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Effect of External pH Perturbations on In Vivo Protein Synthesis by the Acidophilic Bacterium *Thiobacillus ferrooxidans*

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The response of the obligate acidophilic bacterium *Thiobacillus ferrooxidans* to external pH changes is reported. When *T. ferrooxidans* cells grown at pH 1.5 were shifted to pH 3.5, there were several changes in the general protein synthesis pattern, including a large stimulation of the synthesis of a 36-kDa protein (p36). The apparent low isoelectric point of p36, its location in the membrane fraction, and its cross-reaction with anti-OmpC from *Salmonella typhi* suggested that it may be a porin whose expression is regulated by extracellular pH.

Bacteria such as *Escherichia coli* have a number of global regulatory networks that enable them to adapt rapidly and survive periods of adverse environmental conditions (12). These response systems are coordinated in groups of operons under the control of the same regulatory protein (regulons) and in groups of regulons under control of the same environmental stimulus (stimulons). Examples are the heat shock regulon (for a review, see reference 22) and the SOS regulon (for a review, see reference 35). More recently, the possible existence of a pH regulon has been suggested (30), in part on the basis of the presence in *E. coli* of gene fusions induced by external acidification. In addition, the alkaline induction of SOS (28) and heat shock genes in *E. coli* (31) has recently been reported. Nevertheless, a clear understanding of the mechanism of pH homeostasis in bacteria is still missing (6).

Thiobacillus ferrooxidans is an obligate acidophile and is probably the most widely studied acidophilic microorganism (7, 10, 13, 16, 32). It is a chemolithotrophic bacterium which normally grows in the pH range 1.5 to 3.5 when ferrous iron is used as an energy source (7, 10, 13, 16, 32). This presents the bacterium with the problem of maintaining its cytosolic pH close to neutrality. For this, the bacterium possesses a pH gradient the generation of which is almost wholly dependent upon proton extrusion across a proton-impermeable membrane (for a review, see reference 10). These membranes, therefore, maintain pH gradients which differ by orders of magnitude from those found in neutrophilic procaryotes. Since very little is known about the molecular mechanisms by which acidophiles respond to and adapt to pH changes, it appeared to be of interest to study the response of these bacteria to external pH perturbations under stringent acidic conditions.

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The chemolithotrophic microorganisms employed in this study were *T. ferrooxidans* ATCC 19859, from the American Type Culture Collection, and R2 (19, 27). All the microorganisms were grown at pH 1.5 or at pH 3.5 (19) in a modified 9K liquid medium which contained 0.04 g of K_2HPO_4, 33.3 g of $FeSO_4 \cdot 7\ H_2O$, 0.4 g of $MgSO_4 \cdot 7\ H_2O$, and 0.1 g of $(NH_4)_2SO_4$ per liter and no trace metals (33). Growth was at 30°C with rotatory shaking, and it was measured as described previously (19). Under these conditions, the generation time for *T. ferrooxidans* was around 8 to 10 h. Clones of each *T. ferrooxidans* strain were obtained by dilution plating and single-colony isolation on solid medium as described before (19).

Cells grown in 9K medium at pH 1.5 or 3.5 were harvested by centrifugation, washed three times with diluted H_2SO_4 at the corresponding pH, and resuspended at a density of 5×10^9 cells per ml in fresh 9K medium (0.5 ml) at the appropriate pH. These bacterial suspensions were always preincubated for 30 min at 30°C.

For the extracellular pH perturbations, the preincubated cells were centrifuged and were resuspended in 9K medium at their previous growth pH (control cells) or at a higher pH (usually shifted from 1.5 to 3.5) or lower pH (generally shifted from 3.5 to 1.5) (pH-shifted cells). The microorganisms were then incubated at 30°C for 30 min. After this period, between 4 and 8 μCi of $Na_2{}^{14}CO_3$ (55 mCi/mmol) (Amersham International) was added to each sample and the incubation was continued in the presence of the isotope for 90 min more at the same temperature in sealed Eppendorf tubes. At the end of these incubations, aliquots were taken for determination of total radioactivity incorporated into hot-trichloroacetic acid-precipitable material, and the rest of the samples either were used for immunoprecipitation or were directly employed for polyacrylamide gel electrophoresis (PAGE).

