pH-Dependent Catabolic Protein Expression during Anaerobic Growth of *Escherichia coli* K-12

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During aerobic growth of *Escherichia coli*, expression of catabolic enzymes and envelope and periplasmic proteins is regulated by pH. Additional modes of pH regulation were revealed under anaerobiosis. *E. coli* K-12 strain W3110 was cultured anaerobically in broth medium buffered at pH 5.5 or 8.5 for protein identification on proteomic two-dimensional gels. A total of 32 proteins from anaerobic cultures show pH-dependent expression, and only four of these proteins (DsbA, TnaA, GatY, and HdeA) showed pH regulation in aerated cultures. The levels of 19 proteins were elevated at the high pH; these proteins included metabolic enzymes (DhaKLM, GapA, TnaA, HisC, and HisD), periplasmic proteins (ProX, OppA, DegQ, MalB, and MglB), and stress proteins (DsbA, Tig, and UspA). High-pH induction of the glycolytic enzymes DhaKLM and GapA suggested that there was increased fermentation to acids, which helped neutralize alkalinity. Reporter lac fusion constructs showed base induction of *sdaA* encoding serine deaminase under anaerobiosis; in addition, the glutamate decarboxylase genes *gdaA* and *gdaB* were induced at the high pH anaerobically but not with aeration. This result is consistent with the hypothesis that there is a connection between the gad system and GapT metabolism of 4-aminobutanoate. On the other hand, 13 other proteins were induced by acid; these proteins included metabolic enzymes (GatY and AckA), periplasmic proteins (TolC, HdeA, and OmpA), and redox enzymes (GuaB, HmpA, and Lpd). The acid induction of NikA (nickel transporter) is of interest because *E. coli* requires nickel for anaerobic fermentation. The position of the NikA spot coincided with the position of a small unidentified spot whose induction in aerobic cultures was reported previously; thus, NikA appeared to be induced slightly by acid during aeration but showed stronger induction under anaerobic conditions. Overall, anaerobic growth revealed several more pH-regulated proteins; in particular, anaerobiosis enabled induction of several additional catabolic enzymes and sugar transporters at the high pH, at which production of fermentation acids may be advantageous for the cell.

The response to pH includes modulation of catabolism, particularly in the presence of complex carbon sources, such as the tryptone and yeast components of Luria-Bertani medium (LB). Tryptone consists of primarily tryptic peptides and 7.7% (wt/wt) carbohydrates (primarily lactose), whereas yeast extract contains peptides plus 17.5% carbohydrates (primarily glycogen and trehalose) (68). External acids and membrane-permeant acids, whose uptake is amplified by the pH gradient, induce heat shock and oxidative stress proteins, as well as the RpoS regulon (5, 7, 30, 32, 50). The response to pH includes modulation of catabolism, particularly in the presence of complex carbon sources, such as the tryptone and yeast components of Luria-Bertani medium (LB). Tryptone consists of primarily tryptic peptides and 7.7% (wt/wt) carbohydrates (primarily lactose), whereas yeast extract contains peptides plus 17.5% carbohydrates (primarily glycogen and trehalose) (68). External acids and membrane-permeant acids, whose uptake is amplified by the pH gradient, induce heat shock and oxidative stress proteins, as well as the RpoS regulon (5, 7, 30, 32, 50).

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Aerated *E. coli* cultures respond to pH changes by selective expression of numerous stress proteins, redox modulators, and envelope proteins (21, 59, 65). The acid stress chaperones HdeA and HdeB enhance survival in extreme acid conditions (5, 16). The membrane-bound Na<sup>+</sup>H<sup>+</sup> antiporter NhaA protects the cell from excess Na<sup>+</sup> at a high external pH (26, 43). Genes that show pH dependence are often coinduced by other environmental factors, such as growth phase, carbon source, and anaerobiosis (33, 56, 57). External acids and membrane-permeant acids, whose uptake is amplified by the pH gradient, induce heat shock and oxidative stress proteins, as well as the RpoS regulon (5, 7, 30, 32, 50).

