-core increased to 40%, lipid A-cores lacking O antigen increased to 92%, and the average number of O-units per lipid A-core decreased to 1.5, but chains of near normal length were still made (18 to 22 O-units per lipid A-core [Table 2]). Again, we observe a predisposition for normal polymer lengths, even when the system is severely perturbed; this is incompatible with the stochastic model (see Discussion). Similar results were obtained for strain SA120 (Table 2), strain SL1373 (Table 2), and strain SL1377 (data not shown).

As a final note, we found that strain SL1197, which was isolated as a ligase-deficient mutant, is not totally deficient in ligase activity. Although very little O antigen is attached to completed lipid A-core (the average number of O-units per lipid A-core was <1), LPS molecules with a peak frequency of 20 to 22 O-units per lipid A-core were produced (Table 2).

DISCUSSION

Any model describing the mechanism of attaining the observed O-antigen distribution pattern in LPS must incorporate selectively at some stage along the biosynthetic pathway; a completely stochastic model is mathematically inconsistent with the multimodal patterns observed experimentally. Although we have not elucidated the precise stage in biosynthesis at which selectivity occurs, mathematical analysis, experimental observations, and previous contributions outlining the general biosynthetic pathway of LPS allow certain predictions to be made. We will now consider (i) implications and predictions of the mathematical model, (ii) consistency of the model with experimental observation, (iii) possible mechanisms of generating specificity in O-antigen distribution, and (iv) the relationship between O-antigen distribution and virulence as determined by the outcome of interaction with the complement defense system of a host.

The equations presented above completely describe O-antigen distribution in terms of probabilities associated with polymerization and ligation events. We are thus in a position (i) to test specific assumptions regarding the biosynthetic mechanism against experimental observations, (ii) subsequently to exclude those incompatible with observations, and (iii) to solve the equations and determine reaction probabilities when assumptions are compatible with observations. We first tested the assumption that neither polymerase nor ligase has a preference for specific lengths of O-antigen polymer, i.e., $p_k = p$ and $q_k = q$, so that reaction probabilities are independent of length. In this case,

$$\Gamma_k = \left(\frac{p}{p+q}\right)^{k-1} \Gamma_1$$

so that Γ_k decreases steadily with k. Thus a multimodal distribution is impossible.

Note, however, that by making the $\{p_k\}$ length dependent and keeping $q_k = q$, a multimodal distribution is possible. In fact, given such a distribution $\{\Gamma_k\}$, the reaction probabilities satisfy

$$p_k = \frac{q}{\Gamma_k} \cdot [1 - (\Gamma_0 + \Gamma_1 + \ldots \Gamma_k)]$$

In the general case, the reaction probabilities can be recov-

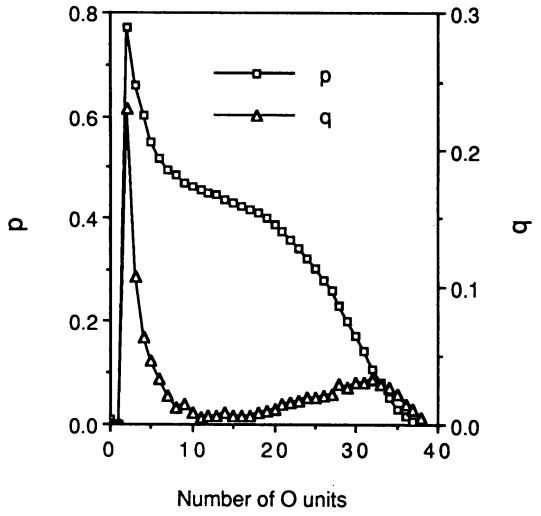

FIG. 5. Use of probability equations to determine p_k and q_k when T_k is defined. T_k was defined as equal for all T_k ($k = 0$ to $k = 38$). The observed outer membrane distribution from strain SL1027 grown at 37°C was used to input $\Gamma_0, \Gamma_1, \ldots \Gamma_{38}$. The p_k and q_k values required to generate the observed distribution in LPS from the outer membrane were calculated and plotted versus O-antigen chain length $T_k = T = 0.026$.