For the preparation of a crude membrane fraction from *T. ferrooxidans*, sulfuric acid-washed cells were further washed (three times) in 50 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and were broken in the presence of lysozyme-EDTA (5). Briefly, lysozyme (30 μg/ml) and EDTA (250 μg/ml) were added to the cells and the mixture was incubated at room temperature for 1 h. The mixture was diluted with 4 volumes of water and was kept at 4°C. The extract was then subjected to sonic oscillation. After the unbroken cells were discarded, the clear lysate was centrifuged at 100,000 × *g* for 2 h. The pellet obtained was used as crude total cell envelope material (5).

PAGE of proteins was done with samples treated as described above. Cells were rapidly cooled and washed

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twice by centrifugation with 200 μl of 10 mM sodium citrate, pH 7, and twice with lysozyme buffer (50 mM Tris hydrochloride [pH 8], 1 mM EDTA). Finally, the cells were resuspended in 10 μl of Laemmli sample buffer (20) and were boiled for 10 min. In some cases, samples were sonicated for two 30-s periods in a Braunsonic oscillator prior to the addition of the sample buffer. Unless stated otherwise, the same amount of radioactivity was applied to each gel lane. To confirm whether the radioactively labeled bands which had been separated by sodium dodecyl sulfate (SDS)-PAGE corresponded to proteins or lipopolysaccharides, we employed proteinase K digestion of proteins (15) as described before (19).

Unless stated otherwise, electrophoresis was performed on sodium dodecyl sulfate–7 to 15% linear gradient polyacrylamide gels as described previously (1, 19). Two-dimensional (2-D) PAGE with equilibrium and nonequilibrium pH gradient electrophoresis (NEPHGE) was performed as described by O'Farrell (25, 26) by employing ampholytes (pH 5 to 7 and 3 to 10) from Bio-Rad Laboratories. NEPHGE gels were electrophoresed for 5.5 h at 400 V. The cell samples, 3 mg (wet weight) or 70,000 to 100,000 cpm, were resuspended in 100 μl of sonication buffer containing RNase and were sonicated and treated with DNase. The mixture was then lyophilized and dissolved in lysis buffer as described previously (8, 25). The second dimension was an SDS–11.5% polyacrylamide gel. Molecular weight standards for PAGE were from Bio-Rad Laboratories and consisted of phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

After electrophoresis, all the gels were processed for staining and autoradiography by using Amplify as described before (1, 19). After drying, the gels were exposed to Kodak X-Omat or Fuji Rx X-ray film for 1 to 3 weeks at −70°C.

For the immunoprecipitation of labeled components, labeled pH-shifted and control cells were incubated as described above and washed in lysozyme buffer. The cells were then treated with O'Farrell's sonication buffer in the presence of 400 μg of lysozyme per ml for 5 min on ice and then subjected to two 30-s sonications. The lysates were incubated in the presence of 80 μg of DNase per ml (25) for 5 more min on ice and were then centrifuged in an Eppendorf microcentrifuge. The supernatants obtained were used for liquid immunoprecipitation with antiserum against whole cells of the R2 strain of *T. ferrooxidans* or with preimmune rabbit serum as described before (19). Finally, the washed immunoprecipitates were dissolved in 25 μl of Laemmli's buffer and were analyzed by SDS-PAGE as described above. For Western immunoblotting, we employed anti-*Salmonella typhi* OmpC serum (diluted 1:1,000) and goat anti-rabbit immunoglobulin G labeled with ^{125}I as the second antibody (3, 18). Pure *S. typhi* OmpC and its antiserum were a kind gift from G. C. Mora.

Effect of a pH shift on the proteins synthesized by *T. ferrooxidans*. When cells grown at pH 3.5 were shifted to pH 1.5, we detected increased synthesis of several proteins of high and low molecular weights (Fig. 1A, thin arrows). It is noteworthy that these same proteins appear under heat shock conditions and in the presence of ethanol (18, 19). Also, the decrease of the synthesis of a 36-kDa protein (p36) was observed (Fig. 1A, thick arrow). On the other hand, when cells of *T. ferrooxidans* grown at pH 1.5 were shifted to pH 3.5, we did not observe any significant changes in the protein pattern, with the notable exception of the increase in synthesis of p36 (Fig. 1B, lane d, thick arrow). Densitometric analysis of the X-ray film region where p36 migrates indicated an increase in synthesis of at least threefold (data not shown). Therefore, these results indicate the reversibility of the effect of the external pH perturbation on p36 synthesis by *T. ferrooxidans*.

