Aeromonas spp. secrete the channel-forming protein proaerolysin across their inner and outer membranes in separate steps using the general secretion pathway. Here we show that treating A. hydrophila or A. salmonicida with the protonophore carbonyl cyanide m-chlorophenyl hydrazone blocks the second step in transport, secretion across the outer membrane from the periplasm, under conditions where the ATP levels in the cell are no different than the levels in control, secreting cells. A threshold for ΔΨ was observed in the region of 120 mV, below which secretion by both species was inhibited. Treatment of cells with arsenate, which lowered ATP levels but did not affect ΔΨ, also reduced secretion from the periplasm, an indication that there is an ATP requirement for this step independent of the requirement for ΔΨ. Secretion across the outer membrane was also arrested by increasing the osmotic pressure of the medium, even though cellular ATP levels and ΔΨ were not affected. This may be due to disruption of some necessary association between the inner and outer membranes.

Many Gram-negative bacteria are able to secrete proteins across their inner and outer membranes. Most of them use a route that has been named the general secretory pathway by Pugsley (1), who first described it while studying the secretion of pullulanase by Klebsiella oxytoca. Proteins secreted by this pathway are expressed with typical amino-terminal signal sequences and their transit across the inner membrane appears to be a Sec protein-dependent process. The second step requires a group of 12 or more genes whose products, with the exception of the outer membrane protein PulD, appear to be associated with the inner membrane. Little is yet known about the mechanism by which this apparatus enables secreted proteins to cross the outer membrane, although evidence is accumulating concerning the function of some of its components. For example, the PilD protein of Pseudomonas aeruginosa (the homolog of PulO, also called XcpA) was shown to be a type IV prepilin peptidase that is responsible for processing the prepilin-like precursors of PulG, H, I, and J proteins and their homologs in bacteria with the general secretory pathway (2). In addition, PulE and its homologues contain a consensus ATP binding site, mutations in which prevent secretion (3–5), and it was shown that the Vibrio cholerae homolog, EpsE, is an autokinase that is attached to the inner membrane via interactions with EpsL (4). Finally, PulD and its homologues are thought to form a pore in the outer membrane, based on structural properties and homology to the pIV protein involved in single-stranded DNA bacteriophage morphogenesis (6, 7).

Aeromonas spp. secrete a number of proteins, including the channel-forming toxin aerolysin, the lipase GCAT, and at least one protease. Proaerolysin secretion by A. hydrophila and by A. salmonicida containing cloned A. hydrophila aerA has been studied in the greatest detail. We have shown that the signal sequence is removed cotranslationally during transit across the inner membrane and that the protein folds and dimerizes in the periplasm before it is released from the cell (8–10). The A. hydrophila genes required for translocation across the outer membrane include exeC–N, homologues of pulC–N (11), a second operon exeAB (12), and the tapD gene, encoding the prepilin peptidase (13).

Both the proton motive force and ATP are known to be required for Sec-dependent secretion across the inner membrane (for a recent review, see Ref. 14), but little is known of the energetics of the second step in secretion via the general secretory pathway. Two of the exe gene products, ExeA and ExeE, contain sequences corresponding to the ATP-binding regions of known ABC transporters, and direct evidence for an energy requirement for secretion across the outer membrane has come from a study by Wong and Buckley (15). They found that CCCP, which permeabilizes the inner membrane to protons causing the collapse of ΔpH and ΔΨ, completely prevented the release of the periplasmic pool of proaerolysin from A. salmonicida. Secretion was also inhibited by lowering the pH of the growth medium. These two observations led them to propose that secretion across the outer membrane requires a proton-motive force. However, in Escherichia coli the loss of ΔΨ due to treatment with CCCP is eventually followed by a reduction of ATP levels. In their study of A. salmonicida, Wong and Buckley (15) did not measure ATP and therefore could not exclude the possibility that it was a diminished supply of high energy phosphate that caused inhibition of secretion (perhaps due to inactivation of ExeA or ExeE), rather than the change in ΔΨ. More recently, Howard et al. (16) found evidence that ExeB, a protein with structural similarity to TonB, and ExeA form an inner membrane complex required for secretion across the

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1 The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenyl hydrazone; EDTA, ethylenediaminetetraacetic acid; GSP, general secretory pathway; IPTG, isopropyl-β-D-thiogalactoside; PBS, phosphate-buffered saline; TPP, tetraphenylphosphonium ion.
outer membrane. Mutations in the ATP binding cassette of ExeA prevented secretion, leading to the hypothesis that the process requires the energy of phosphate bond hydrolysis provided by ExeA and that this is transduced to the outer membrane by ExeB.