ered from the lipopolysaccharide distribution up to a set of constants that depend on the distribution of nascent polymers. We have

$$p_k = \frac{q_k}{\Gamma_k} \cdot [1 - (\Gamma_0 + \Gamma_1 + \ldots \Gamma_k)]$$

$$q_k = \lambda_k \cdot \Gamma_k/[1 - (\Gamma_0 + \Gamma_1)]$$

for some constants (λ_k), which can be chosen subject to the conditions $0 \leq p_k < 1$ and $0 \leq q_k < 1$. These arbitrary constants appear because there are $2N + 2$ reaction probabilities to be determined from $N + 1$ LPS frequencies, i.e., Γ_k, where $k = 0, 1, \ldots N$. If the nascent polymer distribution is also known, then the value of these constants can be determined, thereby yielding a unique set of reaction probabilities corresponding to the experimental data.

At this point, it becomes obvious that a vast array of p and q combinations can be selected, such that all p_k and q_k ($k = 0, 1, 2, \ldots N$) are not equal, but experimental observations are realized. The parameter which will dictate the precise values of p_k and q_k is $\{T_k\}$ ($k = 0, 1, 2, \ldots N$), the frequency distribution of polymerizing intermediates. At present, this frequency distribution is unknown, and repeated attempts in our laboratory to determine these values in wild-type cells have met with failure. In the absence of these data, we can only examine hypothetical cases, but cases which are selected on the basis of mechanistic simplicity and thus do not violate any intuitive criteria regarding enzymatic mechanisms in general. We have written programs to solve for p_k and q_k, after selecting a frequency distribution for polymerizing intermediates $\{T_k\}$ and inputting experimental data for $\Gamma_0, \Gamma_1, \ldots \Gamma_n$. The values of p_k and q_k ($k = 0, 1, \ldots 39$) are given (Fig. 5) for wild-type *S. typhimurium* grown at 37°C, when $T_k = T$ and the observed distribution of O-antigen units in LPS from the outer membrane are used. We stress that these data are presented only to show how the mathematical model can be used to generate p_k and q_k values, the patterns of which can be evaluated in terms of biosynthetic and enzymatic mechanisms of specificity. Rather than present specific test cases, we offer the following conclusions: (i) the simplest cases of endowing either polymerase or ligase with specificity can yield the observed outer membrane distributions with rational patterns for enzyme specificity versus length, and (ii) these simple models can explain experimental observations on partially defective *rfb* mutants, TS *rfa* mutants, and the leaky ligase mutant.

The *rfb* mutants are defective in the production of O-antigen monomers. As previously described, it was found that synthesis of long-chain molecules persisted contrary to expectation. Given a multimodal distribution in wild-type bacteria, an analysis of the model presented suggests an explanation of this phenomenon. If monomer production is suppressed, we can expect the reaction probabilities $\{p_k\}$ to be reduced, and in turn an altered lipopolysaccharide distribution $\{\Gamma_k'\}$ will be seen. In particular, suppose that $p_k' = p_k - \Delta$ ($k = 1, 2, \ldots N - 1$) are the mutant polymerization probabilities, $\Delta > 0$, a constant chosen so that $p_k' > 0$. $p_N' = 0$ as usual. Let us also suppose that the ligase reaction probabilities are unchanged, and for simplicity set $q_i = q$, $i > 0$ and $q_0 = 0$. The difference between the wild-type and mutant distributions can be calculated in terms of $\alpha_k = (\Gamma_k - \Gamma_k')/\Gamma_k$, the relative or percentage change in the frequency of molecules with chain length k. We can suppose this change is a decrease so that $\alpha_k \geq 0$. Now define γ as Γ_1'/Γ_1 and Θ_i as Γ_i/Γ_{i-1} ($i = 2, 3, \ldots$), and set

$$\delta\Theta_i = (\Theta_i - 1) \cdot \Delta/(p_i' + q)$$

Thus, $\delta\Theta_i$ is the change in Θ_i due to the change in reaction probabilities $\{p_k\}$.

