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Lipopolysaccharide Layer Protection of Gram-Negative Bacteria Against Inhibition by Long-Chain Fatty Acids

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Growth, amino acid transport, and oxygen consumption of *Escherichia coli* and *Salmonella typhimurium* are inhibited by short-chain (C_2-C_6) but not by medium or long-chain fatty acids (C_{10}-C_{18}) at concentrations at which these processes are completely inhibited in *Bacillus subtilis*. The resistance of gram-negative organisms is not correlated with their ability to metabolize fatty acids, since an *E. coli* mutant unable to transport oleic acid is still resistant. However, mutants of both *E. coli* and *S. typhimurium* in which the lipopolysaccharide layer does not contain the residues beyond the 2-keto-3-deoxyoctonate core are inhibited by medium (C_{10}) but not by long-chain (C_{18}) fatty acids. Furthermore, removal of a portion of the lipopolysaccharide layer by ethylenediaminetetraacetate treatment renders the organisms sensitive to medium and partially sensitive to long-chain fatty acids. The intact lipopolysaccharide layer of gram-negative organisms apparently screens the cells against medium and long-chain fatty acids and prevents their accumulation on the inner cell membrane (site of amino acid transport) at inhibitory concentrations. These results are relevant to the use of antimicrobial food additives, and they allow the characterization of gram-positive versus gram-negative bacteria and their lipopolysaccharide mutants.

We have shown recently that fatty acids inhibit the growth and oxygen consumption of *Bacillus subtilis* in nutrient medium by inhibiting the transport of amino acids, keto acids, etc., through the cellular membrane (5). The effectiveness of inhibition increased with increasing fatty acid chain length. In contrast, the inhibitory effect on *Escherichia coli* increased only up to a fatty acid chain length of six, hexanoate being as effective as on *B. subtilis;* decanoate inhibited only at a 50-times higher concentration, while long-chain fatty acids had no effect. Two explanations for this discrepancy were considered.

(i) Since *E. coli* can utilize long-chain fatty acids as carbon source, it can convert them to the acyl-coenzyme A (CoA) derivative and then metabolize them by β-oxidation (7). *B. subtilis* cannot utilize them as carbon source and can metabolize only acetate at a high optimal concentration of 50 mM (3, 4). Conceivably, *E. coli* might be able to metabolize rapidly any long-chain fatty acids that attach to the membrane so that they would be effectively removed from the membrane before they could exert any significant inhibitory effect. If that were true, mutants unable to take up or metabolize long-chain fatty acids (7) should be inhibited by them.

(ii) Gram-negative bacteria are typically surrounded by a lipopolysaccharide (LPS) layer (10) which prevents the entry of compounds such as actinomycin D (6) and the action of lysozyme (11). This LPS layer can be partially removed by exposure to ethylenediaminetetraacetate (EDTA), and organisms then become sensitive to actinomycin, lysozyme, and other compounds (8). There are also mutants of *E. coli* (2) and *S. typhimurium* (1) in which the polysaccharide portion is partially or completely absent. Such mutants are much more sensitive than normal strains to carcinogens with high molecular weight (1). If the LPS layer should also prevent the entry of long-chain fatty acids, its partial opening in mutants or by EDTA treatment might make the cells more accessible to fatty acid inhibition.

This paper rules out the first explanation and

shows that the partial opening of the LPS layer in mutants or by EDTA treatment sensitizes the cells to fatty acids of intermediate chain length.

MATERIALS AND METHODS

Bacteria. The strains used are described in Table 1.

Medium and growth conditions. NBP medium contained 8 g of nutrient broth (Difco) per liter and 0.1 M potassium phosphate, pH 6.5. NBP plates contained NBP medium plus 1.5% agar (Difco). The cultures were shaken at 120 strokes per min at 37 C in Erlenmeyer flasks containing ⅕ of their volume of NBP medium.

Lipophilic acids. *n*-Hexanoic acid (C_6) was prepared in distilled water and neutralized to pH 6.5 with KOH. Triton X-100 was dissolved in distilled water. *n*-Octanoic acid (C_8), *n*-decanoic acid (C_{10}), myristic acid (C_{14}), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), and 2,4-dinitrophenol were dissolved in dimethylsulfoxide and added to the NBP medium so that the final concentration of dimethylsulfoxide was equal or below 0.4%. This concentration of dimethylsulfoxide did not inhibit growth of any of the strains used. Nevertheless, the same concentration of dimethylsulfoxide was also added to the control experiments.

