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1 ***ESCHERICHIA COLI* HDEB IS AN ACID-STRESS CHAPERONE**

2

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13 Running title : Periplasmic acid-stress chaperone HdeB

14 Abbreviations: DTT : dithiothreitol ; GAPDH : glyceraldehyde-3-phosphate  
15 dehydrogenase ; ADH : alcohol dehydrogenase ; ANS : 1-anilino-8-naphtalenesulfonate.

16 **ABSTRACT**

17

18 We have cloned, expressed and purified the *hdeB* gene product, which belongs to the *hdeAB*  
19 acid-stress operon. We extracted HdeB from bacteria by the osmotic shock procedure and  
20 purified it to homogeneity by ion exchange chromatography and hydroxyapatite  
21 chromatography. Its identity was confirmed by mass spectrometry analysis. HdeB has a  
22 molecular mass of 10 kDa in SDS-PAGE which matches its expected molecular weight. We  
23 purified the acid-stress chaperone HdeA in parallel in order to compare the two chaperones.  
24 The *hdeA* and *hdeB* mutants both display a reduced viability upon acid stress, and only the  
25 HdeA/HdeB expression plasmid can restore their viability close to the wild-type level,  
26 suggesting that both proteins are required for an optimal protection of the bacterial periplasm  
27 against acid stress. Periplasmic extracts from both mutants aggregate at acidic pH suggesting  
28 that HdeA and HdeB are required for protein solubilization. At pH 2, the aggregation of  
29 periplasmic extracts is prevented by the addition of HdeA, as previously reported, but only  
30 slightly reduced by HdeB. At pH 3, however, HdeB is more efficient than HdeA in preventing  
31 periplasmic protein aggregation. The solubilization of several model substrate proteins at  
32 acidic pH supports the hypothesis that HdeA plays a major role in protein solubilization at pH  
33 2, and that both proteins are involved in protein solubilization at pH 3. Like HdeA, HdeB  
34 exposes hydrophobic surfaces at acidic pH, in accordance with the appearance of its  
35 chaperone properties at acidic pH. HdeB, like HdeA, dissociates from dimers at neutral pH  
36 into monomers at acidic pHs, but its dissociation is complete at pH 3, whereas that of HdeA is  
37 complete at a more acidic pH. Thus, we can conclude that *E. coli* possesses two acid stress  
38 chaperones that prevent periplasmic protein aggregation at acidic pH.  
39

## 40 INTRODUCTION

41  
42 In their natural habitats, bacteria are constantly under assault from a wide array of environmental  
43 stresses, including UV, heat, oxidative, osmotic and pH stresses (5). One of the most frequently  
44 encountered is acid stress (8). Enterobacteria, when travelling through the gastrointestinal tract  
45 encounter an extremely low pH; facultative intracellular pathogens tolerate episodes of low pH within  
46 macrophage phagolysosomes, and fermentative bacteria excrete acidic fermentation products that  
47 trigger an endogenous acid stress (5, 17).

48 In response to acid stress, several mechanisms regulate the homeostasis of bacterial pH. Many  
49 bacteria, including *E. coli*, possess aminoacid (glutamate, arginine, or lysine) decarboxylase systems,  
50 each of which consists of a cytoplasmic decarboxylase which converts its substrate to a related amine  
51 ( $\gamma$ -aminobutyric acid, agmatine or cadaverine, respectively), and an antiporter which exchanges the  
52 imported amino acid for the cytoplasmic amine produced (5, 8, 12, 21, 24). These systems consume  
53 one cytoplasmic proton during amino acid decarboxylation, and one extracytoplasmic proton during  
54 protonation of the exported amine, thus leading to cytoplasmic and periplasmic alkalinization. In  
55 several bacteria, including fermentative bacteria such as *Lactococcus lactis*, the proton-translocating  
56  $F_1F_0$  ATPase can export protons as a consequence of ATP hydrolysis (26). Further protection against  
57 acid stress is obtained by decreasing the permeability of the inner and outer membranes to protons ;  
58 several inner and outer membrane proteins are overexpressed in response to acid stress, including the  
59 cyclopropane fatty acyl phospholipid synthase, OmpC, the LPS biosynthesis enzyme YfbF and the  
60 outer membrane lipoprotein Slp (26). Furthermore, bacteria can reverse their cytoplasmic membrane  
61 potential to an inside-positive potential that slows the influx of protons into the cell (8, 21). They can  
62 also reorient their metabolism towards pathways that decrease proton production or increase amine  
63 production with a consequent alkalinization (underexpression of catabolic sugar enzymes, such as  
64 those of the maltose regulon (27) decreases the production of organic acids, and overexpression of  
65 urease increases ammonia production in *Helicobacter pylori* (2, 7)). Moreover, several protective  
66 proteins may be induced upon acid stress, including the DnaK and GroEL chaperone machines (17)  
67 and several DNA repair enzymes (25).

68 The acid resistance mechanisms of the periplasm are not as well understood as those of the cytoplasm.  
69 Periplasmic proteins are probably more vulnerable than cytoplasmic proteins to acid stress, due to the  
70 relative permeability of the outer membrane porins to molecules smaller than 600 Da (15, 23).  
71 Recently, a periplasmic chaperone, HdeA, which supports acid resistance in *E. coli*, was discovered (9,  
72 14). HdeA exhibits chaperone-like activity at strong acidic pH only (below pH 3), preventing the acid-  
73 induced aggregation of bacterial periplasmic extracts, and of several model substrate proteins such as  
74 alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the  
75 periplasmic ribose receptor (14).

76 In this study, we report the cloning, purification and characterization of HdeB as a novel acid-stress  
77 chaperone, and we show that, whereas HdeA is more efficient than HdeB in protein solubilization at  
78 pH 2, HdeB is more efficient than HdeA at pH 3. Thus, *E. coli* possesses two acid-stress chaperones,  
79 HdeA and HdeB, which are encoded by the acid-stress operon *hdeAB*, and prevent the aggregation of  
80 periplasmic proteins at acidic pH.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditons.** The *E. coli* BL21 (DE3) strain (Novagen, USA) was used for the transformation of the new expression vector constructs containing the genes encoding for HdeB and HdeA, respectively. We generated the genes coding for HdeB and HdeA by amplifying the *hdeB* and *hdeA* genes from *E. coli* genomic DNA (from strain MG1655) by PCR using the forward primers (5'GGTGGTTGCTCTTCACATATGAATATTTTCATCTCTC-3' for *hdeB*, and 5'GGTGGTTGCTCTTCACATATGAAAAAAGTATTAGGC-3' for *hdeA*) containing a *NdeI* site and the reverse primers (5'GGTGGTCTGGGATCCTCATTAATTCGGCAAGTCATT-3' for *hdeB*, and 5'GGTGGTCTGGAATCCTCATCATTACATATCTTTCTYTAAT-3' for *hdeA*) containing a *BamHI* site. The whole *hdeAB* operon was generated by PCR using the *hdeA* forward primer and the *hdeB* reverse primer. The PCR was done in a volume of 50  $\mu$ l, in the presence of 0.5  $\mu$ g of template DNA, 1  $\mu$ g of forward and reverse primers and 5 units of Taq DNA polymerase (Applied Biosystems), for 35 cycles (1 min at 94°C, 2 min at 58°C, 2 min at 72°C) followed by a final cycle (1 min at 94°C, 2 min at 58°C, 10 min at 72°C). The resulting products were digested with *NdeI* and *BamHI*, ligated to the pET-21a (Novagen, USA) *NdeI* and *BamHI* backbone fragment, and transformed into strain BL21 (DE3). The sequences of the cloned genes were confirmed by DNA sequencing (not shown).

