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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 [^{35}S]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 as 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 glyceroglucolipids 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 intestinal 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

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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) 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 (K88ac), 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 $\mu g \cdot ml^{-1}$) resistant.

E. coli RS3087 (*fad-751*::Tn*10 relA1 spoT1 thi-1*) was a gift from The *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, Conn. *fad-751*::Tn*10* is a defect in the *fadAB* cluster, and RS3087 is therefore unable to utilize fatty acids (27). RS3087 was lysogenized with bacteriophage P1*clr* 100CM (18). A lysate was induced at 42°C and used to infect a Luria broth-grown culture of *E. coli* K88 strain Bd1107/7508 (K88ac), which was streptomycin resistant, at a multiplicity of infection of about 1.0 at 30°C. The uninfected culture contained less than 10 CFU, and the infected culture contained 2×10^9 CFU of streptomycin (100 $\mu g \cdot ml^{-1}$)- and tetracycline (10 $\mu g \cdot ml^{-1}$)-resistant bacteria. The toothpicked 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 $\mu g \cdot ml^{-1}$), whereas they grew well on M9 medium containing glucose (1%, wt/vol), streptomycin sulfate (100 $\mu g \cdot ml^{-1}$), and tetracycline hydrochloride (10 $\mu g \cdot ml^{-1}$). 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 removed, and the luminal contents were rinsed out with ice-cold HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-Hanks buffer (8.0 g of NaCl, 0.4 g of KCl, 0.185 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of Na_2HPO_4, 0.35 g of KH_2PO_4, 2.6 g of HEPES; adjusted to pH 7.2). Subsequently, mucus was collected from the ileal region of the small intestine by gentle scraping with a rubber spatula and a small volume of HEPES-Hanks buffer. The collected material was centrifuged twice at 27,000 × *g* for 20 min to remove bacterial and epithelial cells prior to their storage in small aliquots at −85°C. Some of the supernatant was immediately freeze-dried and used for lipid extraction. The dry weight of mucus was 30 $mg \cdot ml^{-1}$; proteins and lipids constituted 10 mg (33%) and 7.2 mg (24%), respectively.

The protein content of the mucus (in milligrams per milliliter) was determined with a Sigma Diagnostic Protein assay kit (P 5656) with bovine serum albumin (Merck) as the standard.

Lipid extraction. Lipids were extracted from the freeze-dried mucus with chloroform-methanol (2:1) (33). The relative proportion of lipids in the mucus was 24% of the dry weight. The lipid fraction was dried under nitrogen and subsequently dispersed in HEPES-Hanks buffer (pH 7.2) at a concentration of 1 $mg \cdot ml^{-1}$ by sonication for 30 min in a water bath sonicator.

Medium and growth conditions. The cells were grown anaerobically overnight in tryptic soy broth (Difco) at 37°C, harvested by centrifugation in a bench top centrifuge at 3,000 × *g* (7 min, 4°C), washed twice with sterile HEPES-Hanks buffer (pH 7.2), and resuspended in anaerobic HEPES-Hanks buffer before being inoculated (10^4 cells ml^{-1}) into anaerobic M9 minimal medium (31) (0.4% glucose), ileal mucus, or lipids extracted from ileal mucus. The mucus and lipid media were prepared with HEPES-Hanks buffer without addition of a carbon or nitrogen source. The mucus medium contained protein at 6.3 $mg \cdot ml^{-1}$ and lipids at 4.5 $mg \cdot ml^{-1}$, and the lipid medium contained extracted lipids at 1 $mg \cdot ml^{-1}$.

Control experiments to investigate whether HEPES-Hanks buffer alone could serve as a carbon or nitrogen source were performed by inoculating (10^4 cells ml^{-1}) either HEPES-Hanks buffer, HEPES-Hanks buffer supplemented with glucose (0.4%, wt/vol), or HEPES-Hanks buffer supplemented with NH_4Cl (0.1%, wt/vol). The control cultures were incubated at 37°C for 4.5 h before CFU were counted on tryptic soy agar plates.

All experiments were carried out in an anaerobic glove box (Formia Scientific Inc.) containing 10% H_2, 5% CO_2, and <0.01% O_2, with the balance being N_2.