Growth and oxygen consumption. Measurement of the inhibition of growth (5) and oxygen consumption (12) of whole cells by fatty acids have been described.

Glycine uptake. Cells grown to an absorbancy at 600 nm (A_{600}) of 0.5 were harvested, washed with 0.1 M phosphate buffer (pH 6.5) containing 100 μg of chloramphenicol per ml, resuspended in the same buffer to an A_{600} of 0.3, and incubated at 30 C for 5 min. Samples (0.5 ml) were placed into test tubes (84 by 15 mm) containing fatty acid or solvent alone (for control), and the uptake was started by the addition of ^{14}C-glycine (0.1 mM, 33 nCi/ml final concentration). The uptake was linear for at least 5 min. For the work reported here, the cells were incubated at 30 C with constant magnetic stirring, and the reaction was stopped after 2 min by the addition of 3 ml 0.1 M potassium phosphate, pH 6.5, containing 2 mM $MgCl_2$, and the suspension was filtered immediately through a membrane filter (Millipore Corp., pore size 0.45 μm). The cells were washed twice with 3 ml of the above buffer, dried, and counted (13). The zero time values were obtained by adding the glycine after the above buffer.

Preparation of EDTA-treated cells. Cells grown to an A_{600} of 0.5 were harvested and treated with 0.5 mM EDTA for 2 min according to Leive (8). The treated cells were diluted with NBP medium for the measurement of growth and O_2 consumption, and with 0.1 M potassium phosphate, pH 6.5, containing chloramphenicol (100 μg/ml) to prevent amino acid incorporation into protein, for uptake studies. Control cells were prepared by the same procedure but without EDTA.

RESULTS

Inhibition of standard strains of B. subtilis and E. coli by fatty acids. Standard strains of *B. subtilis* and *E. coli* were grown in NBP to an A_{600} of 0.5 and then exposed to different fatty acids. Figure 1 shows the inhibition of growth and oxygen consumption. Whereas both organisms were equally sensitive to short-chain fatty acids up to hexanoate, *E. coli* was increasingly less sensitive than *B. subtilis* with increasing chain length. About twice as much octanoate was needed to inhibit *E. coli* to the same extent as *B. subtilis*, while for decanoate the amount needed was already 50 times higher. For oleate, no inhibition was observed up to 5 mM concentration. At these high concentrations, the long-chain fatty acids remained mostly insoluble. Their solubility could be increased by the addition of 0.4% Triton X-100, which does not inhibit the growth of *E. coli*. However, the better-solubilized long-chain fatty acids still did not cause any significant growth inhibition. Triton X-100 lyses *B. subtilis* even when it is

TABLE 1. *Bacterial strains*

Strain	Organism	LPS	Source and description
60015	*B. subtilis*	Absent	Derived from strain 168 of Spizizen; *metC*, *trpC*; rough
50019	*E. coli*	Normal	N. Fiil, K-12, *argA*, *met*, *rel*$^+$; inducible for oleic acid uptake, smooth
50008	*E. coli*	Normal	P. Overath, K-12Ymel *dec-16*; constitutive for the enzymes of fatty acid degradation and for oleic acid uptake, smooth
50009	*E. coli*	Normal	P. Overath, *dec-16 old*D88; acyl-CoA synthetase negative, smooth
50010	*E. coli*	Normal	E. C. Heath, 0111:B4; smooth
50011	*E. coli*	Defective	E. C. Heath, RC-59, *rfa*; no heptose in LPS, blocked from KDO core out
50016	*S. typhimurium*	Normal	B. N. Ames, *his*D3052; smooth
50015	*S. typhimurium*	Defective	B. N. Ames, TA1978, *galE*, *his*D3052, *rfa*; blocked from KDO core out, deep rough