**Construction of the *hdeA* and *hdeB* mutants.** We achieved the one-step inactivation of the *hdeA* and *hdeB* genes using the phage  $\lambda$  recombination (Red) system (4). The strategy is analogous to the PCR-based gene deletion method in yeast, except that we used *E. coli* cells carrying an easily curable, low copy number plasmid pSC101 (replication temperature-sensitive derivative)-based plasmid pKD46 carrying the  $\lambda$  Red recombinase genes expressing the Red system. The basic strategy was to replace a chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR by using primers with 50-nt extensions homologous to the adjacent upstream or downstream flanking regions of the target gene and 20-nt 3' end for the amplification of the kanamycin (*kan*) resistance gene. This replacement was achieved by  $\lambda$  Red-mediated recombination in these flanking homologies (4). Strain BW25113 (*lacI<sup>f</sup> rrnB3  $\Delta$ lacZ4787 hsdR514  $\Delta$ (araBAD)567  $\Delta$ (rhaBAD)568 rph-1*) was used as the parent for construction of the *hdeA* and *hdeB* mutants. The construction of the *hdeA* and *hdeB* disruption mutants is part of the systematic construction of *E. coli* single gene deletion mutants (1). The amounts of HdeA and HdeB in the *hdeA*- and *hdeB*-deficient mutants, and in these mutants complemented with pBAD vectors (see below) were evaluated by 1D- or 2D-gel electrophoresis of osmotic shock fluids prepared from these strains.

**Construction of pBAD vectors.** Construction of pBAD vectors for *in vivo* expression of HdeA and HdeB in the *hdeA*- and *hdeB*-deficient strains was done as follows. The *hdeA* gene, the *hdeB* gene and the *hdeAB* operon were excised from the pET-21a-*hdeA*, pET-21a-*hdeB* and pET-21a-*hdeAB* vectors using *Xba* I and *Hind* III, and ligated to the pBAD33 *Xba* I and *Hind* III backbone fragment (pBAD33 is a pACYC184-derived vector containing the P<sub>BAD</sub> promoter of the *araBAD* operon and the gene encoding the regulator of this promoter, *araC*) (11). The pBAD vectors were induced with 0.05% arabinose.

**Preparation of bacterial extracts, and purification of HdeA and HdeB.** The HdeA and HdeB overproducing strains BL21 (DE3) pET-21a-*hdeA* and BL21 (DE3) pET-21a-*hdeB* were grown at 37°C in 1 liter of Luria-Bertani medium (20) supplemented with ampicillin (50  $\mu$ g/ml) to an OD<sub>600</sub> = 0.5. HdeA and HdeB overexpression was induced with 1 mM IPTG and growth was continued for 3 hours. Cells were harvested by centrifugation at 4°C. The cell pellets were resuspended in 10 ml of 30 mM Tris pH 8.0, 20 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and bacterial periplasmic extracts were prepared according to the osmotic shock procedure described in (13). HdeA and HdeB were each loaded onto a DEAE-Sephacel column equilibrated in 30 mM Tris pH 8.0, 1 mM DTT at 20°C, and eluted with a linear gradient of 0 – 0.5 M NaCl in the same buffer. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and quantified by the Bradford assay. Purified fractions of HdeB and HdeA were each loaded onto a hydroxyapatite column (Bio-Gel HTP from BioRad), equilibrated at 20°C in 30 mM Tris pH 7.5, 1 mM DTT and eluted with a linear gradient of 0 - 50 mM sodium

135 phosphate pH 7.5 in the same buffer. HdeA and HdeB were stored at -70°C. For experiments at  
136 acidic pH, HdeA and HdeB were equilibrated in distilled water by gel permeation on a Bio-Gel P10  
137 column equilibrated in water (BioRad). We performed electrophoresis according to Laemmli, using  
138 16% polyacrylamide gels (Bio-Rad) with Coomassie blue staining (16). All the experiments showing  
139 polyacrylamide gels (Figure 2-5) have been repeated at least three times, leading to similar results.  
140 We used NIH-Image 1.62 software ([rsb.info.nih.gov/nih-image/Default/html](http://rsb.info.nih.gov/nih-image/Default/html)) to quantify protein  
141 bands on polyacrylamide gels.

142

143 **Mass spectrometric identification of HdeA and HdeB.** Excised HdeA and HdeB gel bands were in-  
144 gel digested with mass spectrometry grade trypsin (Roche). Mass spectra were recorded in the positive  
145 ion reflection mode of a matrix -assisted laser desorption ionization-time of flight (MALDI-ToF)  
146 Voyager DE PRO (Applied Biosystems). The peptide masses obtained were searched against the *E. coli*  
147 database (PIR, National Biomedical Research Foundation) with the Mascot engine available online  
148 ([www.matrixscience.com](http://www.matrixscience.com)).

149

150 **Chaperone assays.** The chaperone activity of HdeA and HdeB was assayed by incubating periplasmic  
151 extracts or model substrate proteins for 60 min at 25°C in the presence of HdeA and/or HdeB at acidic  
152 pH, and monitoring the appearance of the proteins in the 15,000 x g pellet or supernatant  
153 (protein were analyzed by SDS-PAGE) (14). Sulfate was used as the anion in order to achieve  
154 effective aggregation of substrate proteins at low pH values (10, 14).

155

156 **ANS binding assay to HdeA and HdeB.** Using a Kontron SFM 25 fluorescence spectrophotometer,  
157 we monitored the binding of ANS (100 µM) to HdeA or HdeB (7 µM) by measuring the increase in  
158 fluorescence intensity of ANS at 25°C upon its binding to protein. Samples were excited at 395 nm,  
159 with emission being recorded between 400 and 600 nm. Fluorescence readings were made for  
160 triplicate samples.

161

162 **Oligomeric forms of HdeA and HdeB.** The molecular weight of HdeA and HdeB was determined by  
163 filtration of the proteins on a TSK G-2000-SW HPLC gel permeation column (Hewlett Packard). Blue  
164 dextran (2 MDa), yellow dextran (20,000 Da), cytochrome C (12,500 Da) and vitamin B12 (1382 Da)  
165 were used as molecular weight standards. For experiments at pH 7.5, the column was equilibrated in  
166 20 mM Tris, pH 7.5, 100 mM NaCl at 20°C, loaded with 20 µl of protein (2.4 mg/ml) and eluted at a  
167 flow rate of 0.5 ml/min. For experiments at pH 3 and pH 2, the column was equilibrated with 150 mM  
168 Na<sub>2</sub>SO<sub>4</sub> adjusted to these pHs with sulfuric acid. Proteins were detected by their absorbance at 280  
169 nm. When both proteins were loaded onto the column, they were detected by SDS-PAGE (on a 18%  
170 acrylamide gel).

