Growth of Escherichia coli K88 in Piglet Ileal Mucus: Protein Expression as an Indicator of Type of Metabolism

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The physiological and molecular responses of enterotoxigenic *Escherichia coli* K88 strain Bd 1107/7508 during growth in piglet ileal mucus and lipids extracted from mucus were studied in terms of growth rate, protein expression, and rate of heat production. *E. coli* K88 multiplied at maximum speed in mucus and in lipids extracted from mucus. By two-dimensional gel electrophoresis of $^{[35S]}$methionine-labelled cells, it was demonstrated that the synthesis of a subclass of 13 proteins was changed at least fourfold during exponential growth in mucus compared with growth in M9 minimal medium. Ten of these proteins were repressed, while three were induced, and one of the induced proteins was identified as heat shock protein GroEL. Furthermore, two-dimensional analysis of *E. coli* K88 cells grown on lipids extracted from mucus revealed a set of lipid utilization-associated proteins. None of these was induced fourfold during exponential growth in mucus. Microcalorimetric measurements (monitoring the rate of heat production) of *E. coli* K88 grown in mucus indicated metabolic shifts in the stationary phase, in which five of the lipid utilization-associated proteins were expressed at a higher level. An isogenic *E. coli* K88 *fadAB* mutant deficient in fatty acid degradation genes grew as well on the wild type on mucus and mucus lipids. The heat production rate curve of the mutant grown in mucus differed from that of the wild type only during the stationary phase. From these results it was concluded that protein expression is influenced when *E. coli* K88 is grown in piglet ileal mucus rather than in M9 minimal medium. Lipids extracted from ileal mucus can serve as a substrate for *E. coli* K88 but appear not to be utilized during exponential growth in mucus. Stationary-phase cells metabolize fatty acids; however, the functional purpose of this is unclear.

Enterotoxigenic *Escherichia coli* (ETEC), which colonizes the small intestine, is a major cause of diarrhea disease in both humans and domestic animals. In the host environment, *E. coli* is exposed to hazardous elements such as antibodies, digestive enzymes, toxins, bacteriocins, and bile salts, etc. The pathogens also have to compete with the established microflora for nutrients and colonization sites. To adapt to the intestinal milieu, the physiology of the bacterial cell might have to be adjusted. Little, however, is known about the molecular and physiological responses of ETEC to in vivo conditions.

Since the use of whole-animal models in studies of host-pathogen interactions is difficult, an in vitro model based on intestinal mucus was used in this investigation. Mucus, which is the viscoelastic gel covering the epithelial cells in the intestine, has in general been regarded as a barrier against colonization of pathogenic microbes (9, 11). However, recent results indicate that it may serve to promote colonization (8). Mucus contains a vast array of compounds, such as high-molecular-weight glycoproteins (mucins), lipids, smaller glycoproteins, and proteins of which some may function as receptors or nutrients. A number of investigators have demonstrated the presence of receptors specific for ETEC in mucus (6, 10, 23, 25, 39), and it can be speculated whether adhesion to these may initiate colonization of the intestine. If subsequent bacterial multiplication in the mucus exceeds the rate of mucus secretion, high bacterial counts could be accumulated within the mucus gel and the infection may thereby be established. In this context, it is interesting that colonization of the mouse large intestine by different *E. coli* strains is dependent on the ability to grow on cecal mucus (24, 38).

Lipids may account for up to 40% of the dry weight of intestinal mucus and can serve as the sole carbon and nitrogen source for different serological variants of *E. coli* (20). Intestinal lipids comprise neutral lipids, glycolipids, and phospholipids. The neutral lipids consist of free fatty acids, cholesterol, cholesterol esters, and mono-, di-, and triglycerides, whereas the phospholipids consist predominantly of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, and to a minor extent, phosphatidylserine. The glycolipids found in mucus are glycerolglucosylamines and glycosphingolipids (34); the latter include galactosylceramide, a putative receptor for *E. coli* K88 (6).