During early-log-phase growth, even well-oxygenated *E. coli* cells initially produce fermentation products such as acetate...
TABLE 1. *E. coli* K-12 strains used in this study

| Strain        | Genotype             | Reference |
|---------------|----------------------|-----------|
| W3110         | F' λ prototroph      | 57        |
| MC4100        | F' araD139 Δ(argF-lac)U169 rpsL150 relA1 flb5301 deoC1 ptsF24 rbsR | 51        |
| EF614         | F' λ IN(mD-rme)ΔlacX74 rpsL gagK2 recD1903::Tn10Δc recDC::putPA1303-Km-gadB::lacZ | 11        |
| JLS0214       | MC4100 trpDC::putPA1303-Km-gadB::lacZ | This study |
| EF615         | F' λ IN(mD-rme)ΔlacX74 rpsL gagK2 recD1903::Tn10Δc recDC::putPA1303-Km-gadA::lacZ | This study |
| JLS0215       | MC4100 trpDC::putPA1303-Km-gadA::lacZ | This study |
| JLS0711       | MC4100 sdaA::lacZ    | This study |

and formate, which at a low external pH can reenter the cell and reach deleterious concentrations (29, 46, 47). For this reason, fermentation pathways respond to pH; for instance, ldhA is induced severalfold by acid in order to produce lactate instead of acetate plus formate (9). A number of proteins induced by acetate and by short-chain fatty acids (5, 7, 30) are also induced by growth at low pH, and the pH gradient drives the fermentation acids back into the cell. For example, the low-oxygen pyruvate-formate lyase Yfd, induced by acetate or formate (7, 30), is also induced during growth on LB at low pH, whereas several acetate-repressible proteins, such as tryptophanase (TnaA) and high-affinity maltose binding protein (MalE), are repressed at low pH (3, 7, 59).

Anaerobiosis amplifies induction of several acid-regulated pathways of catabolism, such as the cadAB, lysU, and adi pathways (34, 48, 53, 61). The absence of oxygen limits the metabolic options available to cells, necessitating increased excretion of weak-acid fermentation products that stress the cell. At the same time, anaerobiosis makes new enzymatic pathways available, such as the pathway for anaerobic beta-oxidation of fatty acids (10). Thus, one would expect anaerobiosis to favor additional pH responses not seen during growth with oxygen.

We describe here a proteomic 2-D gel comparison of *E. coli* protein profiles at low pH and at high pH for cells grown under anaerobiosis. New patterns of gene expression were obtained that substantially augment our picture of pH-dependent protein expression, especially for pathways of catabolism.
RESULTS

For proteomic 2-D gels, *E. coli* K-12 strain W3110 was grown anaerobically in LBK buffered at pH 5.5 or 8.5. The log-phase doubling times were observed to be 31 min (pH 5.5) and 34 min (pH 8.5). At more extreme pH values, such as those tested previously with aerated cultures (59), the growth rate was low and varied greatly. Because pH stress caused growth problems for anaerobic cultures, we focused on comparing protein profiles of acid and base cultures instead of testing across the pH range. Protein profiles were obtained for cultures grown at neutral pH, but they did not reveal any additional pH-dependent expression (data not shown).

It was important to assess the effects of buffers and countercation concentrations under anaerobic growth conditions, as shown previously for aerobic cultures (59). Two alternative buffer strategies were used. In experiment A, the media contained different buffers at each pH in order to minimize the difference in the K⁺ concentrations, and in experiment B, the media included the same buffers at each pH. The composite protein profiles are shown in Fig. 1, and the results of a quantitative analysis of pairwise comparisons are shown in Table 2. The overall patterns of differentially expressed proteins in experiments A and B were largely the same. Six proteins had significant LDE values in experiment A but not in experiment B (Tig, MglB, GapA, GatY, Tsf, and HdeA), whereas two proteins had significant LDE values in experiment B but not in experiment A (MalB and AccB). These differences could reflect buffer effects, but they could also reflect differences in the quality of the spot patterns of the two different gel runs.

At the high pH, 19 proteins showed elevated expression, but only 2 of these proteins, TnaA and DsbA, are induced at high pH aerobically (7, 59). The proteins that showed elevated expression at the high pH under anaerobic conditions included catabolic enzymes (DhaKLM, GapA, HisC, and TnaA) and periplasmic proteins providing substrates for catabolism (ProX, OppA, DegQ, MalB, and MglB), as well as stress proteins (DsbA, Tig, and UspA). On the other hand, the low pH favored expression of 13 proteins, but only 2 of these 13 proteins, GatY and HdeA, are known to be acid induced aerobically (7, 59). One protein, NikA, corresponded to a spot which was low and varied greatly. Because pH stress caused growth problems for anaerobic cultures, we focused on comparing protein profiles of acid and base cultures instead of testing across the pH range. Protein profiles were obtained for cultures grown at neutral pH, but they did not reveal any additional pH-dependent expression (data not shown).