Microcalorimetry. The rate of heat production (dQ/dt) was measured with a multichannel microcalorimeter (Bioactivity Monitor LKB 2277; Thermometric AB, Järfälla, Sweden) of the heat conduction type (36). The calorimeter was fitted with three channels, of which each contained a measuring cell and a reference cell to permit three simultaneous measurements. The voltage signal was recorded by two-channel potentiometric recorders (LKB 2210-022; 1,000-mV range; Pharmacia LKB Biotechnology AB, Bromma, Sweden). Glass ampoules (3 ml) were used to monitor the anaerobic growth of the cultures. The ampoules were sterilized with 70% (vol/vol) ethanol and rinsed with sterile water before being loaded with samples, i.e., 1.5 ml of mucus (6.3 mg of protein per ml) and inoculated with 10^4 cells of the wild type or the isogenic mutant. Reference ampoules were loaded with sterile water (1.5 ml). Sample loading was performed in an anaerobic box, and the ampoules were sealed before being transferred to the calorimeter. The calorimeter was operated at 37.0°C, and the calorimeter response was calibrated for each experiment with reference ampoules in both channels. An external current was supplied to produce a steady-state heat effect of 30 μW within the measuring range of 100 μW.

Radioactive labelling. The labelling procedure was performed in the anaerobic glove box. Cells in 1-ml cultures were labelled at 37°C with 200 μCi of [^{35}S]methionine (specific activity, 1,200 Ci/mmol) for 20 min and subsequently chased with 0.03 M cold methionine for 3 min. The only exception was the late (8 h)-stationary-phase sample, which was labelled for 60 min to obtain enough incorporated material for 2D analysis. Mid-exponential-phase samples, growing in either M9, mucus, or lipid medium, were isotopically labelled at a density of 10^6 cells per ml subsequent to at least six generations of growth in the experimental flasks. Growth, labelling, and 2D analyses were performed with duplicate independent cultures.

The labelled cultures were cooled on ice and thereafter diluted 10 times with ice-cold HEPES-Hanks buffer to restrict aggregation of mucus components. The cells were harvested by centrifugation in a bench top centrifuge at 3,000 × *g* (7 min, 4°C), and the pellet was resuspended in 1 ml of HEPES-Hanks buffer and centrifuged at 13,000 × *g* (20 min, 4°C). 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 min). 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 $MgCl_2$) was added, and the tube was placed on ice for 10 min. The samples were lyophilized and then dissolved in 50 μl of sample buffer (9.9 M urea, 4% [vol/vol] Nonidet P-40, 2.2% [vol/vol] ampholine, 100 mM dithiothreitol, 0.01% [wt/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. 2D-PAGE was run on a Investigator system (Millipore); all of the chemicals and equipment used, essentially as described earlier (26), were supplied by Millipore. The first-dimension gels were 4% Duracryl (0.8% bis)–ampholytes (pH 3 to 10), and the isoelectric focusing was run overnight (18 h) for 18,000 Vh. The isoelectric focusing gels were equilibrated for 2 min in equilibration buffer (3% [wt/vol] SDS, 50 mM dithiothreitol, 500 mM Tris buffer, 0.01% [wt/vol] bromophenol blue) before mounting on the SDS-PAGE slab gels. Second-dimension SDS-PAGE separation was conducted in 11.5% acrylamide (Duracryl; 0.8% bis), and gels were run at 20°C overnight (18 h) at 1,400 mW per gel until the bromophenol blue front reached the bottoms of the gels.

Spot visualization. The gels were fixed for 1 to 2 h in fixing solution (50% [vol/vol] ethanol, 10% [vol/vol] acetic acid). The fixed gels were rinsed in milli-Q water for at least 2 h so that they would attain their maximum size (roughly 25 by 25 cm) before being dried on Whatman 3mm Chr chromatography paper in a Bio-Rad gel drier. Dried gels were exposed to XAR film (Kodak) at −70°C for 3 to 45 days, depending on the amount of radioactivity loaded. Films were developed with an AGFA-CURIX 60 automatic developer.

Scanning and computerized data analysis. The films were scanned with a Howtech 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 Sparc 1 computer (SUN) 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 quantitative and statistical analyses. The computer 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 between gels, log normalization was performed (17), yielding normalization factors in the range of 0.8

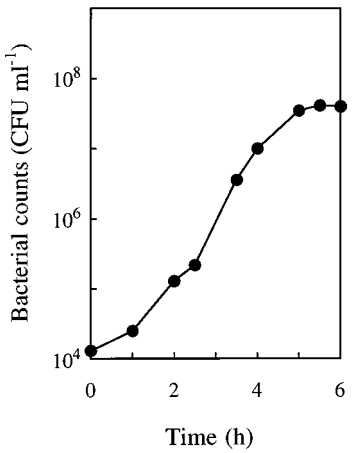

FIG. 1. Anaerobic growth of *E. coli* K88 in lipids extracted from piglet ileal mucus, expressed as increased numbers of CFU per milliliter.

to 1.1. Quantification of the spots indicated as repressed or induced exhibited an average variance of 28% ± 25% (standard deviation).

