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# Phosphatidylserine Found in Intestinal Mucus Serves as a Sole Source of Carbon and Nitrogen for Salmonellae and *Escherichia coli*

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***Salmonella choleraesuis*** **(a pig pathogen),** ***Salmonella typhimurium*** **(a virulent strain in mice), and three strains of** ***Escherichia coli*** **(including a human enterohemorrhagic strain, a human urinary tract isolate, and a human fecal isolate) grew as well in vitro utilizing the lipids derived from mouse cecal mucus as the sole source of carbon and nitrogen as they did in mouse crude cecal mucus. Further analysis of the total lipid extracts of mucus dialysates showed that the acidic lipid fraction supported growth nearly as well as the total lipid fraction. Interestingly, among the many purified acidic lipids from mucus which were tested and analyzed, including several phospholipids, only phosphatidylserine was found to support the growth of all of these enteric bacteria, including** ***Salmonella milwaukee*****, a human pathogen. The possible role of growth on pure phosphatidylserine in the pathogenesis of salmonellae is discussed.**

The mucus gel of the gastrointestinal tracts of humans and animals has been suggested to function as an important barrier to bacterial infection and invasion by entrapping enteric pathogens and inhibiting their attachment to the underlying mucosal cell surface (2). The mucus gel has also been suggested to serve as an important substrate for commensal mucin-degrading bacteria (11, 18) and as colonization sites for other enteric bacteria (6, 7, 13, 16). Mucus occurs as a viscous, sticky, water-insoluble gel which is composed of a high-molecular-weight glycoprotein (mucin) and smaller proteins and glycoproteins, although it is now known that many lipids are also associated noncovalently with the gel layer (17, 21). It has recently been shown that for *Escherichia coli* and *Salmonella typhimurium* to colonize the colons of streptomycin-treated mice, they must be able to grow in the mucus layer (12, 20). The data that we present here indicate that several serovars of *Salmonella* and *E. coli* are able to utilize lipids existing in the mucus layer for growth, of which phosphatidylserine was found to serve as the sole source of both carbon and nitrogen for growth for all of these enteric bacteria.

Dialysates of male CD-1 mouse cecal mucus, which support growth of *E. coli* and *S. typhimurium* as well as crude cecal mucus does in vitro (8), were used as the source of cecal mucus lipids. Lipids were extracted from mucus dialysates in chloroform-methanol-water (4:8:3) (19) or in chloroform-methanol (2:1) (17) and separated into neutral and acidic fractions by high-performance liquid chromatography (HPLC) on silica Iatrobeads (1) and by anion-exchange chromatography on DEAE-cellulose, respectively (15). Each lipid fraction was dried under nitrogen and dispersed by sonication into HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-Hanks buffer, pH 7.4, at 1 mg/ml, and the samples were inoculated with about $2 \times 10^4$ CFU of bacteria per ml. As shown in Table 1, *S. typhimurium* SL7312, a virulent strain, and *E. coli* F-18, a normal human fecal strain (4), grew essentially as well utilizing cecal mucus total lipids as the sole source of carbon and nitrogen as in the cecal mucus dialysate itself for the first 6 h of incubation at 37°C. Moreover, in the presence of total cecal mucus lipids, levels (CFU per milliliter) of about 20% of that reached in cecal mucus dialysates at 24 h were observed (Table 1). The cecal mucus acidic lipids were also effective in promoting growth, whereas the neutral lipid fraction was relatively ineffective (Table 1). Interestingly, the pattern of growth of *Salmonella choleraesuis* 735B, a pig pathogen, was identical to that of *S. typhimurium* in cecal mucus total lipids and acidic lipids (data not shown), and *E. coli* 933 EDL (O157:H7), a human enterohemorrhagic strain, and *E. coli* A55 (O6, $Pap^+$ $Hly^+$), a pathogen causing human urinary tract infections, grew in the total and acidic fractions essentially identically to *E. coli* F-18 (data not shown).