Table 2. Proteins showing differential expression as a function of pH

| Spot | Protein | Expt A | Expt B |
|------|---------|--------|--------|
|      | LDE    | SE     | LDE    | SE     |
| Base induced |        |        |        |        |
| 1    | OppA   | 0.24   | 0.02   | 0.38   | 0.05   |
| 2    | DhaM   | 0.71   | 0.12   | 0.34   | 0.11   |
| 3    | Tig    | 0.64   | 0.13   |        |        |
| 4    | HisD   | 0.81   | 0.10   | 0.48   | 0.03   |
| 5    | DegQ   | 0.58   | 0.22   | 0.27   | 0.04   |
| 6    | MalB   | 0.06   | 0.15   | 0.58   | 0.07   |
| 7    | TnaA   | 0.41   | 0.10   | 0.31   | 0.06   |
| 8    | DhaK   | 0.55   | 0.07   | 0.33   | 0.02   |
| 9    | HdeA   | 0.32   | 0.06   | 0.53   | 0.08   |
| 10   | GatY   | 0.33   | 0.06   | 0.43   | 0.04   |
| 11   | HdeA   | 0.56   | 0.12   | 0.34   | 0.07   |
| 12   | HdeA   | 0.90   | 0.11   | 0.16   | 0.06   |
| 13   | ProX   | 0.80   | 0.03   | 0.71   | 0.07   |
| 14   | UspA   | 0.49   | 0.04   | 0.07   | 0.05   |
| 15   | HisC   | 0.64   | 0.12   | 0.11   | 0.05   |
| 16   | DhaL   | 0.30   | 0.10   | 0.43   | 0.04   |
| 17   | DsbA   | 0.33   | 0.06   | 0.53   | 0.08   |
| 18   | DhaM   | 0.66   | 0.04   | 0.66   | 0.09   |
| 19   | DhaK   | 0.03   | 0.10   | 0.46   | 0.04   |
| 20   | DhaK   | 0.30   | 0.02   | 0.18   | 0.07   |
| 21   | GapA   | 0.44   | 0.09   | 0.03   | 0.10   |
| 22   | YigF   | 0.34   | 0.04   | 0.38   | 0.05   |
| Acid induced |       |        |        |        |
| 5    | TolC   | 0.50   | 0.10   | 0.17   | 0.12   |
| 6    | MglB   | 0.62   | 0.10   | 0.18   | 0.12   |
| 7    | HisC   | 0.60   | 0.09   | 0.36   | 0.09   |
| 8    | Lpd    | 1.00   | 0.00   |        |        |
| 9    | AckA   | 0.60   | 0.09   | 0.36   | 0.09   |
| 10   | UspA   | 0.35   | 0.02   | 0.20   | 0.07   |
| 11   | GapA   | 0.44   | 0.09   | 0.03   | 0.10   |
| 12   | YigF   | 0.34   | 0.04   | 0.38   | 0.05   |

a Proteins were identified by MALDI-TOF, unless indicated otherwise.
b Protein identified by position, based on the method described previously (30, 59).

c Glycine-betaine binding periplasmic protein.

d Periplasmic nickel transporter.

e Flavohemoglobin; nitric oxide dioxygenase.

f Tagatose bisphosphate aldolase.

g Dihydrolipoamide dehydrogenase.

h Acetate kinase.

i Universal stress protein.

j Glyceraldehyde 3-phosphate dehydrogenase A.

k Tolerance to ColE1.

l Periplasmic oligopeptide binding protein.

m Acetyl coenzyme A carboxylase.

n Acyloin dehydratase.

o ColE1 transposase.

p Inorganic pyrophosphatase.

q EF-Ts, transcription elongation factor.

r Outer membrane protein A.