The pI and M_r axes were constructed by using sequence-based, computerized pI and M_r values, taken from the *E. coli* database (37), for some of the identified proteins in the pattern indicated in Fig. 3. In addition, the M_r-pI scale was extended by 2D comparison via comigration of *E. coli* and *Saccharomyces cerevisiae* extracts (4), yielding values for some nonidentified *E. coli* proteins (indicated by asterisks in Fig. 3). The pI axis was constructed by using the following protein pI values: DnaK, 5.07; PtsI, 4.97; TufA, 5.52; 1* (Fig. 3), 6.0. The M_r axis was constructed by using the following protein molecular sizes: DnaK, 69.1 kDa; PtsI, 63.6 kDa; AtpD, 50.2 kDa; TufA, 43.1 kDa; 2* (Fig. 3), 32.0 kDa; 3* (Fig. 3), 18.7 kDa.

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 $6 \cdot 10^4$ 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 utilizion-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

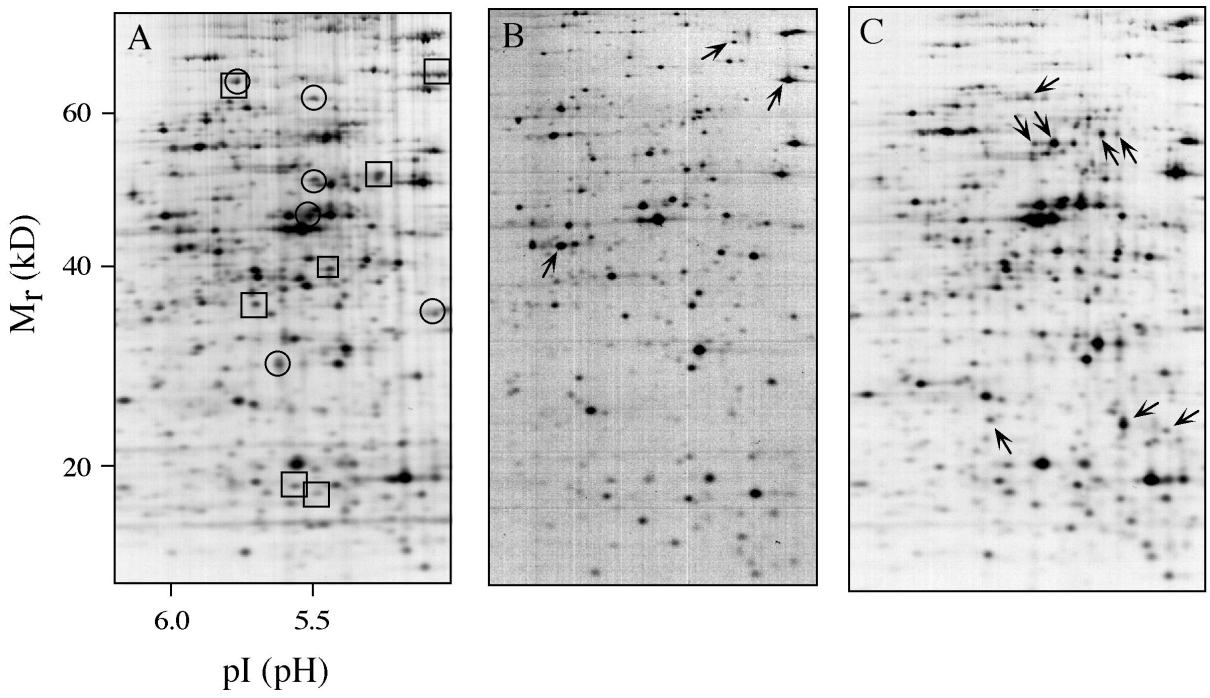

FIG. 2. 2D-PAGE analyses of [^{35}S]methionine-labelled *E. coli* K88 during anaerobic exponential growth in either synthetic minimal medium M9 (A), piglet ileal mucus (B), or lipids extracted from piglet ileal mucus (C). The central portions of the gels are shown (acidic side to the right). Arrows indicate proteins with at least fourfold increased rates of synthesis during growth in mucus (B) and lipids extracted from mucus (C), compared with proliferation in M9 medium. In panel A are indicated proteins whose rates of synthesis decreased by at least a factor of 4 during growth in mucus (circles) or in lipids extracted from mucus (squares), compared with growth in M9 medium.