The bacterial strain used in the work presented here is a K88 fimbria-carrying ETEC strain (*E. coli* K88 strain Bd1107/7508; hereafter called *E. coli* K88) which colonizes the distal part of the small intestine in neonatal and weaned piglets and causes severe diarrhea (15). We have previously demonstrated that *E. coli* K88 binds to K88-specific mucus receptors (5) and multiplies rapidly in piglet ileal mucus (9). The primary goal of this study was to elucidate how *E. coli* cells respond when exposed to mucus. For this purpose, we applied two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) (29), which is a powerful technique for studying physiological alterations in terms of global expression of proteins in bacterial cells (37). With subsequent computer-aided analysis (16, 17), it allows comparison of the responses of many genes to different environmental conditions. The identity of a protein from the
2D map of *E. coli* can be determined by comparison to the established 2D protein database of this organism (37), and thus the physiological implications of resolved expression changes can be hypothesized.

To further investigate the type of metabolism of mucus-grown cells, we used a microcalorimetric technique. The microcalorimeter registers the heat production rate (dQ/dt) of the culture where the (dt) value is influenced by the type of metabolism (3, 19). Thus, the heat production curve may serve as a fingerprint of the physiological status of the bacterial culture and provide information about the metabolic traits exhibited by a pathogen during growth in mucus.

The aim of this investigation was to study the physiological and molecular responses of the pathogen elicited by exposure to piglet ileal mucus and to elucidate if lipids are utilized during growth in mucus. Global protein expression of *E. coli* K88 in ileal mucus and lipids extracted from ileal mucus was studied in relation to protein expression in M9 minimal medium. Furthermore, the rates of heat production by *E. coli* K88 and the isogenic *E. coli* K88 fadAB mutant deficient in the genes for fatty acid degradation were measured during growth in mucus.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* K88 strain Bd 1107/7508 (K88αc), isolated from a piglet with diarrhea and kindly provided by O. Söderlind, National Veterinary Institute, Uppsala, Sweden, was stored at −85°C in 30% (vol/vol) glycerol. The strain was streptomycin (100 μg · ml−1) resistant. *E. coli* RS807 (fad−5,71Δ/mol relA spoT1 thi−1) was a gift from The *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, Conn. fad−5,71Δ/mol relA spoT1 thi−1 is a defect in the fadAB cluster, and RS807 is therefore unable to utilize fatty acids (27). RS807 was lyogenized with bacteriophage P1cr8 100CM (18). A lysate was induced at 42°C and used to infect a *Luria* broth-grown culture of *E. coli* K88 strain Bd 1107/7508 (K88αc), which was streptomycin resistant, at a multiplicity of infection of about 1.0 at 30°C. The uninfected culture continued less than 10 CFU, and the infected culture continued 2 × 106 CFU of streptomycin (100 μg · ml−1) and tetracycline (10 μg · ml−1) resistant bacteria. The top-picked colonies (50 isolates were tested) from the infected culture did not grow on M9 minimal agar (31) containing oleic acid as the sole source of carbon (32) or Luria agar plates containing chloramphenicol (30 μg · ml−1), whereas they grew well on M9 medium containing glucose (1%, vol/vol), streptomycin sulfate (100 μg · ml−1), and tetracycline hydrochloride (10 μg · ml−1). One isolate was used for further experiments and was designated fadAB mutant E. coli K88.

**Mucus collection.** Weaned piglets (35 days old) were sacrificed and the intestines were immediately removed. The ileal part of the small intestine was scraped with a rubber spatula and a small volume of HEPES-Hanks buffer (0.3% [wt/vol] SDS, 5% [vol/vol] mercaptoethanol, 50 mM Tris buffer). The tube was placed in a heat block (100°C, 5 min) and subsequently cooled on ice (1°C). A 20-μl volume of an RNase A (0.25 mg/ml) DNase I (1 mg/ml) (Worthington solution) (nucleases dissolved in 0.5 M Tris [pH 7.0]–50 mM MgCl2) was added, and the tube was placed on ice for 10 min. The samples were homogenized and then divided into two ampoules, and then dithiothreitol (9.9 M urea, 4% [vol/vol] Nonidet P-40, 2.2% [vol/vol] vol/vol) and 18,000 Vh. The isoelectric focusing gels were equilibrated for 2 min in equilibration buffer (3% [vol/vol] SDS, 50 mM Tris buffer, 0.01% [vol/vol] bromophenol blue), placed at room temperature for 30 min, and subsequently centrifuged (13,000 × g, 10 min). The supernatant was withdrawn, and the washed pellet was immediately frozen and stored at −70°C.