Here we show that reduced ATP levels are not responsible for the CCCP effect observed by Wong and Buckley (15), thereby establishing that there is a specific requirement for ΔΨ. We also show that lowering ATP levels by treating cells with arsenate leads to a reduction in secretion under conditions where ΔΨ is not changed, evidence that transfer across the outer membrane requires ATP as well as ΔΨ. Finally, we show that passage across the outer membrane is also prevented by exposing cells to hyperosmotic conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Growth of A. salmonicida—**CB3 pNB5 (17) was grown at 27 °C to an A sixty of 0.4 in Luria Bertani (LB) medium buffered with Davis medium (18). This was supplemented with 0.2% (w/v) glucose and contained 100 µg/ml ampicillin. The cells were then induced with isopropyl-β-thiogalactoside (1 µM) and growth was continued until they reached A sixty, 2. A. hydrophila Ab65 pKW206 (which is pMMB206 containing the aerA gene and its promoter) was grown in the same way except that the growth temperature was 30 °C, isopropyl-β-thiogalactoside was omitted, and chloramphenicol (2.5 µg/ml) replaced ampicillin.

**Tris-EDTA Treatment of the Bacteria and Membrane Potential Measurements—**To measure the potential difference (∆Ψ) across the bacterial inner membrane, the outer membrane must be made permeable to the membrane potential probe [3H]tetraphenylphosphonium bromide. The protocol designed for E. coli (19) was used with the Aeromonas spp. with minor modifications in the following way. One-ml aliquots of cells grown to an A sixty of 0.2 were centrifuged for 1 min at room temperature in a microcentrifuge. The cells were suspended in 200 µl of 100 mM Tris, 1 mM EDTA, pH 8.0 and incubated for 10 min and centrifuged again. The pellets were then resuspended in 400 µl of 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), unless otherwise stated. Where indicated, this buffer also contained 100 µg/ml chloramphenicol (for A. salmonicida), or 20 µg/ml tetracycline (for A. hydrophila), 0.2% glucose, and varying concentrations of CCCP, arsenate, or sucrose. [3H]tetraphenylphosphonium bromide (3.7 GBq/mmol) was then added to a final concentration of 10 nM and the cells were incubated for an additional 15 min (5 or 10 min in the case of the arsenate experiments) at room temperature. Cells were recovered by filtration on GF/F microfiber filters (Whatman) and washed twice with the experimental buffer with or without CCCP, arsenate, or sucrose before counting. Corrections were made for nonspecific binding of the probe as described previously by Chevalier et al. (20), by incubating cells with 40 µM CCCP for 15 min, adding [3H]tetraphenylphosphonium bromide, and then filtering immediately.

The cytoplasmic volumes of A. salmonicida and A. hydrophila were both estimated to be approximately 0.07 µl/2 × 10⁶ cells by comparing the relative sizes of the two bacteria to E. coli by electron microscopy, basing the calculations on the known cytoplasmic volume of the latter (1 µl/10⁶ cells). Aeromonas cell numbers were routinely determined from the relationship 1 × 10⁶ cells/ml = A sixty 1.6.

**Measurement of Cytoplasmic ATP—**One-ml aliquots of cells grown to an A sixty of 0.2 were centrifuged as described above. The pellets were resuspended in 500 µl of the buffer used for the secretion experiments and incubated for the given times. After a 1-min centrifugation, the pellets were each resuspended in 200 µl of ice-cold distilled water to which 20 µl of dimethyl sulfoxide were added to permeabilize the cell envelope (21). The suspensions were then incubated for 10 min on ice. This resulted in complete release of cytoplasmic ATP (22). Following this treatment, the samples were centrifuged for 1 min to remove debris and the supernatants were diluted 10-fold with distilled water. ATP measurements were carried out on 10-µl aliquots using an ATP photometer (SAI Technology, model 2000) and 100 µl of luciferin-luciferase assay medium (10 mg/ml; Sigma). The instrument was calibrated with ATP solutions of known concentrations.