sExtreme-acid periplasmic chaperone.
FIG. 1. pH-dependent protein profiles after anaerobic growth. The horizontal axis represents the approximate pH range of the isoelectric focusing first dimension, and the vertical axis represents the molecular weight (Mw). In the layered view shown two composite images, one representing growth at pH 8.5 (pink) and one representing growth at pH 5.5 (green), are superimposed. Each composite image is based on three 2-D gels from independent replicate cultures. All cultures of E. coli W3110 were grown at 37°C to an OD$_{600}$ of 0.15 in LBK with buffer of the appropriate pH at a concentration of 100 mM as described in Materials and Methods. (A) Cultures grown in LBK buffered with 100 mM MES (pH 5.5) or 100 mM TAPS (pH 8.5). (B) Cultures grown in LBK buffered with a mixture of 50 mM MES and 50 mM TAPS for both pH 5.5 and pH 8.5.
was reported previously to be acid induced aerobically but whose concentration too low for MALDI-TOF identification (59). The acid-induced proteins observed under anaerobiosis included catabolic enzymes (GatY and AckA), periplasmic proteins (TolC, HdeA, and OmpA), and redox proteins (GuaB, HmpA, and Lpd).

**Strain construction.** Our growing picture of pH-regulated catabolism predicts that pH regulates expression of additional pathways of amino acid catabolism. For example, one of the most strongly base-induced proteins in *E. coli* is TnaA (7, 59), which deaminates tryptophan, cysteine, and serine (58, 60). Therefore, we predicted that other enzymes that degrade cysteine or serine, such as the degradative serine deaminase encoded by *sdaA* (62), would also show base induction. A lac reporter fusion to *sdaA* was constructed as described in Materials and Methods. PCR sequence analysis of the fusion strain showed that the *sdaA* promoter, located at positions −154 to 55 in the *E. coli* K-12 genome, was inserted 17 bp from the start of the *EcoRI* restriction site and 260 bp from the *lacZ* sequence in the pRS415 vector. The fusion was then moved into the MC4100 genome (strain JLS0711).

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### DISCUSSION

**Expression of *sdaA*, *gadA*, and *gadB*.** The gene fusions *sdaA::lac*, *gadA::lac*, and *gadB::lac* were tested for expression as a function of pH by using cultures grown with and without aeration. All of the fusions were induced at high pH under anaerobiosis, and induction was enhanced at a higher cell density (Fig. 2). Growth curves indicated that the data for the cultures assayed were obtained during early- or mid-log-phase growth (Fig. 3); growth of cultures at the low and high pHs stopped at a lower cell density than growth of cultures at pH 7. The curves shown in Fig. 3 are for strain JLS 0711; other strains assayed produced similar results (data not shown).

The enhancement of high-pH induction at a higher cell density parallels previous reports of cell density enhancement of expression of *maaA*, *csyK*, and *gabT* (59). The *sdaA::lac* construct also exhibited slight induction by base with aeration. The *gadA::lac* and *gadB::lac* constructs, however, showed high-pH induction only during anaerobic growth at the mid- to late stationary phase.

**FIG. 2.** pH-dependent expression of *lacZ* gene fusions. β-Galactosidase activity is expressed in specific activity units (51). Cultures were grown in buffered LBK to different OD₆₀₀ values, and β-galactosidase activity was assayed as described in Materials and Methods. (A) JLS0711 (*sdaA::lac*); (B) JLS0215 (*gadA::lac*); (C) JLS0214 (*gadB::lac*). The data are means ± standard errors for four independently grown cultures.

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**DISCUSSION**

Our 2-D gel analysis of anaerobic protein profiles revealed a substantial number of pH-dependent proteins that were not observed with aeration. Most of these proteins were catabolic enzymes or catabolite transporters. These new observations may have several explanations. (i) In the absence of oxygen, catabolism generates greater quantities of organic products whose buildup threatens the cell, especially permeant acids at low pH; therefore, greater regulation of catabolism is needed. (ii) Some proteins that show pH regulation with or without oxygen may fail to show up under aeration conditions if their overall expression level is repressed by oxygen; an example is NikA, whose high-pH induction is barely detectable when aeration is used (59) but appeared more strongly in an anaerobic culture. (iii) During anaerobic growth, a number of proteins expressed at high levels when aeration is used may be repressed; the repression of these proteins may reveal the presence of protein spots previously undetected in the gels prepared from aerated cultures.