**Preparation of cell extracts.** The frozen pellets were thawed on ice, and total cell extracts were prepared by adding 180 μl of sodium dodecyl sulfate (SDS) buffer (0.3% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, 50 mM Tris buffer). The tube was placed in a heat block (100°C, 5 min) and subsequently cooled on ice (1°C). A 20-μl volume of an RNase A (0.25 mg/ml) DNase I (1 mg/ml) (Worthington solution) (nucleases dissolved in 0.5 M Tris [pH 7.0]–50 mM MgCl2) was added, and the tube was placed on ice for 10 min. The samples were homogenized and then divided into two ampoules, and then dithiothreitol (9.9 M urea, 4% [vol/vol] Nonidet P-40, 2.2% [vol/vol] vol/vol) and 18,000 Vh. The isoelectric focusing gels were equilibrated for 2 min in equilibration buffer (3% [vol/vol] SDS, 50 mM dithiothreitol, 50 mM Tris buffer, 0.01% [vol/vol] bromophenol blue), placed at room temperature for 30 min, and subsequently centrifuged (13,000 × g, 10 min) before being loaded onto first-dimension gels. 2D PAGE was run on an IPGphor (Amersham Pharmacia Biotech, Uppsala, Sweden) using immobilized pH gradient IPG strips (pH 3–10). The samples were excited at 18,000 Vh.

**Scanning and computerized data analysis.** The gels were scanned with a Howtecht white-light desktop scanner set to a resolution of 200 by 200 μm. The different raw scans were processed to produce a gel image, and spots were detected and quantified. The analysis and subsequent gel matching were carried out on a Spots 1 computer (SU) by using the PDQuest program, version 4.1 Protein and DNA Image Ware Systems Inc.). Spots in the individual gels were manually matched prior to quantitation and statistical analyses. The spot intensity analysis was performed on the spot intensity means of duplicates. Protein synthesis was expressed as parts per million, and the intensity of a spot was normalized to the total intensity of all of the spots in that particular gel. To compensate for minor systematic errors in quantification of the gels, log normalization was performed (17) applying the normalization factors in the range of 0.8
RESULTS

Growth in mucus and lipids extracted from mucus. E. coli K88 multiplied with a generation time of about 20 min on ileal mucus, reaching the stationary phase at $10^8$ cells per ml after 6 h. The cells grew as fast when utilizing lipids extracted from mucus as the sole source of carbon and nitrogen (Fig. 1). Mucus and extracted lipids were dissolved in HEPES-Hanks buffer, and to exclude the possibility that HEPES could serve as a carbon or nitrogen source for E. coli K88, control experiments were performed in which HEPES-Hanks buffer with and without addition of a carbon (glucose) or a nitrogen (ammonium) source was inoculated ($10^4$ cells per ml) and incubated for 4.5 h. All three control cultures exhibited negligible growth and contained about $6 \times 10^4$ cells per ml as determined by CFU counting. Thus, it was concluded that the registered growth in the diluted lipid extracts and mucus was not supported by HEPES.

Protein expression during exponential growth in mucus. 2D-PAGE was used to study the global expression of proteins in E. coli K88 during growth in piglet ileal mucus compared with growth in minimal medium (M9). Cells exponentially growing in mucus or M9 minimal medium were pulsed-labelled with $[^{35}S]$methionine in the mid-exponential growth phase at a density of $10^6$ cells per ml. Growth in M9 minimal medium supported rapid label incorporation; however, we found that radioactive labelling with $[^{35}S]$methionine in ileal mucus was difficult. This may have been due to a high concentration of methionine in the mucus or to a strong impact, during growth in mucus, on the high-affinity transport of this amino acid. As a consequence of the low incorporation of $[^{35}S]$methionine, only the most dominant 150 proteins could be detected, even after extended (up to 45 days) exposure times. During growth of E. coli in mucus, a precipitate was formed which was pelleted with the cells during harvest, and components of this precipitate, or agents associated with mucus-grown cells, interfered with 2D separation. To avoid this problem, we diluted the culture with cold HEPES-Hanks buffer immediately before centrifugation, resulting in less mucus components accompanying the cell pellet.

Computer-assisted analysis of the gels revealed that expression of 13 of the 150 most dominant proteins was changed at least fourfold when E. coli K88 was grown in mucus (Fig. 2) versus M9 minimal medium. Of these 13 proteins, 3 were induced and 10 were repressed.

