members of the Amt family. Here, we report that certain AmtB twin-histidine variants are gain-of-function mutants that served within the Amt family. These we report our certain...
cytoplasmic surface at the described methods (3, 14, 32), and then moving the defined media were prepared by replacing all K
-content of the defined media. The close linkage of glnK and His318 on the conserved twin-histidine element (His168 on the top and Phe415 on the bottom) that separates the periplasmic vestibule of the transport pathway from the conduction pore is shown in red. Whether ammonium crosses the phenyl ring constriction and traverses the conduction pore as NH3 or NH4+
—For strain growth illustrated in Fig. 2, cells grown in LB medium were diluted 100-fold into Na+-based N−C− medium supplemented with 0.4% glucose, 5 mM NH4Cl, and 100 mM KCl. For growth at low pH by inoculation into Na+-based Neidhardt’s MES medium (pH 5.5); all adaptive media contained 1 mM KCl; and cells were finally diluted into either Na+-based Neidhardt’s MES medium (pH 5.5) supplemented with 1 mM KCl, or Na+-based Neidhardt’s medium (pH 5.5) supplemented with 0.1 mM KCl and 0.9 mM NaCl.
Expression and accumulation levels of mutant proteins relative to wild-type AmtB have been reported (14). The three AmtB variants studied here were produced in amounts similar to that of the wild-type strain (60–100%) when glutamine served as the sole nitrogen source (Fig. 2 and Figs. 4–7). However, whereas AmtB1H68D and AmtB1H318E accumulated to 30–40% wild-type levels under this growth condition, AmtB1H68D,1H318E was present in amounts ≤5% of its wild-type counterpart. Similar expression and accumulation patterns were found for these three AmtB variants when low NH3 medium was used for growth studies (Fig. 3).
Transport Assays—Strains were grown in LB medium containing 100 mM KCl and, after overnight incubation, diluted 100-fold into Na+-based N−C− medium supplemented with 0.4% glucose, 5 mM NH4Cl, and 100 mM KCl. After these cultures reached saturation they were again diluted 100-fold into the same Na+-based N−C− medium, except with 3 mM glutamine replacing NH4Cl as the nitrogen source. Following overnight incubation, cultures were finally diluted 200-fold into Na+-based N−C− medium supplemented with 0.4% glucose, 3 mM glutamine, and a mixture of KCl and NaCl (combined concentration of 100 mM). Growth was monitored by changes in absorbance at 600 nm. Strain growth was carried out with aeration at 37 °C.
Growth at low NH3 concentrations (Fig. 3) was performed as previously described (14) with the following differences: cells were first adapted to minimal medium on Na+-based Neidhardt’s MOPS medium (pH 7.4) and subsequently acclimated to growth at low pH by inoculation into Na+-based Neidhardt’s MES medium (pH 5.5); all adaptive media contained 1 mM KCl; and cells were finally diluted into either K+-based Neidhardt’s MES medium (pH 5.5) supplemented with 1 mM KCl, or Na+-based Neidhardt’s medium (pH 5.5) supplemented with 0.1 mM KCl and 0.9 mM NaCl.
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For determination of cytoplasmic K+ pools, cells were collected and processed for inductively coupled plasma atomic emission spectrometry, and processed for inductively coupled plasma atomic emission spectrometry.
Amt Channel Substrate Specificity

FIGURE 2. Effect of K⁺ on AmtB twin-histidine variant strain growth. A and B, strains were cultivated in either K⁺-based N⁻C⁻ minimal medium (contains 200 mM K⁺) (A) or Na⁺-based N⁻C⁻ minimal medium supplemented with 1 mM KCl (B). All media contained 0.04% glucose and 3 mM glutamine. AmtB is expressed under these growth conditions (see "Experimental Procedures" for details). Strain genotypes were wild-type (■), amtb-null (□), H168D (▲), H318D (●), and H168D,H318E (●). The arrow in A denotes the point at which the glucose supply is exhausted by cells expressing AmtB(H168D,H318E) (see C), and the tricarboxylic acid cycle intermediates derived from glutamine begin to be used as the source of carbon. The data, from a single experiment, are representative of findings made in three independent trials. C, glucose consumption by strains illustrated in A. Culture medium glucose concentrations were determined at various times during growth. The times at which strains exhaust their glucose supply are closely approximated by their diauxic shift points in A: 7 h for H168D, 8 h for amtb-null, 8 h for wild type, 8 h for H318D, and 9 h for H168D,H318E. Cell yield per glucose consumed values (shown below in units of A600/mg of glucose per ml) at the diauxic shift point for each strain were 1.2 ± 0.12 for amtb-null, 1.1 ± 0.063 for wild-type, 0.73 ± 0.052 for H168D, 0.62 ± 0.029 for H318D, and 0.55 ± 0.025 for H168D,H318E. Cell yield per glucose consumed values are reported as means ± S.D. for three independent experiments. Symbols used for strain genotypes in C are identical to those in A and B.