171 **Acid stress sensitivity of the *hdeA* and *hdeB* mutants, and of these mutants complemented by**  
172 **plasmids pBAD33-*hdeA*, pBAD33-*hdeB* and pBAD33-*hdeAB*.** Mutant and parental strains were  
173 grown in LB medium containing 0.05% arabinose for 24 hours at 37°C with aeration, in the presence  
174 of the required antibiotics. They were diluted to 10<sup>6</sup> bacteria per ml, and incubated for 90 min in  
175 acidified LB medium (pH 2 or pH 3) at 37°C under aeration (9). A control was done at pH 7. Survival  
176 patterns were determined by plating bacteria overnight on LB agar plates at pH 7.

177

178 **Reagents.** Restriction enzymes were from Invitrogen, and the plasmid extraction kit was from Qiagen.  
179 ADH (from rabbit muscle, 36 kDa) and GAPDH (from *Saccharomyces cerevisiae*, 36 kDa), were  
180 from Sigma, and OppA was purified as described in Richarme and Caldas (22). All other chemicals  
181 were from Sigma and were reagent grade.

## RESULTS

**Expression and purification of HdeA and HdeB.** The BL21 (DE3) strains, transformed with the recombinant expression vector *pET-21a-hdeB* was induced for 3 hours with 1 mM IPTG, and its periplasmic extract was prepared by a modification (13) of the osmotic shock procedure of Nossal and Heppel (20). The HdeB overproducing strain accumulates a periplasmic protein migrating in SDS-PAGE with an apparent molecular weight of 10 kDa, which matches the expected HdeB molecular weight of 9 kDa (after processing of the signal sequence) (**Figure 1, lane 2**). The overexpressed protein represents 29 % in mass of the proteins in the induced periplasmic extract. HdeB was purified, as described in “Materials and Methods”, by two chromatographic steps on a DEAE-Sephacel column and a hydroxyapatite column, (**Figure 1, lane 3**), and its identity was confirmed by mass spectrometry (not shown). We purified HdeA from strain *pET-21a-hdeA*, using the same osmotic shock procedure and chromatographic steps as for HdeB (**Figure 1, lane 4**), and confirmed its identity by mass spectrometry (not shown).

**Acid-induced aggregation of periplasmic extracts from the *hdeA* and *hdeB* mutants.** Periplasmic protein extracts are relatively resistant to acid-induced aggregation (18), and the acid-stress chaperone HdeA contributes to this resistance (9, 14). We investigated the acid-induced aggregation of periplasmic extracts from the *hdeA* and *hdeB* mutants and their parent, by analyzing pellets and supernatants after an acidic treatment at pH 2 or pH 3 for 60 min. The wild-type extract displays a moderate protein aggregation at acidic pH (**Figure 2A**). In contrast, a massive protein aggregation is observed at both pHs in periplasmic extracts from the *hdeA* mutant (as reported previously (14)), and from the *hdeB* mutant. Conversely, a lower quantity of proteins from the two mutants (as compared to their parent) remains in the supernatant at these acidic pHs (**Figure 2B**). For both mutants, protein aggregation is more extensive at pH 2 than at pH 3, and for the *hdeA* periplasmic extract than for the *hdeB* extract. Furthermore, the proteins that appear to aggregate in periplasmic extracts from both mutants are similar. A fraction of HdeA (characterized by N-terminal sequencing) is found in the pellets at pH 2 (slightly above the 7 kDa marker in Figure 3A, except in the HdeA-deficient extract). At pH 7, there is no significant protein aggregation, suggesting that HdeA and HdeB do not function as neutral pH chaperones..

The two mutants display a strong protein aggregation at both pH 2 and pH 3, but HdeA and HdeB are mainly involved in periplasmic protein solubilization, at pH 2 and pH 3, respectively (see below). We checked the expression level of HdeA and HdeB in each mutant. Our 2D-gel electrophoresis analysis (performed as described in (17)) of periplasmic extracts from the *hdeA* and *hdeB* mutants showed that HdeA and HdeB are both undetectable in the *hdeA* mutant (which consequently behaves like a *hdeAB* mutant), and that HdeB is undetectable in the *hdeB* mutant, while HdeA is expressed at around 24% of the wild-type level (not shown). The null expression of the two chaperones in the *hdeA* mutant explains its aggregation phenotype at pH 2 and pH 3. The aggregation phenotype of the *hdeB* mutant cannot be explained by its low HdeA level, since complementation of the mutant by the P<sub>BAD</sub> HdeA expression plasmid (the complemented strain expresses HdeA at a level that is 148% of that of the wild-type) does not significantly modify its aggregation behaviour (not shown).

### **HdeA is more efficient than HdeB in preventing periplasmic protein aggregation at pH 2.**

Periplasmic extracts from the parental strain and from the *hdeA* and *hdeB* mutants, either alone or supplemented with purified HdeA, HdeB, or both, were incubated at pH 2 for 60 min, and their 15,000 x g pellets and supernatants were analyzed. As reported above, protein aggregation at pH 2 increases in extracts from both mutants. The addition of HdeA decreases the aggregation of the *hdeA* (**Figure 3A**) and *hdeB* (not shown) extracts, whereas the addition of HdeB is much less efficient. HdeA and HdeB, together, are no more efficient than HdeA on its own. For the different periplasmic extracts, a high quantity of protein in the supernatant fraction (**Figure 3B**) corresponds to a low quantity in the pellet (**Figure 3A**), and vice versa. Most of HdeA and HdeB remains in the supernatant. We quantified the aggregated proteins in the *hdeB* mutant extract and in its wild-type control using NIH 1.62 software (**Figure 3C**). The profile of the HdeB-supplemented extract is only slightly different from that of the unsupplemented extract, whereas the profile of the HdeA-supplemented extract approaches

236 that of the parental strain extract. These results are consistent with the hypothesis that HdeA is the  
237 main chaperone involved in protein solubilization at pH 2. The important role of HdeA in preventing  
238 protein aggregation at pH 2 has already been discussed (9, 14).

239  
240 **HdeB is more efficient than HdeA in preventing periplasmic protein aggregation at pH 3.**

241 Periplasmic extracts from the parental strain and from the *hdeA* and *hdeB* mutants, either alone or  
242 supplemented with HdeA, HdeB, or both, were incubated at pH 3 for 60 min, and their 15,000 x g  
243 pellet and supernatant were analyzed. As reported above, there is a strong protein aggregation in  
244 extracts from both mutants. HdeA decreases the aggregation of the *hdeA* (**Figure 4A**) and *hdeB* (not  
245 shown) extracts, but HdeB is much more efficient than HdeA. HdeA and HdeB, together, are no more  
246 efficient than HdeB on its own. For the different periplasmic extracts, a high quantity of protein in the  
247 supernatant fraction (**Figure 4B**) corresponds to a low quantity in the pellet, and vice versa. We  
248 quantified the aggregated proteins in the *hdeA* mutant extract and in its wild-type control from Figure  
249 4A using NIH 1.62 software (**Figure 4C**). The protein profiles of the HdeA- and HdeB-supplemented  
250 extracts reflect the greater efficiency of HdeB in solubilizing periplasmic proteins at pH 3. These  
251 results are consistent with the hypothesis that HdeB is the main chaperone for periplasmic protein  
252 solubilization at pH 3, and clarify the poor efficiency of the HdeA chaperone at pH 3 (14, this study).  
253 These results, together with those presented in the preceding paragraph, suggest that, to achieve the  
254 solubilization of periplasmic extracts (the natural substrate of HdeA and HdeB) the task is divided  
255 between the two chaperones, HdeA being involved in protein solubilization mainly at pH 2, and HdeB  
256 mainly at pH 3.