The α_k for $k = 2, 3, \ldots N$ can be written (8) as $\alpha_k = 1 - \gamma\pi_k$ where

$$\pi_k = \left(1 + \frac{\delta\Theta_2}{\Theta_2}\right) \ldots \left(1 + \frac{\delta\Theta_i}{\Theta_i}\right) \ldots \left(1 + \frac{\delta\Theta_{k-1}}{\Theta_{k-1}}\right) \left(1 + \frac{\delta\Theta_k}{\Theta_k}\right)$$

The peaks or modes of the wild-type distribution are of particular interest. If k is a length corresponding to such a mode, the frequencies in some interval to the left of k, $k - 1 \leq i < k$ will be increasing. Thus, $\Theta_i > 1$ in this model. To the right of k, frequencies will be decreasing so that $\Theta_i < 1$ for $k < i < k + m$ for some m. Using the formula for α_k, we infer that α_i is decreasing for $k - 1 \leq i < k$ and increasing for $k < i < k + m$. Thus, corresponding to the mode at k, we have a valley, or low value, of α_i at $i = k$. This means that the lowest percentage decreases in frequency occur at those lengths that correspond to modes in the wild-type distribution. If long lengths appear as modes as they do in a multimodal distribution, they will persist in the mutant distribution since they decrease less than the less frequent shorter lengths. Thus, we can conclude that if there is a preference for the synthesis of certain chain lengths in wild-type bacteria, as is indeed the case since the distribution is multimodal, such preferences persist in the leaky mutants because modes in the distribution decrease the least.

This argument can be generalized to show the case where the $\{q_k\}$ are length dependent and $q_k \geq q_{k-1}$. This assumption means nascent polymers with longer chains are more likely

to undergo a ligase reaction than those with shorter chains. In this case,

$$\delta\Theta_i = \frac{\Delta}{p_i' + q_i} \cdot \left(\Theta_i - \frac{q_i}{q_i - 1}\right)$$

We have

$$\Theta_i = \frac{q_i \cdot T_i}{q_{i-1}\, T_{i-1}} \quad (5)$$

so if k also is a chain length that corresponds to a mode in the wild-type nascent polymer distribution, then $\delta\Theta_i > 0$ for $k - 1 \leq i < k$, because then $T_i \geq T_{-1}$. Thus, α_i will decrease for i in this interval. Since q_i/q_{i-1} by assumption, and $\Theta_1 < 1$ for $k < i \leq k + m$ (since k is a mode in the LPS distribution), we must have $\Theta i < q_i/q_{i-1}$, so $\delta\Theta_i < 0$. Hence, α_i will increase for i in this interval. We can conclude therefore, as we did before, that α_i has a low at $i = k$, or technically speaking, a local minimum at k. So by making the additional assumption that k is also a mode in the nascent polymer distribution, we have been able to deduce the persistence of long-chain synthesis under the more general conditions $q_i \geq q_{i-1}\, i \geq 1$. It is interesting to note that if the reaction probabilities for polymerase reaction are constant, that is, $p_i' = p$ for all $i = 0, 1, \ldots N - 1$, then by equation 1 and equation 5,

$$\Theta_i = \left(\frac{q_i}{q_{i-1}}\right)\left(\frac{p}{p + q_i}\right)$$

Hence, $\Theta_i < q_i/q_{i-1}$ so that $\delta\Theta_i < 0$ and α_i increases in the interval $k - 1 \leq i < k$. We cannot infer the persistence of modal lengths in this case. Indeed, since α_k must in fact increase with k, the longer lengths suffer the largest percentage decreases. This suggests that the selectivity needs to occur in the polymerase reaction, while no selectivity in the transferase reaction is consistent with long-chain persistence. The model has been able to highlight the asymmetry of the two activities.