Gangliosides, sulfatides, and phospholipids make up the majority of acidic lipids of mammalian cells, whereas the majority of neutral lipids are glycosphingolipids (10). Therefore, several phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin [Avanti Polar Lipids, Inc., Alabaster, Ala.]), purified mixtures of standard monosialogangliosides ($GM_1$, $GM_2$, and $GM_3$ [BioCarb Chemicals, Lund, Sweden]), disialogangliosides ($GD_{1a}$, $GD_{1b}$, $GD_2$, and $GD_3$ [BioCarb Chemicals]), and a standard mixture of neutral glycosphingolipids which contained galactosylceramide, lactosylceramide, globotriaosylceramide, globoside, and Forssman glycolipid (BioCarb Chemicals) were tested for their ability to support the growth of *E. coli* and salmonellae. Neither the *E. coli* nor the *Salmonella* strains grew to any great extent on the mixtures of known standard gangliosides or neutral glycosphingolipids (Table 2). Of the purified phospholipids used as the sole source of carbon and nitrogen, only phosphatidylserine supported growth of *S. typhimurium* SL7312 and *E. coli* F-18 to any great extent (Table 3). The other phospholipids were unable to support the growth of these bacteria. Similarly, phosphatidic acid, a combination of phosphatidic acid and L-serine, L-serine

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TABLE 1. Growth of *S. typhimurium* SL7312 and *E. coli* F-18 on cecal mucus lipids as the sole source of carbon and nitrogen

| Substrate | CFU relative to input CFU[a] at: | | | |
|---|---|---|---|---|
| | 6 h | | 24 h | |
| | F-18 | SL7312 | F-18 | SL7312 |
| Dialysate | 264 | 1,506 | 6,436 | 12,875 |
| Total lipids[b] | 620 | 916 | 1,551 | 2,125 |
| Acidic lipids | 158 | 416 | 437 | 625 |
| Neutral lipids | 0.14 | 1.83 | 0.02 | 4.44 |
| HH[c] | 0.55 | 0.85 | 2.53 | 6.55 |

[a] Input CFU per milliliter: *E. coli* F-18, $8.1 \times 10^4$; *S. typhimurium* SL7312, $6.6 \times 10^4$.
[b] Lipids were dispersed in HEPES-Hanks buffer, pH 7.4, at a concentration of 1 mg/ml.
[c] HEPES-Hanks buffer, pH 7.4.

alone, and *O*-phospho-L-serine (Sigma Chemical Co., St. Louis, Mo.) did not support growth, although L-serine and *O*-phospho-L-serine did allow low levels of growth by 24 h (Table 3). Together, the data suggest that phosphatidylserine itself is transported and then metabolized. The human enterohemorrhagic *E. coli* and urinary tract infection-causing strains also utilized phosphatidylserine for growth to the same extent as *E. coli* F-18 (data not shown), and *S. choleraesuis* 735B utilized phosphatidylserine to the same extent as *S. typhimurium* SL7312 (Table 4). Similarly, *S. milwaukee*, a human pathogen, utilized phosphatidylserine for growth as efficiently as the other *Salmonella* strains (data not shown). Phosphatidylserine obtained from Sigma Chemical Co. (bovine brain, 98% pure) or from Avanti Polar Lipids (bovine brain, >99% pure) supported growth of the *Salmonella* and *E. coli* strains in a similar fashion (data not shown).

To determine the amount of phosphatidylserine present in mouse cecal mucus, the acidic lipid fraction was hydrolyzed with 6 N HCl for 18 h at 120°C, and released serine was measured by the PICO-TAG method with phenyl isothiocyanate (5). Derivatized serine was separated and quantified by reverse-phase HPLC with a Supelcosil LC-18 column (3). As shown in Fig. 1, the HPLC profile obtained from the acidic lipid fraction (Fig. 1b) included a peak with a retention value identical to that of the authentic phosphatidylserine standard (Fig. 1a). Comparative analysis of the integrated peak areas indicated that 33.5 μg of phosphatidylserine per mg of total lipid extracted from mouse cecal mucus was present. In addition, cecal mucus dialysate, total lipids, and phosphatidylserine were treated with phospholipase $A_2$ (Sigma Chemical Co.) to cleave the fatty acid from the 2 position of the phospholipids and with phospholipase D (Sigma Chemical Co.) to hydrolyze the two fatty acids from each phospholipid. The enzyme-treated samples were dialyzed against water, and each enzyme-free dialysate was lyophilized, resuspended in water to its original volume, and inoculated at about $5 \times 10^4$ CFU/ml with either *S. typhimurium* SL7312 or *E. coli* F-18. As shown in Table 5, the enzyme-treated samples did not support growth nearly as well as the untreated samples. Therefore, these data further support the view that phospholipids, and more specifically, phosphatidylserine, are primarily responsible for *Salmonella* and *E. coli* growth in mouse cecal mucus dialysates.