257  
258 **Solubilization of model substrate proteins at pH 2 and pH 3.**

259 We examined the aggregation-suppressing function of HdeA and HdeB, using two usual substrates of chaperone activity assays,  
260 alcohol dehydrogenase (ADH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the  
261 periplasmic oligopeptide receptor OppA. Protein aggregation was monitored by analysis of 15,000 x g  
262 supernatants and pellets, after incubation of these proteins at acidic pHs in the absence or presence of  
263 chaperones. In the absence of chaperones, GAPDH remains in the supernatant at pH 7 and 4 (not  
264 shown), but not at pH 3 and 2 (**Figure 5A**). At pH 2, HdeA increases the fraction of soluble GAPDH  
265 from 12% to 81%, whereas HdeB has only a weak effect, and HdeA and HdeB together are no more  
266 efficient than HdeA on its own. At pH 3, HdeA and HdeB, separately, increase the fraction of soluble  
267 GAPDH from 1% to 12% and 8%, respectively, whereas HdeA and HdeB together solubilize up to  
268 55% of GAPDH, suggesting that a synergy occurs between the two chaperones for the solubilization  
269 of this substrate protein to take place. For each experiment, the amounts of GAPDH found in the  
270 supernatant and the pellet are additive (in all the following experiments, the initial amount of each  
271 substrate protein added was quantitatively recovered in its supernatant and pellet fractions).

272 Experiments similar to that shown in Figure 6A were performed with ADH and OppA. For ADH  
273 solubilization, HdeA, but not HdeB, was efficient at pH 2 (98% solubilization); at pH 3, HdeA or  
274 HdeB, alone, were relatively inefficient (less than 10% solubilization) whereas HdeA and HdeB  
275 together solubilized up to 68% of the protein (**Figure 5B**). For OppA, HdeA solubilized 95% and 52%  
276 of the protein at pH 2 and pH 3, respectively, whereas HdeB solubilized 14% and 25% of OppA; the  
277 addition of both proteins at the same time was not significantly more efficient than HdeA alone  
278 (**Figure 5C**). These results are consistent with the hypothesis that HdeA makes a major contribution  
279 for the solubilization of model proteins at pH 2, that the efficiency of each chaperone is variable for  
280 protein solubilization at pH 3, and a synergy between the two chaperones occurs to enable the  
281 solubilization of the two cytoplasmic proteins tested at pH 3 (ADH and GAPDH) to take place.

282  
283 **HdeB exposes surfaces that are less hydrophobic than those of HdeA at acidic pHs.**

284 It is generally believed that molecular chaperones interact with their unfolded substrate proteins via hydrophobic  
285 interactions. HdeA exposes hydrophobic surfaces at acidic pH, but not at neutral pH, in accordance  
286 with the appearance of its chaperone activity at acidic pHs (14). We measured the fluorescence  
287 emission of the hydrophobic probe ANS, in the presence of HdeA or HdeB, at different pHs. As  
288 shown in **Figure 6**, ANS binding to HdeA and HdeB is negligible at pH 8, and increases at pH 3 and  
289 pH 2 (ANS fluorescence is stronger at pH 2 than at pH 3, and stronger with HdeA than with HdeB).  
290 Furthermore, the blue shift of ANS fluorescence is similar for both chaperones at pH 2 (around 30



291 nm), but lower for HdeB (11 nm) than for HdeA (35 nm) at pH 3. This suggests that the surface of  
292 HdeB is less hydrophobic than that of HdeA, especially at pH 3. Such a difference in the exposed  
293 hydrophobicity of these two chaperones might explain their different chaperone properties described  
294 above, and the combination of both chaperones, under certain circumstances, might be required for the  
295 optimal handling of unfolded proteins.

296  
297 **Oligomeric forms of HdeB at neutral and acidic pHs.** HdeB was analyzed by size-exclusion  
298 chromatography on a SW G-2000 HPLC column, as described in Materials and Methods. At pH 7.5,  
299 HdeB (loaded at 2.4 mg/ml) migrates as a dimer with an apparent molecular weight of around 16,000  
300 Da (**Figure 7**), whereas at both pH 3 (**Figure 7**) and pH 2 (not shown), it migrates as a monomer  
301 (9,000 Da). At pH 7.5, HdeA (loaded at 2.4 mg/ml) migrates as a dimer (as previously reported (14))  
302 with an apparent molecular weight of around 17,000 Da (not shown), whereas at pH 3 it migrates as a  
303 mixture of monomers and dimers (with an apparent molecular weight of around 14,000 Da) and at pH  
304 2 as a monomer (10,000 Da) (not shown), as reported previously (14). Thus, like HdeA, HdeB  
305 dissociates from dimers to monomers at acidic pHs, but, whereas the dissociation of HdeB is complete  
306 at pH 3, that of HdeA is not. This difference might explain why HdeB is a more efficient chaperone  
307 than HdeA at pH 3.

308 We also checked whether HdeA and HdeB form a complex at pH 3. When a mixture of HdeA and  
309 HdeB (2.4 mg/ml, each) was loaded onto the SW G-2000 HPLC column equilibrated at pH 3, HdeA  
310 migrated (as it does when alone) as a mixture of monomers and dimers with an apparent molecular  
311 weight of around 14,000 Da,, and HdeB migrated (as it does when alone) as a monomer of around  
312 9,000 Da (not shown).