**High-pH-induced proteins during anaerobic growth.** The high-pH induction of several more catabolic enzymes fits into our growing picture of pH-regulated catabolism during growth in complex medium under conditions that may resemble the growth conditions in the intestine (Fig. 4). Our general model is that low pH favors production of alkaline amines that counteract acidification plus CO₂, an acid that readily diffuses and is removed rapidly by the host circulation, whereas high pH favors production of fermentation acids plus NH₃, which diffuses and is removed. This model is consistent with acidic induction of amino acid decarboxylases and alkaline induction of deaminases and sugar breakdown.
We show here that high pH induced the three major components of the dihydroxyacetone (Dha) kinase system (DhaK, DhaL, and DhaM) (20, 44). The Dha kinase system transfers phosphate from the phosphotransferase system to DhaM and then through Dha kinase (DhaLM) to Dha, a catabolic product of sugars and amino acids. The phosphorylated Dha is then converted to glyceraldehyde 3-phosphate by GapA, which is also induced at high pH; from there, breakdown leads to fermentation products. Unlike other phosphotransferase systems, Dha kinase acts entirely within the cytoplasm, without involving vectorial transport; thus, the catabolite and its acidic fermentation products are maintained in the cytoplasm.

Besides enzymes, two sugar transporters were induced at high pH, the maltose oligosaccharide porin MalB (4, 17, 36) and the galactose binding protein MglB (25). These transporters should be useful for uptake of the hydrolyzed glycogen and lactose in LBK. In general, glycolysis and fermentation of available sugars should proceed more rapidly at high pH, at which the fermentation acids either buffer the internal pH or exit the cell down the pH gradient. Interestingly, several sugar porins, including MalB and OmpF, exhibit channel closure at pH values below 5, at which even low concentrations of fermentation acids can endanger the cell (4, 42).

Amino acid catabolism at high pH favors deaminases, such as TnaA (7). The high-pH induction of TnaA, which deaminites Trp, Cys, and Ser, led us to test the pH dependence of expression of serine deaminase. The SdaA protein did not show up on our 2-D gels, which separated only a subset of E. coli proteins. Nevertheless, an sdaA: lac fusion showed strong induction at high pH. The high-pH induction required anaerobiosis, which is consistent with our prediction that anaerobic conditions turn on modes of pH regulation of catabolism that are not seen under aerobic conditions. Serine deamination may also play a role in the stationary phase, when the pH of LB rises above pH 9 (53), since mutants with increased stationary-phase survival show enhanced catabolism of serine (69).

The gadA and gadB reporters showed increased expression as the pH increased across the pH range (Fig. 2). The high-pH induction of gadA:: lac and gadB:: lac required a high cell density and anaerobiosis. These results confirmed the previous report of elevated GadA levels at pH 9 (7). In other studies, expression of gadA and gadB may have been induced by acid in the early stationary phase (12), although a gadX mutant actually showed acid repression of gadA and gadB (37). The high-pH induction of gad is interesting in view of the role of this gene in resistance to acid (11, 12, 38, 63, 65). However, gadC mutants show defective acid resistance only when they are grown at pH values above 7; thus, the role of gad in acid resistance appears to be especially important for cultures grown at high pH before exposure to extreme acid conditions (24).

The complexity of the gad response may be related to the fact that unlike the other acid-induced decarboxylases (CadA, Adi, and SpeF), which generate amines, GadA and GadB generate an amino acid, 4-aminobutanate (GABA), which can be directed into alternative pathways (Fig. 4). At high pH, GABA is directed into production of succinate by GabT (59). Succinate is a nonpermeant acid that could neutralize internal alkalization or be converted to other fermentation acids.

Also induced at high pH were the histidine biosynthesis components HisC and HisD. HisC catalyzes amino transfer from L-histidinol-phosphate to 2-oxoglutarate, forming glutamate (19, 23). The role of HisC during high-pH induction may be related to its interaction with the pH-dependent GABA-glutamate system.

The DegQ periplasmic endoprotease (31) cleaves misfolded proteins by recognizing specific peptide folds usually buried within the three-dimensional protein structure. Other protein-folding agents induced at high pH include UspA and Tig. Both base stress and acid stress cause problems with protein folding, which are addressed by different chaperones and proteases; at low pH, HdeA was induced, as observed previously in aerobic cultures (59).

Low-pH-induced proteins during anaerobic growth. Fewer catabolic proteins were induced under acidic conditions than at
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