*E. coli* and salmonellae are known to utilize long-chain fatty acids as the sole source of carbon after a long lag period (14) and to use L-serine as the sole source of carbon and nitrogen (9). To our knowledge, however, nothing regarding the ability of salmonellae and *E. coli* to utilize lipids as the sole source of carbon and nitrogen is known. In the present study, we have focused on those lipids present in cecal

TABLE 2. Growth of *E. coli* F-18 and *S. typhimurium* SL7312 on a mixture of standard acidic glycosphingolipids (gangliosides) and neutral glycosphingolipids

| Substrate | CFU relative to input CFU[a] at: | | | |
|---|---|---|---|---|
| | 6 h | | 24 h | |
| | F-18 | SL7312 | F-18 | SL7312 |
| Monosialogangliosides[b] | 0.69 | 0.89 | 0.10 | 3.25 |
| Disialogangliosides | 0.75 | 1.52 | 0.02 | 5.45 |
| Neutral glycosphingolipids | 0.92 | 1.39 | <0.02 | 5.16 |
| HH[c] | 0.85 | 0.81 | 0.21 | 1.69 |

[a] Input CFU per milliliter: *E. coli* F-18, $5.2 \times 10^4$; *S. typhimurium* SL7312, $6.4 \times 10^4$, where data are presented as the ratios of CFU at 6 and 24 h to the input CFU.
[b] Mixtures of purified standards were dispersed in HEPES-Hanks buffer, pH 7.4, at a concentration of 1 mg/ml.
[c] HEPES-Hanks buffer, pH 7.4.

TABLE 3. Growth of *S. typhimurium* SL7312 and *E. coli* F-18 on selected purified phospholipids

| Substrate[a] | CFU relative to input CFU[b] at: | | | |
|---|---|---|---|---|
| | 6 h | | 24 h | |
| | F-18 | SL7312 | F-18 | SL7312 |
| Phosphatidylserine | 6.78 | 21.91 | 203 | 1,319 |
| Phosphatidylcholine | 1.70 | 1.10 | 6.65 | 3.47 |
| Phosphatidylethanolamine | 0.74 | 0.49 | 0.52 | 0.70 |
| Phosphatidylinositol | 1.60 | 0.66 | 0.91 | 0.51 |
| Sphingomyelin | 0.65 | 0.36 | 0.30 | 0.12 |
| Phosphatidic acid + L-serine | 1.26 | 0.55 | 0.52 | 0.19 |
| Choline chloride | 3.42 | 2.44 | 2.63 | 4.71 |
| L-Serine | 1.62 | 1.71 | 21.53 | 36.06 |
| *O*-Phospho-L-serine | 2.41 | 3.62 | 31.13 | 36.52 |
| HH[c] | 1.39 | 0.78 | 0.74 | 0.45 |

[a] Unless otherwise noted, all substrates were suspended in HEPES-Hanks buffer, pH 7.4, at 1 mg/ml.
[b] Input CFU per milliliter: *E. coli* F-18, $2.3 \times 10^4$; *S. typhimurium* SL7312, $4.7 \times 10^4$.
[c] HEPES-Hanks buffer, pH 7.4.

TABLE 4. Growth of *S. typhimurium* SL7312 and *S. choleraesuis* 735B on selected purified phospholipids

| Substrate[a] | CFU relative to input CFU[b] at: | | | |
|---|---|---|---|---|
| | 6 h | | 24 h | |
| | SL7312 | 735B | SL7312 | 735B |
| Phosphatidylserine | 29.18 | 28.10 | 222 | 533 |
| Phosphatidylcholine | 2.06 | 5.48 | 3.10 | 5.28 |
| Phosphatidylethanolamine | 0.43 | 0.81 | 1.42 | 1.95 |
| Phosphatidylinositol | 0.43 | 1.24 | 0.80 | 3.38 |
| Phosphatidic acid | 0.20 | 1.38 | 1.04 | 2.33 |
| HH[c] | 0.37 | 1.48 | 0.88 | 2.48 |

[a] Phospholipids were dispersed in HEPES-Hanks buffer, pH 7.4, at a concentration of 1 mg/ml.
[b] Input CFU per milliliter: *S. typhimurium* SL7312, $4.9 \times 10^4$; *S. choleraesuis* 735B, $2.1 \times 10^4$.
[c] HEPES-Hanks buffer, pH 7.4.