313 **Acid stress sensitivity of the *hdeA* and *hdeB* mutants and of these mutants complemented by**  
314 **pBAD33-*hdeA*, pBAD33-*hdeB* and pBAD33-*hdeAB*.** Cultures of the mutants, and of their parent  
315 were incubated for 90 min at 37°C at either pH 2 or pH 3 (a control was done at pH 7), as described  
316 under “Materials and Methods”, and their survival patterns were observed on LB plates at pH 7. The  
317 *hdeA* mutant, which contains neither HdeA nor HdeB (see above), displays 4% and 3% survival rates  
318 at pH 3 and pH 2, respectively. The HdeB mutant, which contains a reduced amount of HdeA (24% of  
319 the wild-type level (see above)) and no HdeB displays 8% and 7% survival rates at pH 3 and pH 2,  
320 respectively. (Figure 8). This suggests that, both genes are required for resistance of *E. coli* to these  
321 acidic pHs (the involvement of *hdeA* in *E. coli*'s resistance at pH 2 has already been reported (9)).  
322 However, since the *hdeA* mutant expresses neither HdeA nor HdeB, and since the *hdeB* mutant  
323 expresses 24% of the wild-type level of HdeA, in order to determine the respective contribution of  
324 each chaperone in acid-stress resistance, we complemented these mutants with pBAD vectors  
325 expressing HdeA or (and) HdeB under the control of the arabinose P<sub>BAD</sub> promoter.  
326 The viability of the *hdeA* mutant complemented with pBAD33-*hdeA* (this strain contains HdeA (140%  
327 of the wild-type level), but no HdeB) increased to 12% (a 3-fold increase) and 18% (a six-fold  
328 increase) after the pH 3 and pH 2 stresses, respectively. This suggests that HdeA alone affords some  
329 protection *in vivo* against acid stresses at these pHs.  
330 The viability of the *hdeA* mutant complemented with pBAD33-*hdeB* (this strain contains HdeB (175%  
331 of the wild-type level) but no HdeA) increased to 16% (a 4-fold increase) and 11% (a 4-fold increase)  
332 after the pH 3 and pH 2 stresses, respectively. This suggests that HdeB alone affords a moderate  
333 protection of bacteria against acid stresses at these pHs.  
334 The viability of the *hdeB* mutant complemented with pBAD33-*hdeA* (this strain contains HdeA (148%  
335 of the wild-type level) but no HdeB) increased to 9% and 16% after the pH 3 and pH 2 stresses,  
336 respectively. These viabilities are not very different (especially at pH 3) from those of the  
337 unsupplemented *hdeB* mutant (8% and 7%, respectively). Importantly, this suggests that the low  
338 viability of the uncomplemented *hdeB* mutant is not a consequence of its reduced HdeA level (25% of  
339 the level of the parental strain) but results indeed from an HdeB deficiency.  
340 The viability of the *hdeB* mutant expressing the *hdeB* plasmid (this strain contains HdeA (28% of the  
341 wild-type level) and 125% of the wild-type HdeB level) increased to 28% and 18% after the pH 3 and  
342 pH 2 stresses, respectively.  
343 The viabilities of the *hdeA* and *hdeB* mutants expressing the *hdeAB* plasmid attained 50-75% of the  
344 wild-type level, suggesting that the P<sub>BAD</sub> expression system satisfyingly complements the strains  
345 deficient in acid-stress chaperones.  
346 In short, these viability experiments suggest that both HdeA and HdeB are required for an optimal  
347 protection of bacteria against acid stress *in vivo*, at either pH 3 or pH 2 (especially since the *hdeA*- and  
348 the *hdeB*-deficient strains display a lower viability when complemented with a single chaperone than  
349 when complemented with both chaperones).

**DISCUSSION**

We have cloned, overexpressed, purified and characterized HdeB as a novel acid-stress chaperone. HdeB was found in the periplasm in a soluble form. It was purified by osmotic shock, followed by two chromatographic steps on DEAE-Sephacel and hydroxyapatite columns, and identified by MALDI-ToF mass spectrometry. We purified HdeA in parallel in order to compare the two chaperones.

A periplasmic extract from the *hdeB* mutant aggregates at pH 2 and pH 3, like an extract from the *hdeA* mutant (9). The aggregation defect of the *hdeB* mutant does not result from the lower expression (24% of the wild-type level) of HdeA since complementation of this mutant by the HdeA expression plasmid did not rescue its aggregation defect. Thus, HdeB is important for the solubility of the bacterial periplasm at acidic pH.

At pH 2, HdeA is the main chaperone involved in the *in vitro* solubilization of periplasmic extracts and of the model substrate proteins used in our study (the chaperone properties of HdeA at a pH around 2 have already been described by others (9, 14)). HdeB is much less efficient than HdeA in solubilizing periplasmic extracts at pH 2, and its best performance at this pH was the 25% solubilization of OppA. Furthermore, HdeB did not increase the efficiency of HdeA at pH 2.

At pH 3, HdeB solubilizes periplasmic extracts more efficiently than HdeA. It can prevent the aggregation of periplasmic extracts in the absence of HdeA, and thus functions as an acid-stress chaperone by itself. HdeA or HdeB, separately, are relatively inefficient in solubilizing GAPDH and ADH (less than 10% of these proteins were solubilized), and there is a functional synergy between them (leading to 52% and 68% solubilization of GAPDH and ADH, respectively). The occurrence of a synergy between HdeA and HdeB seems to depend on the particular substrate protein used (the synergy observed for the solubilization of the cytoplasmic proteins ADH and GAPDH is not obvious for that of OppA), or on chaperone / substrate ratios (at lower chaperone concentrations, we also observed some synergy between HdeA and HdeB for the solubilization of periplasmic extracts at pH 3, unpublished results).

Like HdeA, HdeB displays more hydrophobic surfaces at acidic pHs than at neutral pH, as judged from ANS fluorescence spectra. The surface of HdeB, however, appears less hydrophobic than that of HdeA, and such a difference might explain their respective roles in the renaturation of unfolded proteins.

Like HdeA, HdeB dissociates from dimers at neutral pH to monomers at acidic pHs. The dissociation of HdeB into monomers, however, is complete at pH 3 whereas that of HdeA is not. The easier dissociation of HdeB into monomers possibly explains its better chaperone properties at pH 3. Although HdeA and HdeB appear to cooperate in the solubilization of several protein substrates at pH 3, we could not detect the formation of a stable heterodimer between the two chaperones at this pH.

An *hdeB*-deficient strain, like an *hdeA*-deficient strain (9), displays an increased sensitivity to acid stress at pH 2 and pH 3 (and its sensitivity to acid stress is not a consequence of its reduced HdeA level), suggesting that HdeB, like HdeA, is involved in resistance to acid stress. *In vivo*, there seems to be a requirement for both chaperones for an optimal resistance of bacteria to acid stresses at either pH 3 or pH 2, in accordance with their co-expression from the same operon. This contrasts somewhat with results obtained *in vitro* suggesting that HdeA is more efficient at pH 2 and HdeB at pH 3. In fact, there are many differences between the *in vivo* and *in vitro* situations, including differences in protein concentrations and in the physico-chemical properties of the medium.

HdeA and HdeB are general chaperones (like DnaK, GroEL, small Hsps and periplasmic chaperones that also function as general chaperones (3, 6)), since they reduce the aggregation of many different periplasmic proteins. Furthermore, whereas HdeA and HdeB display a different pH specificity *in vitro* (pH 2 for HdeA, pH 3 for HdeB), they solubilize roughly the same proteins, since the profile of aggregated proteins at pH 2 in the presence of HdeA is similar to that of aggregated proteins at pH 3 in the presence of HdeB. Finally, the *hdeA* and *hdeB* mutants do not present a protein aggregation phenotype at neutral pH, suggesting that HdeA and HdeB are not involved in protein solubilization at neutral pH (this has previously been reported for HdeA (14)). Computational and structural

403 experiments will allow us to understand further the different specificities of these two chaperones  
404 which have been designed by enterobacteria to prevent periplasmic protein aggregation at acidic pH.