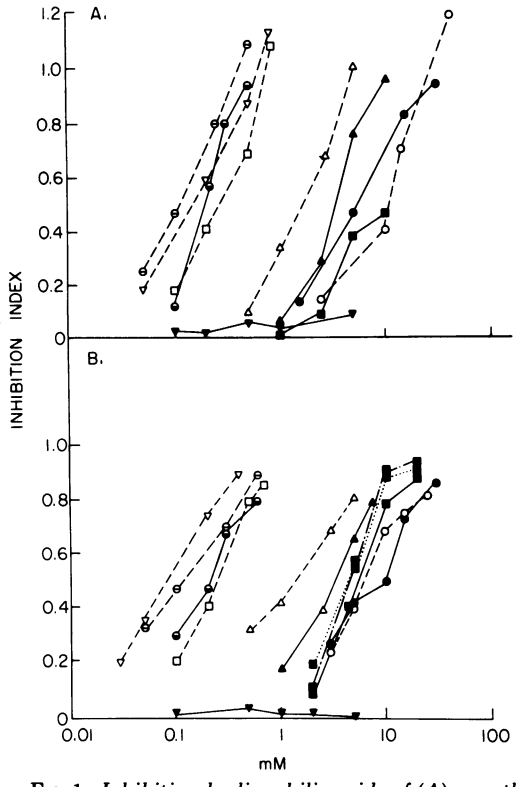

FIG. 1. *Inhibition by lipophilic acids of (A) growth and (B) oxygen consumption. E. coli strains (closed symbols):* (——) *50019,* (·····) *50008,* (-·-·-) *50009 (for growth of 50008 and 50009, see Fig. 2). B. subtilis strain (open symbols):* (----) *60015.* (○●) C_6, (△▲) C_8, (□■) C_{10}, (▽▼) *oleate, and* (⊖⊖) *DNP. The inhibition index:* $I = 1 - \Delta A/\Delta A_c$, *where* $\Delta A = A_{600}$ *at 1 h after addition of compound minus* A_{600} *at time of addition;* ΔA_c *is the difference of* A_{600} *in the control culture (no compound added).*

used at concentrations as low as 0.05%, which is not sufficient to significantly solubilize long-chain fatty acids. The protection of gram-negative bacteria gainst Triton X-100 presumably is due to the presence of the LPS layer and is likely to be reduced or to disappear when mutants or treatments are used that open up this layer. For this reason, Triton X-100 was not used further except when specifically mentioned.

The uptake of L-glycine was similarly inhibited by all fatty acids in *B. subtilis*, whereas only the short-chain fatty acids were effective in *E. coli* (Table 2).

Comparison of E. coli mutants differing in fatty acid metabolism. To determine whether the metabolism of fatty acids influences their inhibitory effect on *E. coli*, we have employed two mutants (Table 1). One mutant (50008) is constitutive in its ability to convert the fatty acid to its acyl-CoA derivative and to degrade it by the beta-oxidation enzymes. The other mutant (50009) was derived from this strain but lacks the acyl-CoA synthetase activity and is, therefore, unable to take up or metabolize long-chain fatty acids (7). Figure 2 shows that both strains were equally inhibited by hexanoate (20 mM), whereas they were both not inhibited by decanoate (2 mM) or oleate (1 mM)) at concentrations which completely inhibit the growth of *B. subtilis*. The inhibition of oxygen consumption by decanoate also showed the same concentration dependence for both mutants as well as for the earlier used strains of *E. coli* (50019) in which the metabolism of long-chain fatty acids is inducible (Fig. 1). We

TABLE 2. *Inhibition of glycine uptake*

Strain	Inhibition Index[a]		
	Hexanoate (20 m)	Decanoate (2 mM)	Oleate (1 mM)
60015	0.78	0.99	0.96
50008	0.79	0.10	0.09
50009	0.78	0.12	-0.04
50019	0.63	0.04	-0.04

[a] Minus sign (-) indicates an apparent (but not real) stimulation of uptake rate over control value (due to experimental variability).

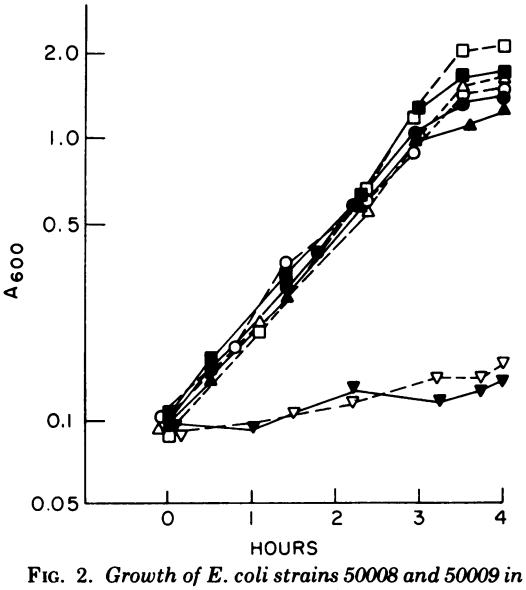

FIG. 2. *Growth of E. coli strains 50008 and 50009 in the presence of fatty acids. Symbols: open symbols with dotted line = strain 50008, closed symbols with solid line = strain 50009;* (○●) *no addition;* (▽▼) *20 mM* C_6; (△▲) *2 mM* C_{10}; *and* (□■) *1 mM oleate.*

conclude, therefore, that the inability of fatty acids to inhibit gram-negative bacteria does not result from the ability of the cells to take up or metabolize the fatty acid.