Protein expression during exponential growth in lipids extracted from ileal mucus. The relative rate of synthesis of 16 individual proteins was changed at least fourfold when cells were grown in lipids extracted from mucus compared with growth of cells in M9. Eight of the lipid utilization-associated proteins (LUAPs) were shown to be induced (Fig. 2). To elucidate whether lipids are used during exponential growth of E. coli K88 in mucus, the protein pattern obtained from lipid-grown cells was compared with the protein profile of mucus-grown cells.

The proteins whose expression changed at least fourfold during exponential growth in mucus or lipids are listed in Table 1. The data show that there was no overlap between mucus- and lipid-induced proteins, while the synthesis of two of the repressed proteins was significantly decreased in both mucus and lipids.

Identification of proteins expressed during growth in mucus. Some of the most dominant proteins were identified by comparing positions of spots on the 2D gels with a reference 2D image of the E. coli gene-protein database (37). Although the setup and apparatus for 2D-PAGE were the same as those used for the gene-protein database, the differences between the patterns of protein spots in our gels and the database were in some instances considerable, especially for low-abundance spots. Therefore, it was only possible to identify the most dominant and characteristic proteins. The positions of these are shown in Fig. 3, and the proteins are listed in Table 2. The heat shock protein GroEL was within the group of proteins whose levels of synthesis were altered more than fourfold during growth in mucus.

Rate of heat production of E. coli K88 during growth in mucus. Occurrence of metabolic shifts during anaerobic growth of E. coli K88 in piglet ileal mucus was investigated by measuring the rate of heat production with a multichannel microcalorimeter. In Fig. 4b, it can be seen that during the exponential phase, there are no indications of metabolic shifts. However, in the stationary phase there was a small peak at 6.3 h and a large peak at 7.5 h, indicating possible alterations of metabolism.

Expression of LUAPs by E. coli K88 in different phases of growth in mucus. The detected increases in the rate of heat production by stationary-phase cells instigated further analyses of the protein expression during this phase. The cells were $[^{35}S]$methionine labelled at two additional times during growth in mucus chosen on the basis of the heat production curve, i.e., the early stationary phase (6 h) and the late stationary phase (8 h).

The patterns obtained from E. coli cells sampled at the different growth phases in mucus (exponential and early and late stationary phases) were compared with those obtained from exponential-phase cells grown in lipids extracted from mucus. Induced LUAPs are indicated in Fig. 5, where minor parts (A and B) of these gels are compared with corresponding parts of gels obtained with cells grown in M9 minimal medium and in lipids extracted from mucus. These gels show that some
of the LUAPs were significantly induced in the stationary phase.

**Regulatory classes of responsive proteins.** The quantification of the proteins that exhibited at least fourfold changed synthesis rates during exponential growth in mucus and lipids is shown in Table 3. They were divided into four main classes: those induced at least fourfold during exponential growth on mucus (I) or lipids (II) and those repressed at least fourfold during growth on mucus (III) or lipids (IV). A further grouping within these classes was based on the pattern of expression in the different phases of growth in mucus and during exponential growth in lipids. In class I, two of the proteins showed similar patterns, i.e., sample spot proteins (SSP) 7605 and 8504, of which the latter was, as shown above, identified as GroEL. This protein was induced in exponential-phase and late-stationary-phase cells but not in early-stationary-phase cells during growth in mucus. The heat shock protein DnaK (Mr, 69.1 kDa; pl, 5.1) showed a similar pattern of induction in mucus but was not induced more than threefold (data not shown). The third protein, SSP 2303, was found to be strongly induced (19-fold) during exponential growth on mucus but repressed during later phases (data not shown). None of the proteins in class I fulfilled the criterion of fourfold induction during growth on lipids.

Class II proteins were divided into the following subgroups: IIA, those induced in lipids to a higher degree than in the stationary phase in mucus (three proteins); IIB, those induced in lipids to a lower degree than in the stationary phase in mucus (three proteins); and IIC, those induced only in lipids (two proteins). The proteins in subgroups IIA and IIB were induced between 8- and 20-fold, while the LUAPs in subgroup IIC were more strongly induced, i.e., 30- and 80-fold. In each of the subgroups IIA and IIB, one protein was also induced