**Cell Fractionation and Measurement of Proaerolysin Activity—**One-ml aliquots of the cells grown to A sixty 0.2 were centrifuged and resuspended in the specified medium containing chloramphenicol (100 µg/ml) or tetracycline (20 µg/ml) and incubated at 25 °C for the given times. Cells were then centrifuged (1 min, room temperature), and aliquots of the supernatants were taken for measurement of proaerolysin activity. The cells were resuspended in 1 ml of 33 mM Tris-Cl, pH 7.5, 20% sucrose, 1 mM EDTA and incubated for 5 min. After a 2-min centrifugation, the pellets were osmotically shocked by rapid resuspension in 200 µl or 1 ml of ice-cold distilled water. After a further 5-min incubation, the suspensions were centrifuged and aliquots of the supernatants were taken for measurement of proaerolysin concentration.

Proaerolysin activity in A. salmonicida was measured essentially as described by Wong and Buckley (15). Proaerolysin activity in A. hydrophila was measured in the same way except that rat erythrocytes, which are more sensitive to aeroysin, were substituted for human erythrocytes (23).

**RESULTS**

**CCCP Inhibits Secretion of Proaerolysin from the Periplasm of A. salmonicida and A. hydrophila—**We have previously shown that A. salmonicida pNB5 contains a sizeable pool of cell-associated proaerolysin (approximately 1.5 µg in 1 ml of culture with an A sixty of 2.0), virtually all of which is located in the shockable fraction. The second step in secretion, transport across the outer membrane, can be measured by following the decline in this pool with time or by following the appearance of proaerolysin in the medium. Washed cells resuspended in fresh medium containing chloramphenicol to prevent synthesis of new protxin are used (15, 17). Changes in periplasmic proaerolysin levels in the presence and absence of CCCP are compared in Fig. 1. In the absence of CCCP, the amount of proaerolysin associated with the cells decreased with time, so that after 20 min, only 10% remained. There was a corresponding increase in the amount of protxin outside the cells as we found before (not shown). In contrast, when cells were treated with 40 µM CCCP, most of the proaerolysin remained in the periplasm. This is consistent with the results of our previous experiments in which we used a higher concentration of CCCP (15). We also examined the effect of CCCP on the release of periplasmic proaerolysin from A. hydrophila cells containing the plasmid pKW206. These cells contain a smaller periplasmic pool of proaerolysin (50–100 ng of proaerolysin in 1 ml of cells at an A sixty of 2.5). When suspended in fresh medium containing tetracycline, these cells secrete the entire pool of accumulated proaerolysin within 5 min (data not shown). Again the addition of CCCP inhibited secretion, but in this case, even in the presence of 40 µM CCCP, 40–50% of the protein could be recovered in the supernatant (compare Fig. 2).

**Relationship between the Transmembrane Potential and Secretion of Proaerolysin from the Periplasm—**When the trans-
membrane potential of *A. salmonicida* was determined by measuring the accumulation of the radiolabeled lipophilic cation tetraphenylphosphonium bromide, the value obtained was 170 ± 10 mV (negative inside; mean ± S.D. of three independent measurements), in good agreement with the membrane potentials that have been reported for other Gram-negative bacteria (24, 25). As with *E. coli* (26), ΔΨ declined when *A. salmonicida* was incubated with increasing concentrations of CCCP. The values obtained were 165, 123, 112, 107, 88, and 0 mV for 0, 2, 5, 10, 20, and 40 μM CCCP, respectively.

In a parallel experiment, secretion of proaerolysin was measured using cells treated with the same concentrations of CCCP for 5 min. Treatment with Tris-EDTA was omitted to minimize reduction of the periplasmic pool, which would otherwise have occurred as a result of secretion during the incubation (*A. salmonicida* cells would release 75% of the periplasmic pool during the 15 min needed for the treatment and subsequent centrifugation steps and *A. hydrophila* would release all of it). The results in Fig. 2, A and B, illustrate the dependence of the secretion of proaerolysin on the membrane potential. It may be seen that when ΔΨ reached 120 mV, secretion started to decrease (more proaerolysin remained in the periplasm), and when it fell below approximately 90–100 mV, periplasmic levels no longer changed. Thus a threshold membrane potential of approximately 120 mV is required for efficient secretion of proaerolysin from both bacteria.