405

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## LEGENDS TO FIGURES

**Figure 1. Purification of HdeA and HdeB.** Protein samples were separated by dodecylsulfate polyacrylamide gels (16%), and stained with Coomassie brilliant blue. *Lane 1*, periplasmic extract from uninduced strain BL21(DE3), *pET-21a-hdeB*; *lane 2*, periplasmic extract from BL21(DE3), *pET-21a-hdeB* induced for 3 hours with IPTG; *lane 3*, 5  $\mu$ g of purified HdeB; *lane 4*, 5  $\mu$ g of purified HdeA. The positions of molecular weight markers are indicated on the left.

**Figure 2. Acid-induced aggregation of periplasmic extracts.** SDS-PAGE analysis of the pellet (A) and supernatant (B) fractions of periplasmic extracts (20  $\mu$ g each) from the *hdeA* mutant, *hdeB* mutant and their parent, after an acid treatment at pH 2 or at pH 3 for 60 min (a control was made at pH 7).

**Figure 3. Prevention of periplasmic protein aggregation at pH 2.** SDS-PAGE analysis of the pellet (A) and supernatant (B) fractions of periplasmic extracts (20  $\mu$ g) from the *hdeA* mutant and its parental strain, after incubation for 60 min at pH 2, either alone or in the presence of purified (15  $\mu$ g each) HdeA, HdeB or both (7.5  $\mu$ g each), C) Densitometric scan of the protein distribution of periplasmic extracts from the wild-type strain, and the *hdeB* mutant supplemented with HdeA or HdeB, as indicated. We checked that the intensities of protein bands (excluding the HdeA/B bands) in pellets and supernatants add up to similar amounts in all cases.

**Figure 4. Prevention of periplasmic protein aggregation at pH 3.** A) SDS-PAGE analysis of pellet (A) and supernatant (B) fractions of periplasmic extracts (20  $\mu$ g) from the *hdeA* mutant and its parental strain, after incubation for 60 min at pH 3, either alone or in the presence of purified (15  $\mu$ g each) HdeA, HdeB or both (7.5  $\mu$ g each), B) Densitometric scan of protein distribution of periplasmic extracts from the wild-type strain and the *hdeA* mutant supplemented with HdeA or HdeB, as indicated. We checked that the intensities of protein bands (excluding the HdeA/B bands) in pellets and supernatants add up to similar amounts in all cases.

**Figure 5. Solubilization of GAPDH, ADH and OppA at acidic pH.** A) SDS-PAGE analysis of the supernatant and pellet fractions of GAPDH (10  $\mu$ g) that was subjected to treatment for 60 min at pH 3 or 2, either alone or in the presence of (5  $\mu$ g each) HdeA, HdeB, or both. B) SDS-PAGE analysis of the supernatant fractions of ADH (10  $\mu$ g) that was subjected to treatment for 60 min at pH 3 or 2, either alone or in the presence of (5  $\mu$ g each) HdeA, HdeB, or both. C) SDS-PAGE analysis of the supernatant fractions of OppA (10  $\mu$ g) that was subjected to treatment for 60 min at pH 3 or 2, either alone or in the presence of (5  $\mu$ g each) HdeA, HdeB, or both. The molecular weights (monomeric form) of HdeA, HdeB, GAPDH, ADH and OppA are 10 kDa, 9 kDa, 36 kDa, 36 kDa and 61 kDa, respectively.

**Figure 6. ANS fluorescence of HdeB and HdeA at neutral and acidic pHs.** The intensity of ANS fluorescence (100  $\mu$ M, in H<sub>2</sub>SO<sub>4</sub> solution of pH 2 or pH 3, or in 10 mM Tris pH 8) in the presence of 7  $\mu$ M HdeA or 7  $\mu$ M HdeB was measured after excitation at 395 nm.

**Figure 7. Oligomeric forms of HdeB at neutral and acidic pHs.** For experiments at pH 7.5, the column was equilibrated in 20 mM Tris, pH 7.5, 100 mM NaCl at 20°C, loaded with 20  $\mu$ l of HdeB (2.4 mg/ml) and eluted at a flow rate of 0.5 ml/min. For experiments at pH 3 and pH 2, the column was equilibrated with 150 mM Na<sub>2</sub>SO<sub>4</sub> adjusted to these pHs with sulfuric acid, and loaded with 20  $\mu$ l of HdeB or (and) HdeA (2.4 mg/ml each), equilibrated at the pH of the column). Proteins were detected by their absorbance at 280 nm. Blue dextran (2 MDa), yellow dextran (20,000 Da), cytochrome C (12,500 Da) and vitamin B12 (1382 Da) were used as molecular weight standards.

**Figure 8. Acid stress sensitivity of the *hdeA* and *hdeB* mutants, and of these mutants complemented with plasmids pBAD33-*hdeA*, pBAD33-*hdeB* and pBAD33-*hdeAB*.** The mutants, either uncomplemented (0) or complemented with pBAD33-*hdeA* (A), pBAD33-*hdeB* (B) or pBAD33-*hdeAB* (AB) and their parent were subjected to acid stress in LB medium adjusted to pH 3

525 (light grey) or pH 2 (dark grey) for 90 min at 37°C, as described in “Materials and Methods”, and  
526 survival patterns were determined by plating bacteria overnight on LB agar plates at pH 7. The results  
527 are the average  $\pm$  SEM of three experiments. The colony counts of the mutants were normalized to  
528 those of the parental strain. All the strains displayed similar viabilities at pH 7.