<table>
<thead>
<tr>
<th>Change and SSP no.*</th>
<th>M_r/pl</th>
<th>Growth in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mucus</td>
</tr>
<tr>
<td>Fourfold induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2303</td>
<td>40.7/5.9</td>
<td>×</td>
</tr>
<tr>
<td>7605</td>
<td>67.0/5.2</td>
<td>×</td>
</tr>
<tr>
<td>8504</td>
<td>61.6/5.1</td>
<td>×</td>
</tr>
<tr>
<td>3110</td>
<td>23.0/5.7</td>
<td></td>
</tr>
<tr>
<td>4503</td>
<td>58.8/5.6</td>
<td>×</td>
</tr>
<tr>
<td>5403</td>
<td>53.3/5.5</td>
<td>×</td>
</tr>
<tr>
<td>5415</td>
<td>53.3/5.5</td>
<td>×</td>
</tr>
<tr>
<td>6410</td>
<td>53.2/5.4</td>
<td>×</td>
</tr>
<tr>
<td>6511</td>
<td>54.3/5.3</td>
<td></td>
</tr>
<tr>
<td>7112</td>
<td>22.4/5.3</td>
<td></td>
</tr>
<tr>
<td>8110</td>
<td>21.0/5.2</td>
<td>×</td>
</tr>
<tr>
<td>Fourfold repression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>207</td>
<td>32.6/&gt;6</td>
<td>×</td>
</tr>
<tr>
<td>2003</td>
<td>10.0/5.8</td>
<td>×</td>
</tr>
<tr>
<td>3203</td>
<td>33.9/5.7</td>
<td>×</td>
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<tr>
<td>3601</td>
<td>63.3/5.7</td>
<td>×</td>
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<td>4007</td>
<td>18.4/5.6</td>
<td>×</td>
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<td>4104</td>
<td>27.7/5.6</td>
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<td>5005</td>
<td>17.9/5.5</td>
<td>×</td>
</tr>
<tr>
<td>5305</td>
<td>37.9/5.4</td>
<td>×</td>
</tr>
<tr>
<td>5405</td>
<td>43.9/5.4</td>
<td>×</td>
</tr>
<tr>
<td>5406</td>
<td>50.8/5.5</td>
<td>×</td>
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<tr>
<td>5507</td>
<td>60.8/5.5</td>
<td>×</td>
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<td>6001</td>
<td>9.7/5.4</td>
<td>×</td>
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<tr>
<td>7404</td>
<td>51.0/5.3</td>
<td>×</td>
</tr>
<tr>
<td>8202</td>
<td>32.6/5.1</td>
<td>×</td>
</tr>
<tr>
<td>8611</td>
<td>63.6/5.0</td>
<td>×</td>
</tr>
<tr>
<td>9207</td>
<td>28.9/5.0</td>
<td>×</td>
</tr>
</tbody>
</table>

* The SSP number is a unique number automatically assigned to each spot by the PDQuest software.
during exponential growth in mucus, i.e., SSP 6410 and 8110; however, they did not fulfill the criterion of fourfold induction.

Classes III and IV contain the 16 proteins which were repressed. Two of these were repressed more than fourfold during growth in both mucus and lipids, i.e., SSP 3601 and 7404. However, several of the proteins in subgroups IIIA and IVA showed low levels of expression in mucus and lipids but did not reach the fourfold criterion in both media (data not shown).

**TABLE 2.** Synthesis of identified proteins during growth in mucus or lipids

<table>
<thead>
<tr>
<th>Gene</th>
<th>SSP no.</th>
<th>(M_r/pI)</th>
<th>Protein</th>
<th>Level in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mucus</td>
</tr>
<tr>
<td>eno</td>
<td>4407</td>
<td>44.0/5.6</td>
<td>Enolase</td>
<td>1.3</td>
</tr>
<tr>
<td>atpD</td>
<td>8405</td>
<td>50.2/5.1</td>
<td>ATP-synthase-F1 sector, (\beta) subunit</td>
<td>1.4</td>
</tr>
<tr>
<td>mopA</td>
<td>8504</td>
<td>61.6/5.1</td>
<td>GroEL</td>
<td>4.3</td>
</tr>
<tr>
<td>tig</td>
<td>8505</td>
<td>53.8/5.1</td>
<td>Trigger factor</td>
<td>2.7</td>
</tr>
<tr>
<td>rpsA</td>
<td>8605</td>
<td>68.5/5.1</td>
<td>Ribosomal subunit protein S1</td>
<td>1.7</td>
</tr>
<tr>
<td>dnaK</td>
<td>8609</td>
<td>69.1/5.1</td>
<td>DnaK, HSP70</td>
<td>3.2</td>
</tr>
<tr>
<td>ptsI</td>
<td>8611</td>
<td>63.6/5.0</td>
<td>phosphoeneolpyruvate-protein phosphotransferase</td>
<td>0.9</td>
</tr>
<tr>
<td>dnaN</td>
<td>9301</td>
<td>37.4/&lt;5</td>
<td>DNA-directed DNA polymerase III-(\beta)</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Level during exponential-phase growth in mucus or lipid relative to level in M9 minimal medium.
Subgroup IIIB proteins were repressed in all growth phases in mucus but not in lipids, in contrast to the proteins in group IVB, which were repressed only in lipids. Subgroup IIIC contains two proteins which were repressed in the exponential phase but induced in the stationary phase during growth in mucus.