There are two reports of novel chain length patterns which deserve comment. Certain strains of *Aeromonas salmonicida* and *Aeromonas hydrophila* contain LPS with highly preferred chain length patterns clustering around 18 and 10 O repeats, respectively (4). This case highlights the possibility that strong selectivity can occur in the transferase of some organisms, and in terms of the model, q_L approaches 1, where L is the preferred O-repeat lengths, and q_j approaches 0 for those chain lengths which do not appear in the final product. These preferred chain lengths may be required for proper assembly of other cell surface components (4). In addition, a mutant of *Salmonella anatum* was isolated and found to contain LPS with greater than normal O-antigen chain lengths (15). Those authors speculated that a leaky defect in core biosynthesis might result in longer O-antigen chains because of their longer residence time in the polymerizing pool, although we did not observe such results with leaky core mutants of *S. typhimurium*. However, there is no reason to assume that all gram-negative bacteria require the same O-antigen distribution patterns on their surface. In view of the important role of LPS in the structure, function, and assembly of the outer membrane, one could well imagine unique chain length requirements and organism-specific regulatory mechanisms for attaining the required patterns during growth.

Another alternative for generating multimodal distribution patterns which fit experimental data involves selectivity at the level of enzyme or substrate compartmentalization, rather than enzyme-substrate specificity. In the absence of enzyme-substrate specificity, one can intuitively predict and mathematically prove that (i) an increase in the level of lipid A-core or ligase activity leads to shorter O-antigen polymers, the converse being true for decreases, whereas (ii) an increase in polymerase or concentration of lipid-linked O monomers leads to longer O-antigen polymers, the converse being true for a decrease in these components of the overall reaction. If specific membrane regions involved in O-antigen synthesis were sequestered and stably differentiated with respect to a specific component of the overall reaction, then it follows that these regions could produce different distributions of O antigen attached to lipid A-core without involvement of enzyme-substrate specificity. Rather than elaborate on all possibilities, we now present our overall conclusions in relation to experimental observations of wild-type and mutant cells.

The simplest mechanism which explains our experimental results involves specificity of either polymerase or ligase for certain O-antigen polymer lengths. Severe inhibition (90%) of O-antigen monomer production (leaky *rfb* mutants) or severe inhibition (90%) of ligase activity or lipid A-core production would lead to very short or very long O-antigen chains, respectively, in mature LPS if purely stochastic events were to occur. This is certainly not the case (Fig. 3 and 4; Table 2). Since specificity alone can explain these data, we need not invoke changes in polymerase or ligase levels as effectors which regulate polymer length via a sensory system. Furthermore, when considering compartmentalization as a selectivity mechanism, we require (i) a mechanism for generating and maintaining compartmentalization and (ii) a sensory system for regulation of system components in response to perturbation of biosynthesis by mutation.

Nothing is known regarding the spatial organization of membrane-bound components involved in LPS biosynthesis. Macromolecular complexes may exist whereby the position of an enzyme influences its reaction kinetics, or the complexes might contain different concentrations of components (enzyme or substrate) which would alter product output. In view of recent data describing conformational analysis of O-antigen polymers (2), one must also consider transmission of information regarding polymer length to an enzyme via structural changes which may occur as the polymer grows in length. Recent work describing chemicoenzymatic synthesis of O-antigen polymers offers the possibility of examining this process in vitro (13). Other biological systems pertinent to a discussion of regulation of O-antigen distribution include regulation of fatty acid chain length (3), nascent lipomannan and cell wall polymer length (20), capsule polysaccharide polymer length (17), teichuronic acid polymer lengths (9), polyribitol phosphate chain length (5), and dolichol phosphate synthesis (1) to name a few.

Why do gram-negative bacteria make LPS with such precise patterns of O-antigen distribution? Rough mutants of *S. typhimurium* and *Escherichia coli* (lacking O antigen) are killed by complement (23), but the precise mechanism by which O-antigen protection is realized was not elucidated until recently (11). We now know that in the absence of antibody (i) the presence of O antigen precludes stable insertion of the C_{5-9} membrane attack complex and thus precludes killing (10), and (ii) that LPS with long polysaccharide chains is the primary target for covalent attachment of C_3 (11). Although divalent antibody to O antigen renders cells susceptible to complement killing and alters the site of covalent C_3 attachment (10), an increase in the percent

coverage of lipid A-core molecules will negate this effect and render cells resistant (6). Together these data reflect the importance of O-antigen distribution and describe in precise terms the mechanisms by which such distributions are attained and serve to evade the host immunodefense system.

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