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 (M_r, 69.1 kDa; pI, 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 1. Cellular response of *E. coli* K88 during exponential growth in ileal mucus or lipids extracted from mucus compared with growth in M9 minimal medium

Change and SSP no.[a]	M_r/pI	Growth in:	
		Mucus	Lipids
Fourfold induction			
2303	40.7/5.9	×	
7605	67.0/5.2	×	
8504	61.6/5.1	×	
3110	23.0/5.7		×
4503	58.8/5.6		×
5403	53.3/5.5		×
5415	53.3/5.5		×
6410	53.2/5.4		×
6511	54.3/5.3		×
7112	22.4/5.3		×
8110	21.0/5.2		×
Fourfold repression			
207	32.6/>6	×	
2003	10.0/5.8		×
3203	33.9/5.7		×
3601	63.3/5.7	×	×
4007	18.4/5.6		×
4104	27.7/5.6	×	
5005	17.9/5.5		×
5305	37.9/5.4		×
5405	43.9/5.4	×	
5406	50.8/5.5	×	
5507	60.8/5.5	×	
6001	9.7/5.4	×	
7404	51.0/5.3	×	×
8202	32.6/5.1	×	
8611	63.6/5.0		×
9207	28.9/<5	×	

[a] The SSP number is a unique number automatically assigned to each spot by the PDQuest software.