Fatty acid inhibition of mutants defective in the LPS layer. The function of the LPS layer in regard to the inhibition by fatty acids was examined in two strains of *E. coli*, one of which (50010) has the normal LPS, whereas the other (50011) cannot incorporate heptose into the LPS, i.e., it is blocked beyond the 2-keto-3-deoxyoctonate (KDO) core (E. C. Heath, personal communication). Whereas the growth of both strains in NBP liquid was inhibited by 20 mM hexanoate, only that of the LPS mutant (50011) was inhibited by 2 mM decanoate; however, 1 mM oleate did not inhibit either strain, although it completely inhibited the growth of *B. subtilis* (Table 3). The same observation was made for two *S. typhimurium* strains, one of which (50016) has the normal LPS, whereas the other (50015) has a deletion which eliminates the whole galactose operon and, in addition, lacks some enzyme needed for the elongation of the polysaccharide beyond the KDO core (1). Again, growth of the normal strain was not inhibited by 2 mM decanoate, while that of the LPS mutant was completely inhibited (Table 3). Since the latter strain (50015) requires histidine for growth, several histidine-independent revertants were isolated from it as control; their growth was also inhibited by 2 mM decanoate. Oleate (1 mM), however, did not inhibit the growth of any of these gram-negative strains.

On NBP plates containing 2 mM decanoate, the KDO mutant of *S. typhimurium* and the *B. subtilis* strain could not grow, just as NBP liquid, but the KDO mutant of *E. coli* grew as well as on plates having no decanoate (Table 3). Only with 5 mM decanoate in NBP plates could the KDO mutant of *E. coli* also not grow, while the normal strains of *E. coli* or *S. typhimurium* grew well. Triton X-100 was also less effective on plates than in liquid medium. Triton X-100 (1%) in NBP plates did not inhibit the growth of the KDO mutants, while even 0.4% completely inhibited that of *B. subtilis* (Table 3). The growth of both KDO mutants was inhibited in NBP liquid containing 0.4% Triton X-100, while the growth of normal *E. coli* and *S. typhimurium* was unaffected (Table 3).

The effect of different decanoate concentrations on oxygen consumption and glycine uptake by the above strains is shown in Fig. 3. Both reactions were clearly more inhibited in the two deficient mutants than in the two strains having the normal LPS. Nevertheless, *B. subtilis* was still more sensitive than the LPS mutants of *E. coli* or *S. typhimurium*.

Sensitization of gram-negative cells by EDTA treatment. To remove part of the LPS layer from *E. coli* cells, a strain (50009) containing the normal LPS layer was grown to an A_{600} of about 0.5, washed, and treated by EDTA as described in Materials and Methods. Figure 4 shows the growth response of treated and untreated cells to decanoate and linoleate or oleate. The cells were clearly sensitized by the EDTA treatment so that they showed either a growth inhibition or cell lysis. At some later time, depending on the fatty acid concentration, growth resumed, apparently because some of the cells had reformed their LPS layer and thus had become resistant again. While this result agreed with the above mutant observations, concentrations of linoleate or oleate exceeding 0.5 mM were increasingly less effective

TABLE 3. *Inhibition of growth*

Strain	Organism	LPS layer	Growth[a] NBP plate			Inhibition index[b] NBP liquid			
			No addition	Decanoate (2 mM/5 mM)	Triton X-100 (1%)	Hexanoate (20 mM)	Decanoate (2 mM)	Oleate[c] (1 mM)	Triton X-100 (0.4%)
50008	*E. coli*	Normal	+	++	+	0.70	0.07	−0.08	0.02
50009	*E. coli*	Normal	+	++	+	0.73	0.06	0.06	0.05
50010	*E. coli*	Normal	+	++	+	0.83	0.09	−0.36	0.10
50019	*E. coli*	Normal	+	++	+	0.88	0.08	0.05	0.04
50016	*S. typhimurium*	Normal	+	++	+	0.78	0.12	−0.30	0.05
50011	*E. coli*	Defective	+	+−	+	0.86	1.70	−0.23	1.35
50015	*S. typhimurium*	Defective	+	−−	+	0.85	1.37	−0.40	1.60
60015	*B. subtilis*	Absent	+	−−	−[d]	0.96	1.32	1.20	1.50

[a] Growth on NBP plates after incubation at 37 C for 16 h.
[b] Defined in legend to Fig. 1.
[c] Minus sign (−) indicates an increase in the growth rate.
[d] Even 0.4% was sufficient to inhibit growth.