**Effect of the CCCP-induced Collapse of ΔΨ on the Cytoplasmic ATP Concentration**—Experiments with *E. coli* have shown that depolarization of the cells using a protonophore can affect the level of cytoplasmic ATP (27). It was thus necessary to consider that the reduction in the concentration of high energy phosphate was the actual cause of the block in secretion we observed, rather than the change in ΔΨ. To determine whether this was the case, cytoplasmic ATP was measured at various times in control *A. salmonicida* pNB5 cells and in cells treated with 40 μM CCCP, using the conditions defined in Fig. 1. The results in Fig. 3 show that the ATP level in cells treated with the ionophore for 10 min was the same as the level in control cells. Because the results in Fig. 1 and our previous data (15) show that within this period, control cells secreted proaerolysin but CCCP-treated cells did not, we can conclude that secretion was blocked because of the decline in ΔΨ, not because of the protonophore indirectly lowered energy levels below those in untreated cells.

**Effect of Changes in Cellular ATP on Secretion**—Recent results have suggested that ExeB, a TonB-like protein, and ExeA, a second inner membrane protein containing a P type ATP binding site, may regulate the rate of secretion of proaerolysin across the outer membrane (16). To determine whether aerolysin secretion from the periplasm also depends on high energy phosphate as this would predict, we studied release from the periplasm in cells treated with arsenate to reduce cellular ATP levels. We found that the ATP concentrations in *A. hydrophila* pKW206 were 7.2, 3.2, 4.0, 1.7, and 0.5 mM when cells were treated with 0, 1, 2.5, 5, and 10 mM arsenate, respectively. Fig. 4 shows that the decrease in the ATP pool caused by arsenate treatment was accompanied by a reduction in the secretion of proaerolysin. Only 40% of the initial proaerolysin pool was secreted when the ATP level decreased below 1.7 mM. ΔΨ was measured in EDTA-treated cells incubated for 5 min in the presence of the same concentrations of arsenate used to lower ATP levels, to determine whether the inhibitor also affected the membrane potential in this time period. Fig. 4 shows that ΔΨ remained practically constant between 158 and 150 mV, whatever the ATP concentration in the cells. Thus we can conclude that secretion is inhibited as a consequence of a decrease in cellular ATP independent of any change in ΔΨ.

**Decreasing the pH of the Medium Results in a Decline in ΔΨ**—Previous data from Wong and Buckley (15) have shown that reducing the medium pH results in a decrease in proaerolysin secretion by *A. salmonicida*. It is possible that this is due to an effect on ΔΨ, because it is known that decreasing pH<sub>mat</sub> decreases ΔΨ in *E. coli* (28), as well as in other bacteria (24). To determine whether this was the case, ΔΨ was measured with cells resuspended in buffers ranging from pH 5.5 to 8, containing glucose and chloramphenicol. The results are presented in Fig. 5. It may be seen that when pH<sub>mat</sub> fell below 6.8, ΔΨ was reduced to values near the threshold required for secretion (see Fig. 2). Thus it is possible that lowering the medium pH re-
Indicated pH containing glucose and CAM for LB medium was treated with Tris-EDTA and suspended in buffer of the supernatant. The pellet was used to measure cytoplasmic ATP. \( \Delta \Psi \) was determined on aliquots of the cells first treated with Tris-EDTA and then resuspended in medium containing arsenate.

Precise description of the energetics of protein secretion from binary observations of Wong and Buckley (15) and allow a more perosmotic block was not due to a decrease in cellular ATP in a \( \Delta \Psi \)-independent manner. A. hydrophila pKW 206 was incubated for 5 min in medium containing glucose, tetracycline, and different concentrations of arsenate, buffered at pH 7. The cells were centrifuged, and secreted proaerolysin was measured in the supernatant. The protonophore CCCP inhibited secretion from A. hydrophila as well as from A. salmonicida. The reduction in the apparent release of aerolysin appeared to be smaller in A. hydrophila; however, this is likely because it has a much smaller periplasmic pool of proaerolysin than A. salmonicida, which produces considerably more protein from the cloned gene. As a result, if the rate of secretion is approximately the same in both species, a larger fraction of the A. hydrophila pool leaves the periplasm during the time taken for the manipulations required in each experiment. Thus, within 5 min, 50% of the proaerolysin is released by A. salmonicida (Fig. 1), whereas A. hydrophila secretes all of its periplasmic pool in this time. If we consider that complete response to CCCP treatment may require at least 1–2 min, this could account for the fact that we measured 80% retention of proaerolysin in A. salmonicida following CCCP treatment, but only 50% in A. hydrophila under the same conditions.