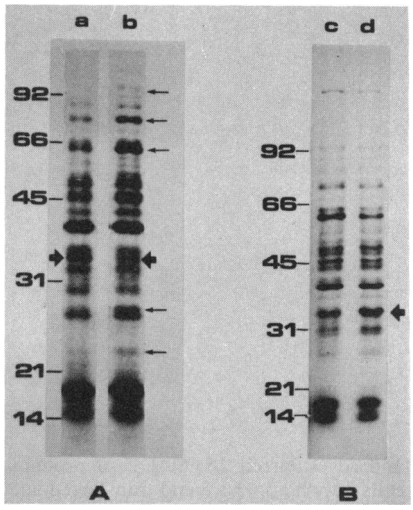

FIG. 1. Effect of extracellular pH shifts on the pattern of protein synthesis by *T. ferrooxidans*. Cells grown at pH 3.5 (A) were kept at this pH (lane a) or were shifted to pH 1.5 (lane b). Bacteria grown at pH 1.5 (B) were kept at the same pH (lane c) or were transferred to pH 3.5 (lane d). Incubations were done in the presence of $Na_2{}^{14}CO_3$ as described in the text, and the proteins synthesized were analyzed by SDS-PAGE and fluorography. Numbers to the left indicate molecular mass markers in kilodaltons. Thick arrows indicate the pH-regulated 36- to 40-kDa protein. Thin arrows indicate proteins with electrophoretic mobilities equivalent to those of the heat shock proteins from *T. ferrooxidans*.

2-D PAGE analysis of the proteins synthesized during external pH shifts. To further characterize the proteins synthesized in response to external pH perturbations, we analyzed them by 2-D PAGE (Fig. 2). Several changes were observed when the cells were shifted from pH 1.5 (Fig. 2A) to 3.5 (Fig. 2B). Some proteins greatly increased their rate of synthesis at pH 3.5 (Fig. 2, thin arrows in spots 5 and 6), or they were detected only after the pH shift (thin arrows in spots 1, 4, and 8). Other protein spots greatly diminished after the pH shift (thick arrows in spots 2, 3, 7, and 9 through 12). The increase in proteins 5 and 6 was striking. The molecular mass range of these proteins was estimated to be 36 to 40 kDa, and it is most probable that they were the p36 protein previously observed by SDS-PAGE. Spot 6 showed smearing, suggesting that it may be a chemically modified protein or a polypeptide tightly bound to a component which is not completely dissociated under the electrophoretic conditions employed. To clarify this point, we separately excised spots 5 and 6 and the smeared "tail" of this last protein and subjected them to in situ proteolysis with protease V8 (9, 18) (data not shown). We found that none of the peptide patterns obtained with these proteins matched that obtained with the pH-induced p36 extracted from a slab SDS-polyacrylamide gel, suggesting that p36 was not entering the standard O'Farrell 2-D polyacrylamide gel.