**Growth and rate of heat production of E. coli fadAB mutant in mucus.** When *E. coli* K88 was grown anaerobically in mucus, LUAPs were induced and the rate of heat production was increased in the early and late stationary phases. These findings indicated metabolic shifts in which utilization of lipids was involved. It has previously been reported that *E. coli* can utilize long-chain fatty acids as the sole carbon source after a long lag phase (27). *E. coli* K88 could multiply on fatty acids (oleic acids) (data not shown), and therefore an *E. coli* K88 fadAB mutant deficient in the fatty acid degradation genes was constructed to elucidate whether fatty acids were utilized during the stationary phase by mucus-grown cells. Growth of this isogenic mutant on mucus and mucus lipids was studied, and it was found that the mutant grew as well as the wild type on mucus (Fig. 6a). The heat production rate curve of the mutant was very similar to that of the wild type (Fig. 6b) during exponential growth. The mutant and the wild type had the same growth rate and biomass yield, and thus the heat production per unit of biomass produced was equivalent in these cultures. This indicated that the two cultures had the same type of metabolism, suggesting that *E. coli* K88 did not utilize fatty acids during exponential growth in mucus. However, the increase in heat production during the stationary phase registered earlier in the wild type was absent in the mutant (Fig. 6b), indicating that fatty acids were used by the wild type during the stationary phase. These results support the 2D gel data.

**DISCUSSION**

Because of difficulties in performing molecular studies on microbes in whole animals, studies on bacterial gene expression under conditions like those that exist in vivo have, in general, been conducted with in vitro systems using host cells (1, 2, 7, 13, 14). In the experiments reported here, we used growth in porcine ileal mucus to simulate conditions in the piglet small intestine.

*E. coli* K88 is exposed to a variety of challenges in the piglet intestine, including interactions with hazardous components and competition for, as well as adaptation to, nutrients available in the host environment. We found that 13 of the 150 proteins detected were strongly regulated during growth in mucus compared with growth in minimal medium. Reports on the interaction of salmonellae with host epithelial cells (14) and macrophages (1, 7) have also demonstrated induction and repression of several proteins. Among the mucus-responsive proteins with at least fourfold changes in expression, we identified, by comparison with the established 2D-PAGE *E. coli* protein database (37), the heat shock-induced chaperonin GroEL on our 2D maps and found its synthesis to be upregulated during exponential growth in mucus. Both GroEL and DnaK are, however, reportedly induced in rich media with at least fourfold changes in expression, we identified, by comparison with the established 2D-PAGE *E. coli* protein database (37), the heat shock-induced chaperonin GroEL on our 2D maps and found its synthesis to be upregulated during exponential growth in mucus. We were also able to locate one additional stress-induced protein, the product of the dnaK gene, which was induced about threefold during growth in mucus.

The regulatory patterns of these proteins when the cells were grown in M9 (exponential phase), mucus (exponential and early and late stationary phases), and lipids (exponential phase) (Table 3) were similar to the pattern of *S. typhimurium* (12) and have, in fact, been suggested as putative *Salmonella typhimurium* (12) and *Helicobacter pylori* (21) virulence factors. Regarding *S. typhimurium*, it was shown that a 66-kDa heat shock protein was responsible for binding of the pathogen to intestinal mucus. Whether heat shock proteins contribute to the virulence of *E. coli* K88 is, however, unclear.