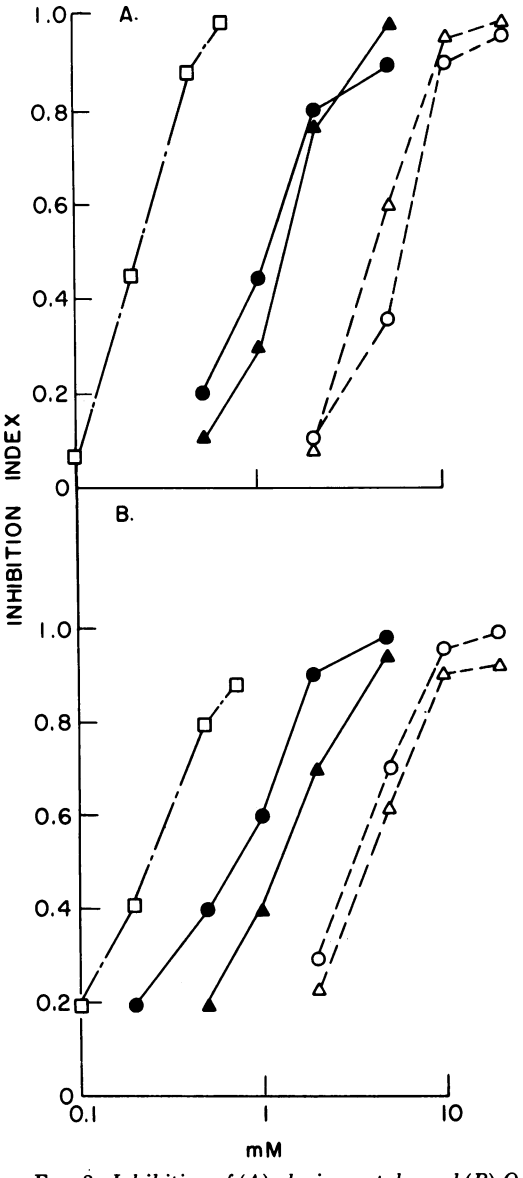

FIG. 3. *Inhibition of (A) glycine uptake and (B) O_2 consumption by n-decanoate. E. coli strains: O, 50010; ●, 50011. S. typhimurium strains: Δ, 50016; ▲, 50015. B. subtilis strain: □, 60015.*

in causing growth inhibition or cell lysis, as if these concentrations protected the cells by forming a lipid layer around them.

Since the effect of EDTA on gram-negative cells was rapidly repaired in growth media, it should be easier to measure it under conditions under which growth does not resume. For this purpose the uptake of glycine was determined in EDTA-treated cells in the presence of chloramphenicol. Hexanoate, decanoate, and myristate

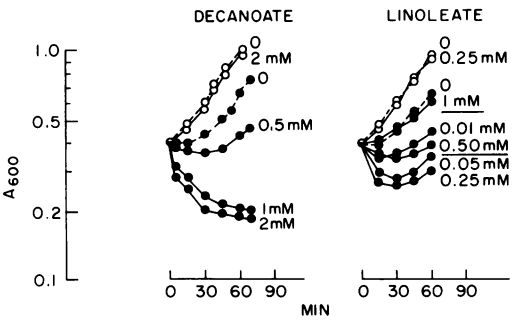

FIG. 4. *Growth of EDTA-treated cells of E. coli strain 50009 in the presence of fatty acids. The cells, with or without EDTA treatment, were suspended in NBP medium at an A_{600} of 0.4. Samples (20 ml) were placed in Erlenmeyer flasks containing n-decanoate, linoleate, or their solvent dimethylsulfoxide alone. The cultures were shaken at 37 C, and the A_{600} was followed. Symbols: O, control cells; ●, EDTA-treated cells, ----, dimethylsulfoxide (0.1%); ——, fatty acid. Inhibitory effect of oleate was similar to that of linoleate.*

inhibited with increasing effectiveness, and even oleate showed up to 50% inhibition (Fig. 5).

EDTA treatment also sensitized the oxygen consumption of *E. coli* to the long-chain fatty acids (Table 4).