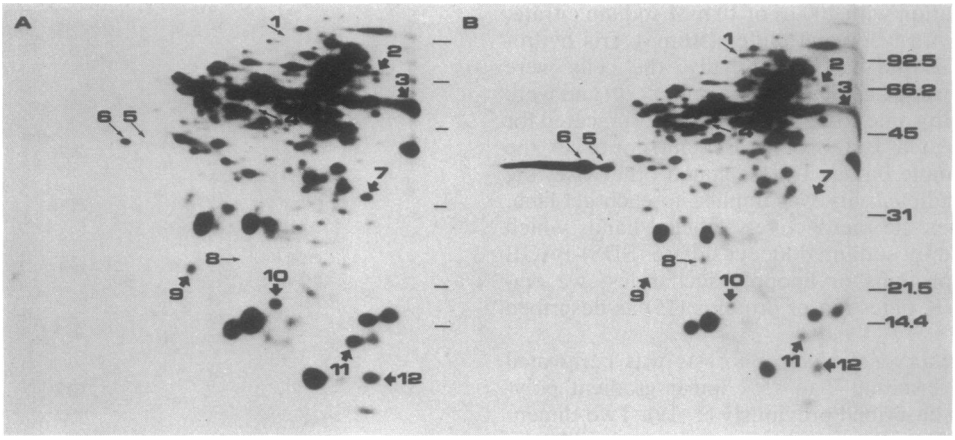

FIG. 2. Standard O'Farrell 2-D PAGE of proteins synthesized by *T. ferrooxidans* after an external pH shift. Total proteins from *T. ferrooxidans* cells grown at pH 1.5 and transferred and labeled at the same pH (A) or after being shifted to pH 3.5 (B) were separated by the O'Farrell system and subjected to autoradiography. The acid side (pH 5) of the isoelectric focusing gel is on the right, and the pH 7 region is on the left. Thin arrows indicate some of the main proteins whose synthesis increases after the pH shift. Thick arrows indicate some of those proteins whose synthesis decreases after the pH shift. Proteins were numbered arbitrarily from higher to lower molecular mass. Numbers to the right indicate the molecular mass standards in kilodaltons.

To test this idea, the total labeled proteins from *T. ferrooxidans* were separated by 2-D NEPHGE (Fig. 3). It is clear that at pH 3.5 there was a large induction of a protein with a molecular mass of about 36 to 40 kDa (Fig. 3B, thick arrow) and an apparent isoelectric point of about 3.5 and which was not seen in the equilibrium 2-D PAGE system. On the basis of its isoelectric focusing mobility and molecular weight, the tentative position for protein spot 6 is indicated for comparison in this electrophoretic system. Since outer membrane porins are acidic, with a molecular mass in the range of 30 to 50 kDa (4, 24), and since they are regulated on the basis of extracellular environmental conditions, we compared the migration of the *T. ferrooxidans* pH-regulated protein with that of the porin OmpC from *S. typhi* (Fig. 3C). A similar migration position was observed, suggesting a structural relatedness between the proteins.

Cellular location of the pH-induced 36-kDa protein. To confirm whether p36 was in fact a membrane protein, we prepared a crude total-envelope fraction from *T. ferrooxidans* cells radioactively labeled after a shift in pH from 1.5 to 3.5. The results obtained are shown in Fig. 4. Lanes a show the total protein pattern of cells labeled at the indicated pH and the thick arrow shows the pH-regulated protein in the 36- to 40-kDa region. Lanes b show the analysis of the crude membrane fraction from the same cells. It is apparent that five to seven main bands were present in this membrane preparation. The thick arrow shows the migration of the pH-regulated protein, indicating that it was found associated with the total membrane fraction. One of these membrane proteins was a major cell protein with a molecular mass of about 40 kDa (thin arrows) whose synthesis did not significantly change with pH. In connection with this, a 40-kDa heat-modifiable outer membrane protein in *T. ferrooxidans* has recently been described, and it has been suggested to be a porin (27). Some other proteins which have higher molecular weights and which are apparently associated with the membrane also showed a decrease in their synthesis after the extracellular pH shift (Fig. 4, lanes b).

When whole, intact cells of *T. ferrooxidans* grown at pH 1.5 were labeled with ^{125}I in the presence of Iodogen (Sigma Chemical Co.) and the total proteins were analyzed by SDS-PAGE and autoradiography, we obtained several major labeled components (Fig. 4, lane c). This labeling technique detects mainly externally exposed proteins, and as expected, several of them comigrate with the proteins present in the crude membrane preparation. In addition, a high-molecular-weight component (about 95 kDa) is heavily iodinated on the surface of *T. ferrooxidans* (dot in lane c). In this regard, it is interesting that most gram-negative bacteria possess trimers of porins in the outer membrane with a molecular mass in the range of 90 to 100 kDa (4, 23, 24).

We have previously prepared a polyclonal antiserum against whole Formalin-fixed *T. ferrooxidans* cells (19). These antibodies recognized some lipopolysaccharides and mainly a few major proteins which are assumed to be located on the surface of the bacteria (3). This antiserum was used to immunoprecipitate cell extracts obtained from ^{14}C-labeled cells grown at pH 3.5. Gel analysis of the immunoprecipitated proteins showed that only five major proteins were present (Fig. 4, lane d). These proteins, as expected, comigrated in general with the proteins present in the total crude membrane fraction, suggesting their localization on the surface of the bacteria.