Earlier studies have demonstrated that *E. coli* is capable of utilizing lipids extracted from mouse cecal mucus for growth (20). Similarly, we found that *E. coli* K88 grew excellently on...
lipids extracted from piglet ileal mucus (Fig. 1). However, we did not know if lipids are a preferred substrate when E. coli K88 cells are grown in ileal mucus. Although lipids constituted up to 24% of the dry weight of the piglet ileal mucus, other carbon sources seemed to be preferred by E. coli K88, since none of the LUAPs were induced to the same extent during exponential growth in mucus. However, Krivan et al. (20) have suggested that phosphatidylserine is an important carbon and nitrogen source for both E. coli and salmonellae during growth in cecal mucus. One may therefore hypothesize that lipids are more essential substrates for E. coli in the large intestine than in the small intestine.

Several proteins whose identities are known were expressed differently during exponential growth in M9 minimal medium, ileal mucus, and lipids derived from ileal mucus (Table 2), and these differences may indicate how E. coli K88 cells experience the intestinal milieu. Enolase, which has been demonstrated to be induced by glucose starvation (28) and anaerobiosis (35), was found to be induced almost twofold during growth in lipid extracts, indicating a possible lack of glucose. Phosphotransferase I is an enzyme in the complex carbohydrate phosphotransferase system which is responsible for uptake and phosphorylation of a large number of carbohydrates (30). Expression of phosphotransferase I was repressed 10-fold during growth in lipids, while the level of synthesis in mucus was almost equal to that obtained in minimal medium supplemented with glucose as the carbon source. Thus, it can be suspected that the carbon source utilized during growth in the lipid fraction was different from that utilized during growth in mucus. It has been proposed that phosphatidylserine, which may serve as the sole source of carbon and nitrogen during growth in cecal mucus, is transported into the cell and metabolized intracellularly (20).

To further elucidate the metabolic traits of E. coli K88 grown in mucus, we applied the technique of microcalorimetry. When the rate of heat production by E. coli K88 (Fig. 4b) was monitored, no signs of metabolic shifts were detected during exponential growth. However, in the stationary phase, the rate of heat production was significantly increased. The protein pattern from stationary-phase cells demonstrated that five LUAPs were induced in the stationary phase (Fig. 5), indicating utilization of lipids. Two of the LUAPs, SSP 6410 and 8110, also responded by induction during exponential growth. However, in the stationary phase, the rate of heat production was significantly increased. The protein pattern from stationary-phase cells demonstrated that five LUAPs were induced in the stationary phase (Fig. 5), indicating utilization of lipids. Two of the LUAPs, SSP 6410 and 8110, also responded by induction during exponential growth.

To clarify whether fatty acids were utilized by mucus-grown E. coli K88 cells in the stationary phase, we constructed an E. coli K88 fadAB mutant deficient in the genes involved in β-oxidation of fatty acids. When the rates of heat production of the isogenic strains (Fig. 4 and 6) were compared, it was evident that E. coli K88 did not utilize fatty acids during exponential growth. It was shown, however, that fatty acids were tentatively exploited by stationary-phase cells. The increased rate of heat production of the parent strain in the stationary phase did not result in increased numbers of CFU. This may be explained.

FIG. 5. Examples of synthetic profiles from the gel portions of some of the proteins associated with exponential growth on lipids extracted from mucus (LUAPs). (A) pH 5.7 to 5.0; M_r, 48 to 60 kDa. (B) pH 5.8 to 5.0; M_r, 17 to 24 kDa (acidic side to the right). I, exponential growth in M9; II, exponential growth in mucus; III, early stationary phase growth in mucus; IV, late stationary phase growth in mucus; V, exponential growth in lipids. Arrows indicate the locations of proteins of interest.
either by concomitant death of cells or, alternatively, by the possibility that the energy transformed was used for maintenance or simply wasted. In this context, it would be of interest to study the effect of fatty acid utilization on the persistence of enterotoxigenic E. coli strains in the intestine.

The comparison between the rate of heat production of the wild type and the isogenic mutant support the 2D data, in which LUAPs were found to be induced in the stationary phase during growth in mucus. Thus, one may suspect that some of the induced LUAPs were involved in fatty acid degradation.

In conclusion, E. coli K88 experienced factors in mucus that influenced the expression of specific proteins. Heat shock proteins DnaK and GroEL belong to the group of proteins induced during growth in mucus. Lipids, including fatty acids, did not seem to be utilized during exponential growth in ileal mucus but may be involved in metabolism during the stationary phase.

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