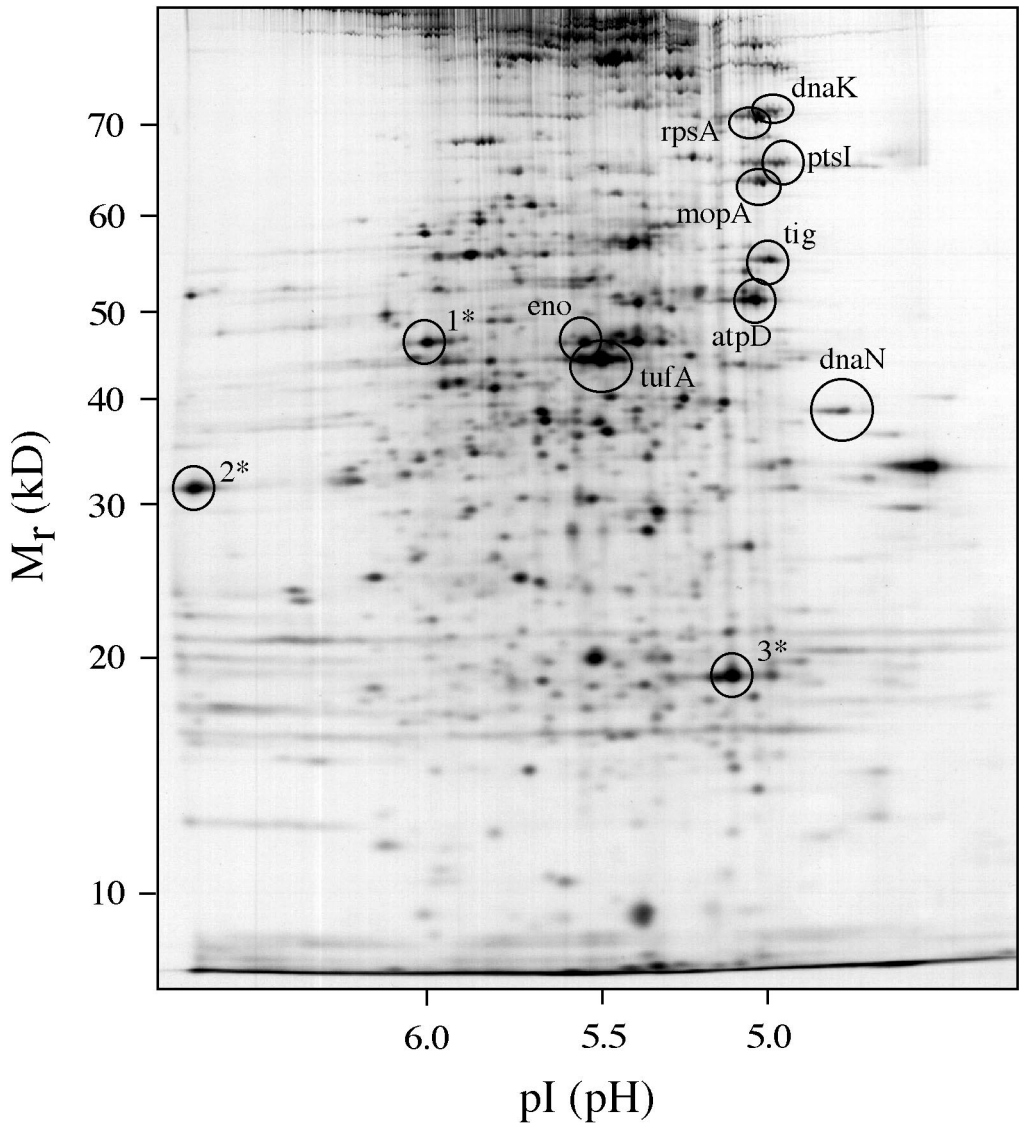

FIG. 3. Proteins identified in the 2D pattern of *E. coli* K88. This 2D gel was obtained with cells labelled during anaerobic exponential growth in M9 medium. Identification was done by comparison with the 2D-PAGE *E. coli* protein database (38), and for each identification the corresponding gene designation is indicated. For explanations of the gene names, see Table 2. The spots indicated with asterisks were used to establish the M_r and pI axes (see Materials and Methods).

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

Gene	SSP no.	M_r/pI	Protein	Level[a] in:	
				Mucus	Lipid
eno	4407	44.0/5.6	Enolase	1.3	1.8
atpD	8405	50.2/5.1	ATP-synthase-F1 sector, β subunit	1.4	2.0
mopA	8504	61.6/5.1	GroEL	4.3	2.1
tig	8505	53.8/5.1	Trigger factor	2.7	1.6
rpsA	8605	68.5/5.1	Ribosomal subunit protein S1	1.7	1.2
dnaK	8609	69.1/5.1	DnaK, HSP70	3.2	1.7
ptsI	8611	63.6/5.0	phosphoenolpyruvate-protein phosphotransferase	0.9	0.1
dnaN	9301	37.4/<5	DNA-directed DNA polymerase III-β	1.1	1.0

[a] Level during exponential-phase growth in mucus or lipid relative to level in M9 minimal medium.

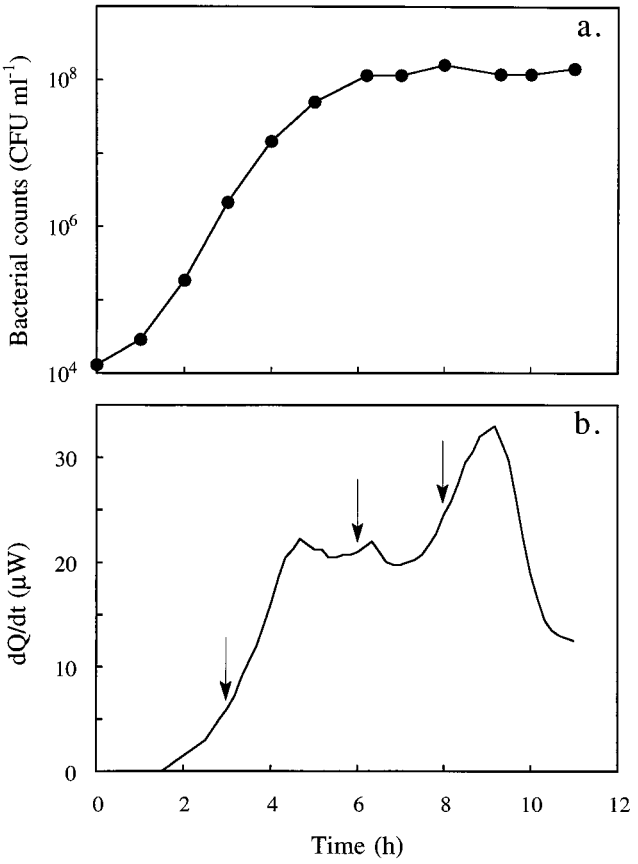

FIG. 4. Growth (a) and rate of heat production (b) of *E. coli* K88 during anaerobic growth in piglet ileal mucus. Growth was measured as the increase in CFU per milliliter, and the rate of heat production (*dQ/dt*) was measured with a microcalorimeter. The arrows indicate times of [^{35}S]methionine labelling of the *E. coli* K88 culture during growth: 3 h, exponential growth phase; 6 h, early stationary phase; 8 h, late stationary phase.