Reaction of the crude membrane fraction from *T. ferrooxidans* with an antiserum against *S. typhi* OmpC. The results described above suggested the presence of porinlike proteins in *T. ferrooxidans*. To confirm the presence of such polypeptides, we separated the proteins from the membrane fraction of *T. ferrooxidans* by SDS-PAGE, and after transferring them to nitrocellulose, we probed them with antiserum against *S. typhi* OmpC (Fig. 5). The membranes from cells transferred from pH 3.5 to pH 1.5 showed a single 40-kDa band cross-reacting with the antiserum (Fig. 5, lane a). The membranes from cells shifted from pH 1.5 to 3.5 showed this same band, but in addition, they contained a 36-kDa cross-reacting polypeptide (Fig. 5, lane c, lower arrow) which comigrated with OmpC from *S. typhi* (lane b). Since the bands are faint, a densitometric analysis of the 30- to 40-kDa region was also included. Although the heterolo-

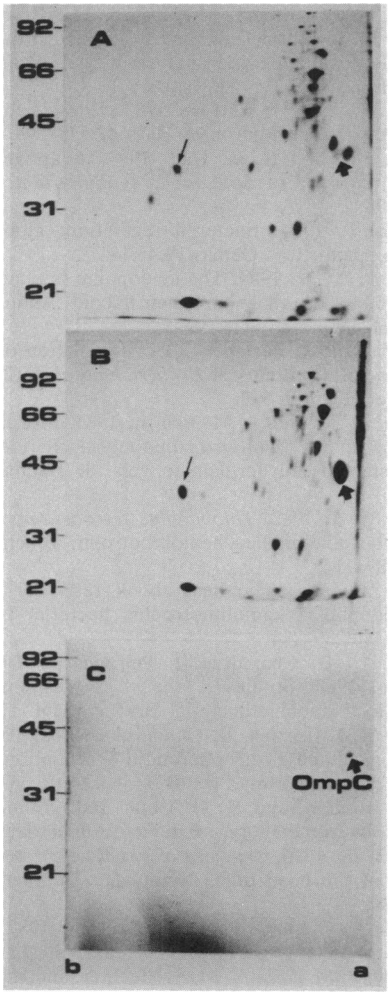

FIG. 3. NEPHGE of *T. ferrooxidans* total proteins synthesized at different pHs. Cells labeled at pH 1.5 (A) or after a shift to pH 3.5 (B) and pure *S. typhi* OmpC (C) were separated by nonequilibrium 2-D PAGE with a pH gradient between 3.0 (a) and 10.0 (b) and were then either subjected to autoradiography (A and B) or stained with Coomassie blue (C). Numbers to the left indicate molecular mass markers in kilodaltons. The thick arrows point out the similarity of migration of the indicated spots. Thin arrows indicate the tentative position of protein spot 6.

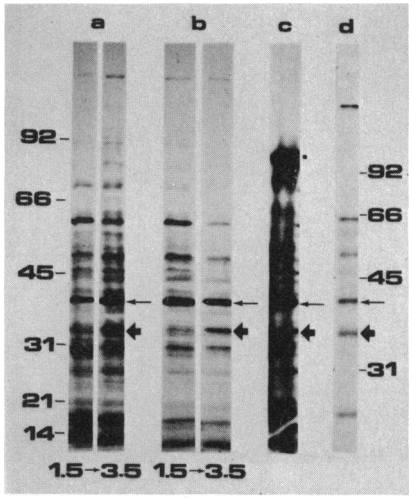

FIG. 4. Cellular location of some proteins from *T. ferrooxidans*. Total labeled cells (lanes a), a crude membrane fraction (lanes b) from cells labeled at pH 1.5 or labeled after transfer to pH 3.5, as indicated, and total proteins from intact *T. ferrooxidans* cells labeled with ^{125}I (lane c) were separated by SDS-PAGE. In a separate electrophoretic run, a low-speed supernatant from lysozyme-treated cells labeled after transfer at pH 3.5 was immunoprecipitated with an antiserum against whole *T. ferrooxidans* cells and the immunoprecipitate was also separated by SDS-PAGE and was then subjected to autoradiography (lane d). The arrows indicate the proteins in the 36- to 40-kDa range referred to in the text.

gous immunological reaction was not strong, it confirmed the porinlike nature of a 40-kDa protein and a pH-regulated 36-kDa protein in *T. ferrooxidans*.