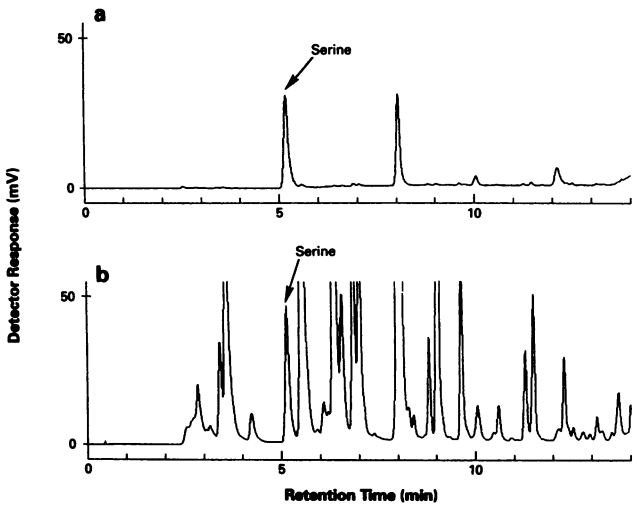


FIG. 1. Results of HPLC of serine hydrolytically released from phosphatidylserine. Standard phosphatidylserine (1 μg) (a) and the acidic lipid fraction of mouse cecal mucus (20 μg) (b) were hydrolyzed and derivatized with phenyl isothiocyanate and were injected onto a Supelcosil LC-18 column (250 by 4.6 mm). The column was eluted with a linear gradient of ammonium acetate-trimethylamine as described previously (3) by using a UV detector operated at 254 nm.

mucus preparations and have demonstrated that salmonellae and *E. coli* are capable of utilizing cecal mucus total lipids, the corresponding acidic lipid fraction, and phosphatidylserine as the sole sources of carbon and nitrogen and that they do so without an extended lag period. Moreover, in the case of *S. typhimurium*, it will be of interest to determine whether the ability to utilize phosphatidylserine is required for pathogenesis in the mouse intestine. For example, it is possible that the ability to metabolize the phosphatidylserine present in intestinal epithelial cell membranes is required for successful invasion by salmonellae of intestinal epithelial cells. Interestingly, these findings may also explain why salmonellae survive and grow in chicken eggs, which are rich in phospholipids, including phosphatidylserine.

TABLE 5. Effect of phospholipase treatment on growth of *S. typhimurium* SL7312 and *E. coli* F-18

| Strain | Phospholipase[a] | CFU relative to input CFU[b] in the indicated substrate | | |
|---|---|---|---|---|
| | | Dialysate | Total lipids | Phosphatidylserine |
| SL7312 | None | 388 | 162 | 43.3 |
| | $A_2$ | 2.52 | 1.02 | 1.81 |
| | D | 2.28 | 2.74 | 4.05 |
| F-18 | None | 479 | 221 | 87.2 |
| | $A_2$ | 39.7 | 10.7 | 4.14 |
| | D | 45.5 | 5.51 | 5.86 |

[a] Samples were treated with either none or 1 U of each phospholipase for 6 h at 37°C. Untreated samples were incubated without enzymes for 6 h at 37°C and then were dialyzed, lyophilized, resuspended, and inoculated identically to the phospholipase-treated samples.

[b] Input CFU per milliliter: *S. typhimurium* SL7312, $4.2 \times 10^4$; *E. coli* F-18, $2.9 \times 10^4$. Incubation was at 37°C for 6 h.

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## ADDENDUM IN PROOF

Data obtained after acceptance of this paper show that *E. coli* and salmonellae can also utilize phosphatidylserine as the sole source of phosphate for growth.

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