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. 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 SSP 7605 (M_r, 67.0; pI, 5.2) in class I, and thus one can suspect that this protein also belongs to a heat shock family. In agreement with Lee et al. (22), we have previously shown that the piglet ileal lumen contents contain growth-inhibitory compounds (5). In contrast, piglet ileal mucus supported extensive growth and the cells did not seem to be growth restricted or evidently stressed. It was therefore surprising that the heat shock proteins GroEL and DnaK were induced in cells during exponential growth in mucus. Both GroEL and DnaK are, however, reportedly induced in rich media compared with minimal media (37). The increased synthesis of these proteins in mucus could thus depend on the fact that the growth rate in mucus exceeds that in M9 minimal medium. However, the level of induction during *E. coli* K88 growth in lipids was only half of that obtained during growth in mucus, even though mucus and lipids supported growth at the same rate. One may therefore hypothesize that mucus contains additional factors which regulate the synthesis of heat shock proteins GroEL and DnaK. The heat shock proteins have attracted increased interest during the past few years as having a role in pathogenicity 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

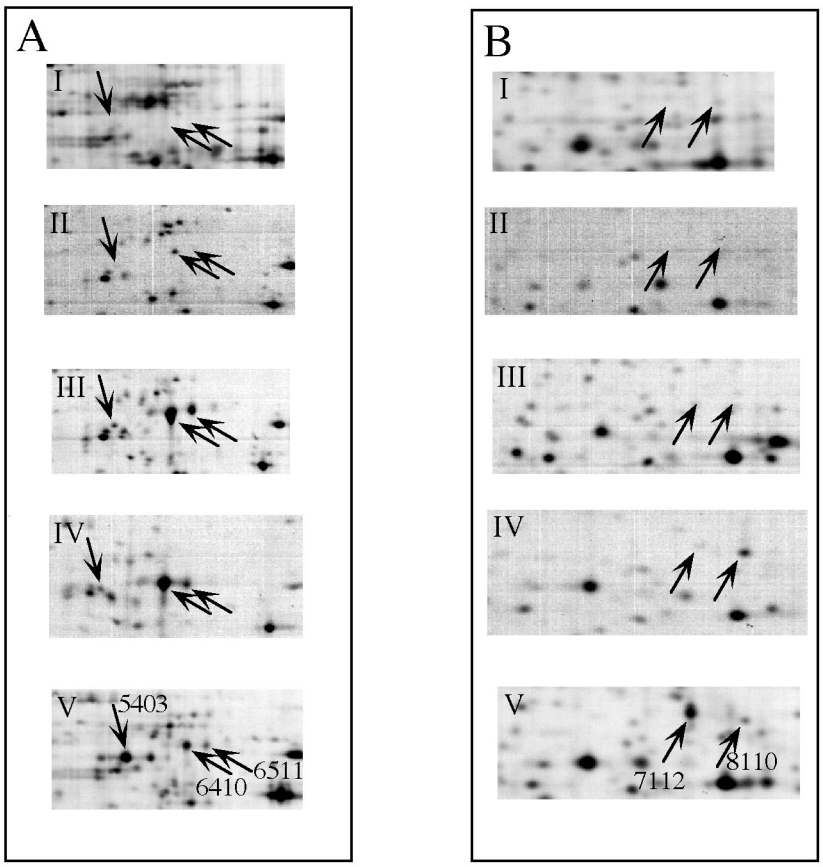

FIG. 5. Examples of synthetic profiles from the gel portions of some of the proteins associated with exponential growth on lipids extracted from mucus (LUAPs). (A) pH 5.7 to 5.0; M_r, 48 to 60 kDa. (B) pH 5.8 to 5.0; M_r, 17 to 24 kDa (acidic side to the right). I, exponential growth in M9; II, exponential 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.

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 in mucus (Table 3); however, the level of synthesis was much lower than the high level of induction observed in the early and late stationary phases.