Our experiments show that inhibition of secretion by CCCP was likely due to the decrease of \( \Delta \Psi \) that results from treatment with the protonophore, rather than to an indirect effect on cellular high-energy phosphate because during the time period of our experiments, ATP levels in the CCCP treated cells were the same as levels in control cells (Fig. 3). Conversely, our results with arsenate-treated cells indicate that secretion also depends upon cellular ATP levels. The observation that both ATP and \( \Delta \Psi \) are required for secretion across the outer membrane is comparable to that made by Marino et al. (29) in a study of translocation of lipopolysaccharide from the inner membrane to the outer membrane of E. coli. They found that translocation is blocked by decreasing the ATP pool with arsenate, under conditions where the protonmotive force is not reduced, but they also found that translocation could be prevented by decreasing \( \Delta \Psi \) with a protonophore under conditions where the ATP level was not modified.
Because both ExeA and ExeE contain ATP binding sites in their primary structures, they are obvious candidates to explain inhibition of secretion by reduced ATP levels. Howard et al. (16) recently pointed out a resemblance between ExeB and TonB, which opens gated ports for the inward movement of ligands across the outer membrane (30). The authors proposed that hydrolysis of ATP by ExeA may cause a conformational change in ExeB leading to the opening of a secretion port in the outer membrane. Ligand uptake involving TonB is known to depend on $\Delta \Psi$ in some unknown manner (30), and the $\Delta \Psi$ dependence of secretion we observe here may extend the similarity between TonB and ExeB further. Interestingly, in experiments comparable to ours, Pugsley and coworkers$^2$ have observed that secretion of PulA by E. coli containing the cloned Klebsiella pul genes is not affected by reducing cellular ATP, although as with aerolysin secretion, it is inhibited by declines in $\Delta \Psi$. In contrast to secretion by Aeromonas spp., pullulanase secretion by K. oxytoca or by E. coli does not appear to require a gene product comparable to ExeA. Thus, one way to rationalize our results with those of Possot et al.$^6$ is to argue that it is the function of ExeA rather than ExeE that is sensitive to the reductions in cellular ATP caused by the arsenate treatment. This explanation is also in accordance with previous proposals (based on homologies between pul genes and type IV pilin assembly genes) that PulE and its homologs provide energy for the assembly of the secretion apparatus rather than for the secretion process itself (3, 5).

Experiments with other bacteria have shown that although increasing $\Delta \mathrm{pH}$ by lowering the pH of the external medium results in a decrease in $\Delta \Psi$, the proton motive force remains quite constant (180 mV) over a large range of external hydrogen ion concentrations (24). If this is the case with Aeromonas spp., then one explanation for the inhibition of secretion we observe with reduced medium pH is that the process depends specifically on the $\Delta \Psi$ component of the proton motive force. However, it is also possible that the pH effect is independent of the proton motive force effect we have observed. This might be more consistent with the recent observation made by Possot et al.$^6$ in their study of pullulanase secretion by E. coli. They found that inhibition of secretion by lowering the pH of the medium is irreversible, in contrast to CCCP inhibition. Perhaps in both E. coli and Aeromonas spp., the structures of secreted proteins or of some critical component of the secretory machinery are altered by low pH, reducing the apparent rate of secretion.

Secretion was also prevented when cells were exposed to hyperosmotic conditions. This was not a consequence of energy depletion, because neither $\Delta \Psi$ nor cellular ATP levels changed during the incubation. The inner membrane contracts away from the outer membrane under hyperosmotic conditions (31), and this physical effect may account for the inhibition of secretion, either through a general disruption of the secretion apparatus or by prevention of a necessary contact between the TonB-like ExeB protein and the outer membrane. It is certainly conceivable that contact between the inner and outer membranes is required for secretion because there is evidence of such a requirement both for incorporation of lipopolysaccharide into the outer membrane and for colicin import by E. coli (27, 32). However, Houssin et al. (33) have shown that proton motive force-driven transport, facilitated diffusion, and ATP-driven transport in E. coli are also inhibited by an increase in osmotic pressure, although neither the membrane potential nor ATP is decreased. The authors argued that inhibition may be due to conformational changes in cytoplasmic membrane proteins induced by deformation of the membrane. In the same way, structural changes in inner membrane components of the Aeromonas secretory machinery could lead to inhibition of secretion.

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