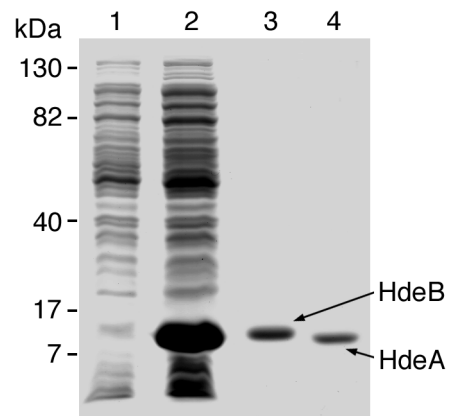


Figure 1

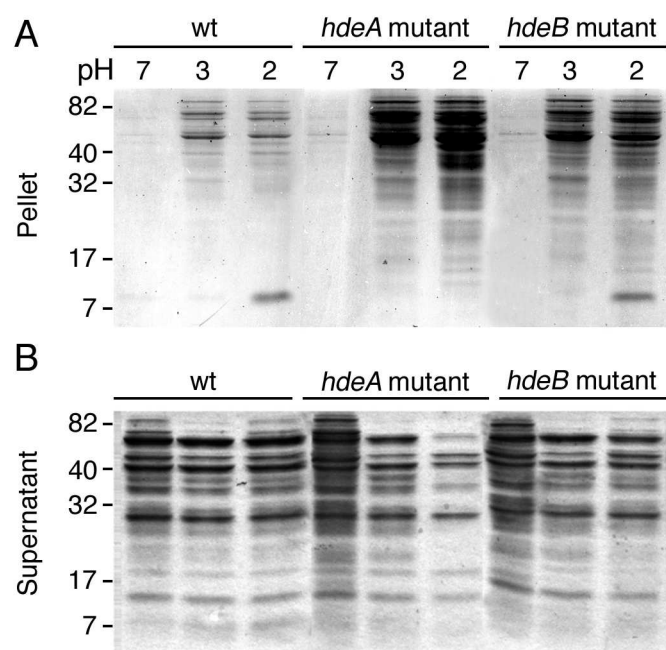


Figure 2

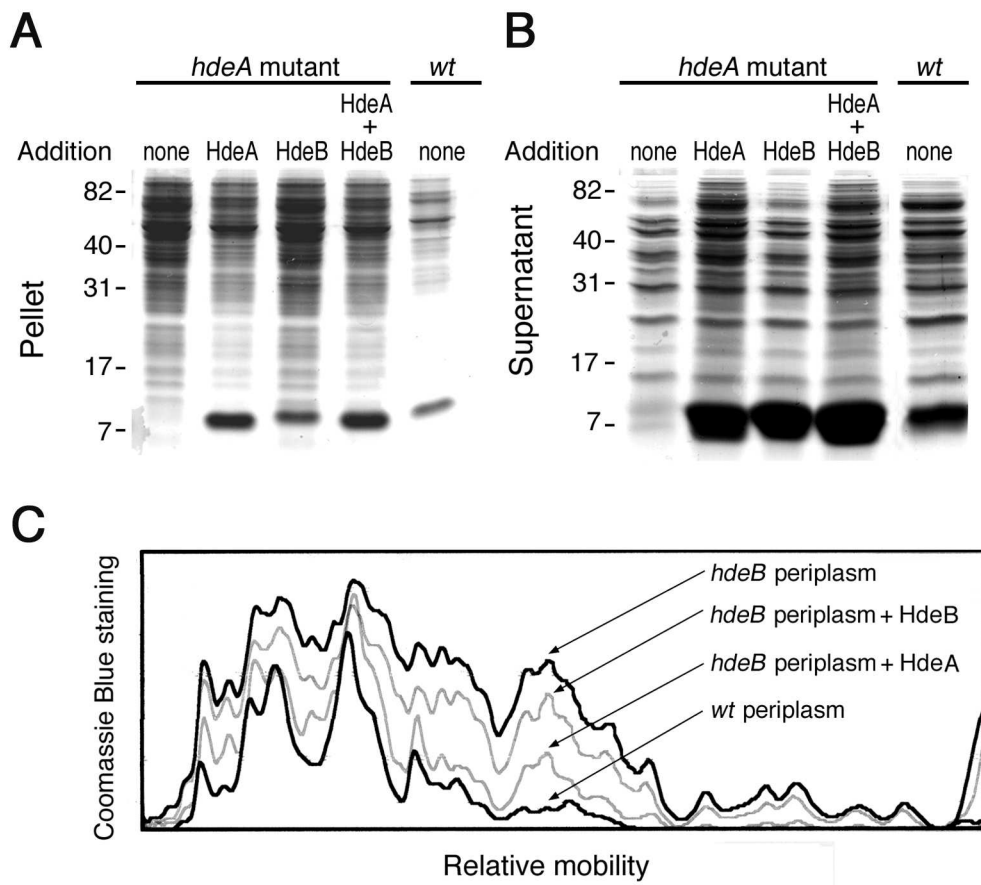


Figure 3

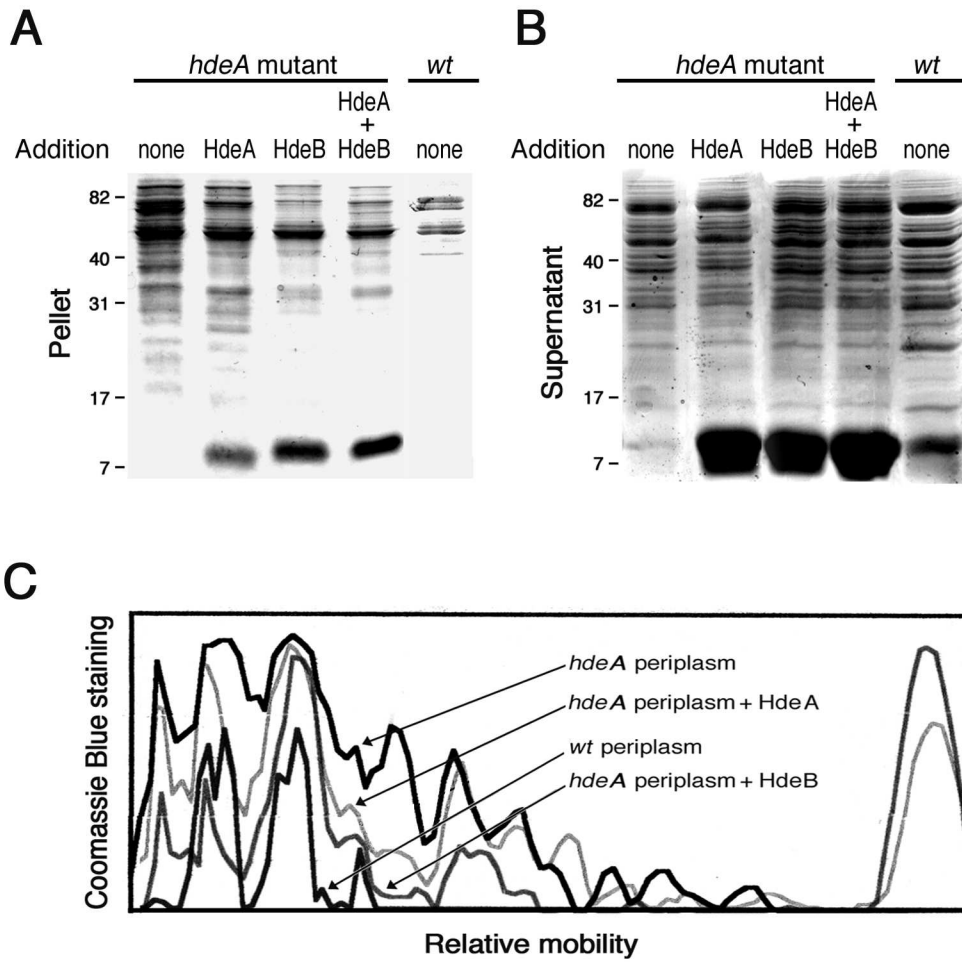


Figure 4