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

TABLE 3. Classification of regulatory mucus or lipid growth-responsive proteins[a]

Regulatory class	Typical kinetic profile	Proteins in class SSP nb.	Mr / pI	Max. synth. rate (ppm)
I. INDUCED DURING GROWTH IN MUCUS				
I		2303 7605 8504	40.7 / 5.9 67.0 / 5.2 61.6 / 5.1*	4428 3176 29815
II. INDUCED DURING GROWTH IN LIPIDS				
II A		3110 4503 5403	23.0 / 5.7 58.8 / 5.6* 53.3 / 5.5	2073 3520 9230
II B		6511 6410 8110	54.3 / 5.3* 53.2 / 5.4 21.0 / 5.2	5069 19492 5174
II C		5415 7112	53.3 / 5.5 22.4 / 5.3*	3554 9835
III. REPRESSED DURING GROWTH IN MUCUS.				
III A		207 5507 3601 7404	32.6 / > 6 60.8 / 5.5* 63.3 / 5.7 51.0 / 5.3	2937 1610 2782 4188
III B		5405 4104 6001 8202	43.9 / 5.4 27.7 / 5.6 9.7 / 5.4 32.6 / 5.1*	8169 4731 18584 2549
III C		9207 5406	28.9 / < 5 50.8 / 5.5*	6104 3448
IV. REPRESSED DURING GROWTH IN LIPIDS				
IV A		3203 3601 4007 5005 5305 7404	33.9 / 5.7* 63.3 / 5.7 18.4 / 5.6 17.9 / 5.5 37.9 / 5.4 51.0 / 5.3	1969 2782 1852 1649 1673 4188
IV B		2003 8611	10.0 / 5.8 63.6 / 5.0*	1217 3838

[a] For classification of proteins, see the text. The histograms show the typical kinetic profiles of the classes. The bars represent rates of synthesis during growth in (from left to right) M9 minimal medium (exponential phase), mucus (exponential phase), mucus (early stationary phase), mucus (late stationary phase), and lipids extracted from mucus (exponential phase). SSP numbers are unique numbers automatically assigned to the spots by the PDQest software. The asterisks in the M_r/pI column indicate the proteins whose expression profiles are shown in the histograms.

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.

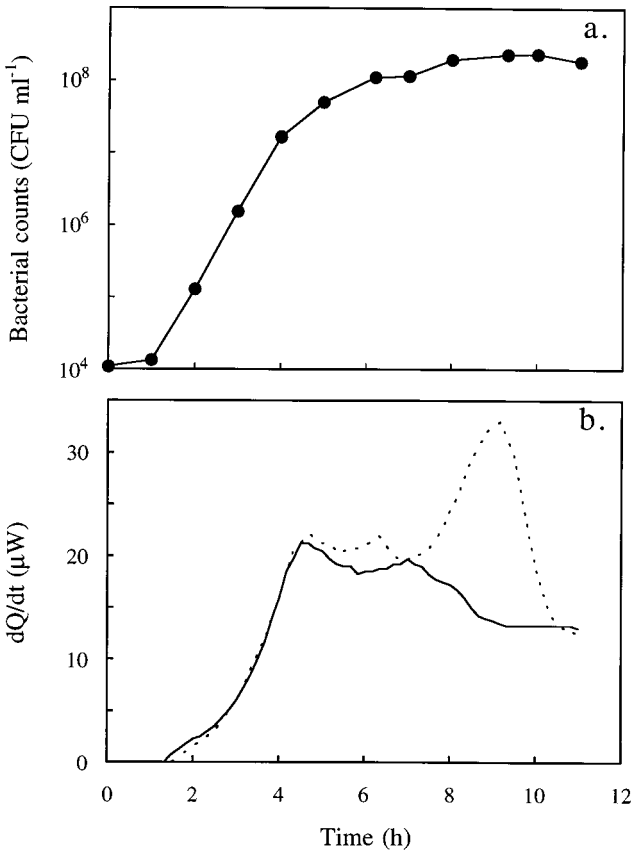

FIG. 6. Anaerobic growth (a) of *E. coli* K88 *fadAB* mutant and rates of heat production (b) of *E. coli* K88 *fadAB* mutant (solid line) and the isogenic parent *E. coli* K88 (dotted line) during growth in piglet ileal mucus. Growth was measured as the increase in the number of CFU per milliliter, and the rate of heat production (*dQ/dt*) was measured with a microcalorimeter.

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