Conclusions. In the present report, we have shown that *T. ferrooxidans* responds to external pH changes by regulating the synthesis of several of its cellular components. pH-regulated gene expression has also been observed in a few heterotrophic microorganisms (11, 14, 28, 30, 31), especially by using the *lac* fusion technique (14, 29, 30).

It is a well-established phenomenon that bacteria respond to different kinds of stress by inducing the synthesis of a common set of proteins (21). We have shown before that *T. ferrooxidans* responds to a variety of environmental stresses by altering the production of specific sets of proteins (17, 18). Thus, we have previously found that a shift of extracellular pH from 3.5 to 1.5 activated a response similar to the heat shock response in *T. ferrooxidans*. On the other hand, an extracellular pH shift from 1.5 to 3.5 failed to induce the heat shock-like response, indicating that the pH stress response seen in the reverse situation (a shift from pH 3.5 to 1.5) is specific to the abrupt increase in acidification. A similar situation was recently described for *E. coli*, in which an alkaline extracellular shift induced the heat shock response, whereas an acidic shift did not elicit the same response (31). It is not known at present whether these external pH changes directly induce these stress responses or if these stress responses occur through changes in other membrane parameters (such as polarization) which could be altered during the pH shift.

For *E. coli*, it has recently been shown that modifying the pH of the culture medium while osmolarity and ionic strength are kept constant greatly influences the relative amounts of cell envelope proteins. Thus, growth at low pH caused a decrease in the content of protein OmpF, protein LamB, and a 30-kDa protein and a concomitant increase in protein OmpC (14).

Our results strongly support the existence of a pH-regulated synthesis of a porinlike p36 protein in *T. ferrooxidans*. The size of the protein, its acidic character, and its location in the membrane fraction support the porin nature of the pH-induced polypeptides. In addition, we found an immunological cross-reaction with an *S. typhi* anti-OmpC serum by Western blotting analysis. Although *T. ferrooxidans* is not closely related to *S. typhi* or *E. coli*, this result is not totally unexpected, since a strong homology between porins from different microorganisms has been observed (2, 4, 34). Obviously, a definitive proof of the porin nature of the membrane proteins studied here would be the formation of pores in black lipid films (4, 23, 24).

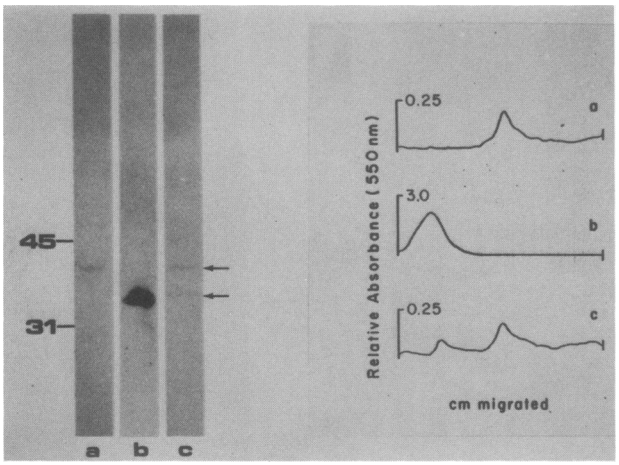

FIG. 5. Western blot analysis of *T. ferrooxidans* envelope preparations with anti-OmpC antibody. Total membrane fraction proteins electrophoresed in an SDS–7 to 15% gradient polyacrylamide gel were electroeluted onto nitrocellulose. The nitrocellulose was then exposed to a 1:1,000 dilution of a rabbit antiserum against *S. typhi* OmpC and then to goat anti-rabbit immunoglobulin G antibody labeled with ^{125}I, and the nitrocellulose was then developed by autoradiography. Membrane preparations were from *T. ferrooxidans* grown at pH 1.5 (lane a) or transferred to pH 3.5 (lane c). *S. typhi* OmpC was used as the standard (lane b). Arrows indicate the two *T. ferrooxidans* protein bands recognized by the antiserum. Numbers indicate molecular mass standards in kilodaltons. The densitometric analysis of the X-ray film is also shown.

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