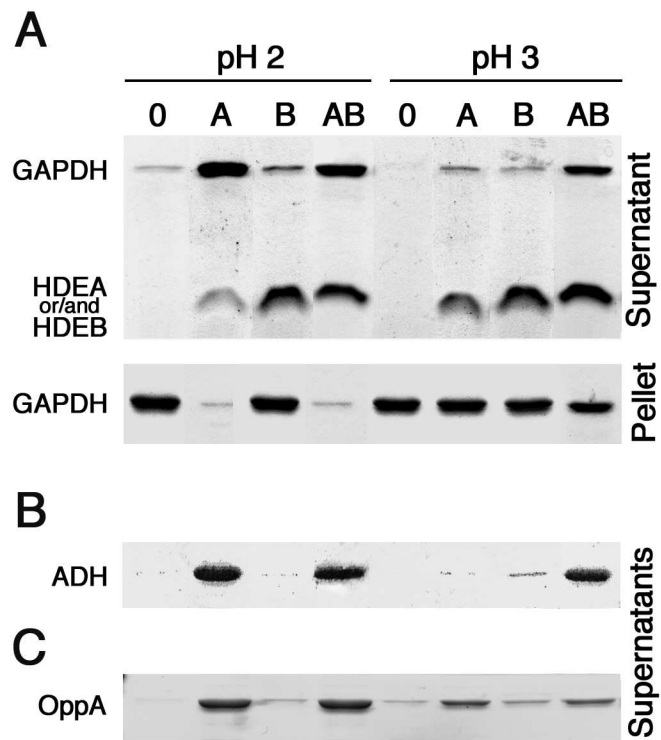


Figure 5

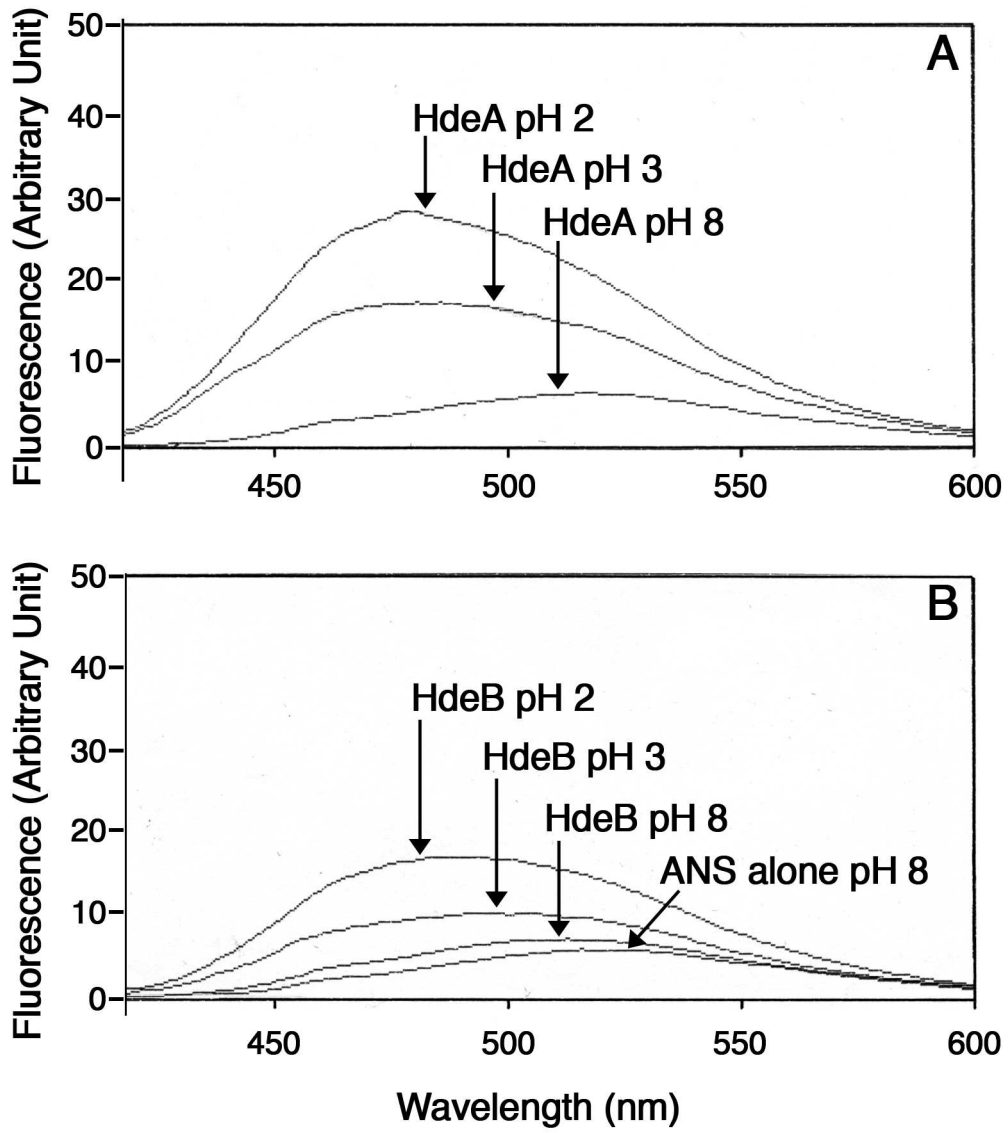


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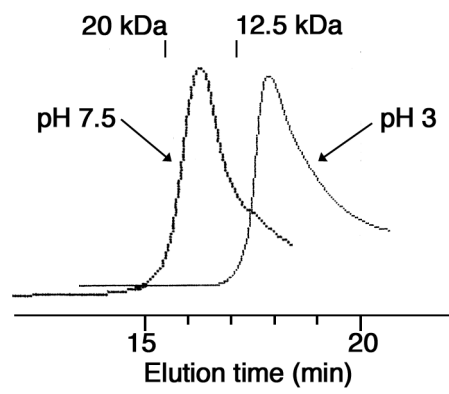


Figure 7

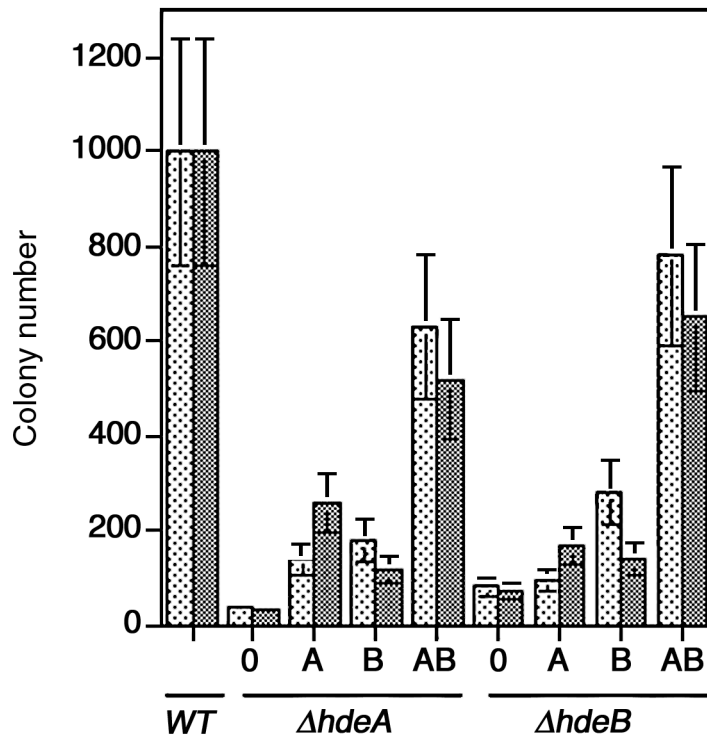


Figure 8