DISCUSSION

It is apparent from our results that the LPS layer of gram-negative bacteria protects them against the inhibition by intermediate and long-chain fatty acids. This protection probably is essential for the survival of bacteria in the intestinal tract in which such fatty acids are produced by the digestion of fats. However, these bacteria are not resistant to short-chain fatty acids up to hexanoate and only slightly resistant against octanoate. Short-chain fatty acids, properly neutralized, may therefore be useful for the treatment of infection by gram-negative bacteria.

In liquid nutrient broth, mutants in which the LPS is stripped down to the KDO core can be inhibited to the same extent as the normal strains by about ¼ of the decanoate concentration, irrespective of the process whose inhibition is measured (growth, oxygen consumption, or amino acid uptake). *B. subtilis* is still more sensitive than either of these strains. On nutrient plates, inhibition of the KDO mutants requires a higher decanoate concentration (5 mM) either because some decanoate binds to the agar or because the solid agar surface retards cell lysis. Both plates or liquid growth

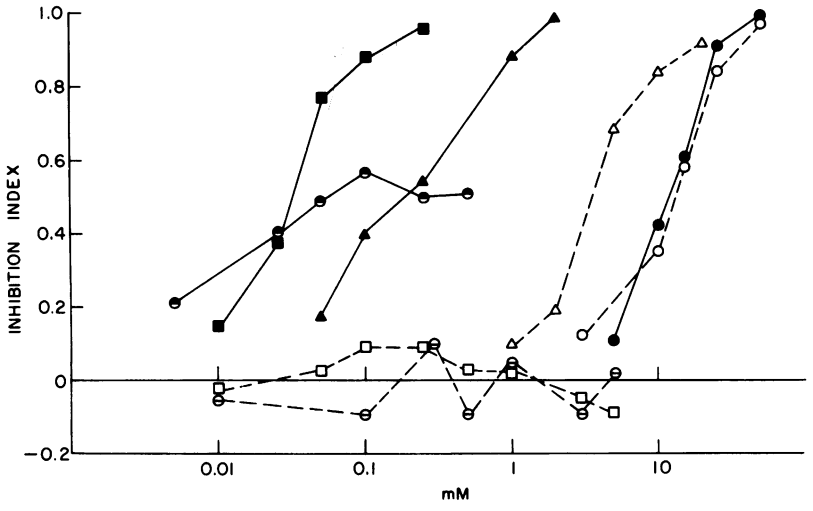

FIG. 5. *Inhibition by fatty acids of glycine uptake by E. coli strain 50009. Symbols: open symbols with dotted lines, control cells; closed symbols with solid line, EDTA-treated cells;* ○●, C_8; △▲, C_{10}; □■, C_{14}; *and* ⊖⬬, *oleate.*

TABLE 4. *Inhibition of oxygen consumption*[a]

Cells	Inhibition index		
	Decanoate (2 mM)	Myristate (1.5 mM)	Oleate (1 mM)
Control	0.02	0	0
EDTA treated	0.95	0.91	0.82

[a] *E. coli* strain (50009) was grown in NBP to an A_{600} of 0.5. After EDTA treatment (or without it = control), the rate of oxygen consumption was measured in presence of the stated compounds.

media can be used to distinguish between normal gram-negative strains and their LPS-defective mutants. However, oleate or linoleate do not inhibit the LPS mutants (or the normal strains), whereas *B. subtilis* is completely inhibited by a 1 mM concentration of these compounds. This finding can be used to distinguish between gram-positive and -negative strains. The lack of an LPS layer in gram-positive strains may also render them particularly useful for the detection of mutagens or carcinogens with high molecular weight.

The normal gram-negative strains can be sensitized to fatty acids by treatment with EDTA, which removes up to 20% of the LPS and up to 15% of phosphatidylethanolamine (9). Following this treatment, the uptake of amino acids and the consumption of oxygen are inhibited even by the long-chain fatty acids. Growth is also transiently inhibited (or some cells are lysed) until a sufficient number of cells have reformed their LPS layer to become again resistant; then growth resumes. The holes produced in the LPS layer by EDTA treatment apparently are slightly larger than those present in LPS mutants that are stripped down to the KDO core, because the mutants do not become sensitive to long-chain fatty acids. EDTA is sometimes used as a food additive in addition to lipophilic acids, possibly because it enhances the inhibitory effect of the lipophilic acids on gram-negative organisms; whether consumption of EDTA is harmless to humans will depend on the amount of metals present in the gut.

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