spectroscopy (ICP-AES)² (see below). To analyze K⁺ uptake, resuspended cells were washed twice and finally suspended at an A600 = 1.0 in Na⁺-based N⁻C⁻ medium containing 100 mM NaCl, and then treated with 5 μM 2,4-dinitrophenol for 30 min. Treatment with this protonophore results in the rapid loss of cellular K⁺ (39, 40). K⁺-depleted cells were harvested, washed twice with Na⁺-based N⁻C⁻ medium supplemented with 100 mM NaCl, resuspended at an A600 = 1.0 in the same medium supplemented with 0.2% glucose, and held on ice until use. To initiate tests of K⁺ transport, cell suspensions were preincubated for 20 min at 37 °C prior to the addition of an equal volume of Na⁺-based N⁻C⁻ medium supplemented with 0.2% glucose and suitable mixtures of KCl, NH₄Cl, and NaCl (combined concentration of 100 mM). After a 1 min incubation aliquots were collected on Millipore filters (0.45-μm pore size; type HAWP), rinsed twice with 5 ml of Na⁺-based N⁻C⁻ medium supplemented with 100 mM NaCl, and dried. Washed cells (on the filter) were then incubated at room temperature (~25 °C) for 1 h in 5 ml of 1 M HNO₃ containing 50 μM NaCl to extract K⁺. Samples were cleared of cell debris by passage through Millipore filters (0.45-μm pore size; type HAWP) and analyzed for K⁺ by ICP-AES. To determine whether AmtB-mediated K⁺ transport required an energy source (Fig. 7A), glucose was eliminated from all assay buffer preparations. For assays with carbonyl cyanide m-chlorophenylhydrazone (CCCP), this compound or an equal volume of ethanol vehicle was added 5 min prior to the initiation of K⁺ uptake tests. Strain growth, K⁺ depletion, and K⁺ uptake assays were carried out with aeration at 37 °C.

Apparent kinetic constants (half-saturation constant values and maximal transport rates) for AmtB-mediated K⁺ uptake were estimated by curve fitting initial transport rates to the Michaelis-Menten equation using the program CurveExpert Professional, version 1.6.2. The concentration range of K⁺ used to approximate these kinetic constants was 0.5–50 mM. Inhibition constant (Kᵢ) values for ammonium inhibition of AmtB-mediated K⁺ transport were determined using linear Dixon plots (41), with the assumption of competitive inhibition between ammonium and K⁺. For energy requirement assays and Kᵢ determinations (Fig. 7), K⁺ was present at twice half-saturation constant values for AmtB(H168D) and AmtB(H168D,H318E) in the glnK⁺ background, and 40 mM for AmtB(H318D) and AmtB(H168D,H318E) in the glnK⁻ background. Transport values measured in the absence of ammonium using these K⁺ concentrations were 180 ± 38 nmol/ml per A600 per min for AmtB(H318D) in the glnK⁺ background, 210 ± 51 nmol/ml per A600 per min for AmtB(H168D,H318E) in the glnK⁺ background, 140 ± 21 nmol/ml per A600 per min for AmtB(H318D) in the glnK⁻ background, and 96 ± 6.3 nmol/ml per A600 per min for AmtB(H168D,H318E) in the glnK⁻ background. The concentration range of ammonium used to approximate Kᵢ values was 1 μM to 10 mM.

Glucose Determination—Samples from cultures illustrated in Fig. 2A were subjected to filtration (0.22-μm pore size; Millipore type Millex-GV) to remove cells. Glucose concentrations in cell-free medium were determined enzymatically using a glucose oxidase/peroxidase-coupled assay monitoring the oxidation of o-dianisidine (Sigma-Aldrich product GAGO-20).

RESULTS

K⁺-dependent Growth Defect in AmtB Twin-histidine Variant Strains—A number of E. coli AmtB mutant proteins carrying acidic residues at the His¹⁶⁸/His³¹⁸ twin-histidine site cause a pronounced growth defect when expressed under nitrogen-limiting conditions in media devoid of ammonium and of high K⁺ content (Fig. 2, A and C). This defect is characterized by a decreased carbon yield relative to both the wild-type and amtb-null strains. We reasoned that the twin-histidine variants exhibiting the growth defect were wasting energy in the futile active transport of K⁺. Several mutants were selected to test this hypothesis. Two of these, AmtB(H168D) and AmtB(H318D), have near wild-type ammonium transport activity even in the
presence of \( \sim 20 \text{mM} \) \( K^+ \) (Fig. 3 and Ref. 14). Like wild-type AmtB, the AmtB\(^{H168D}\) protein also transports the ammonium analog methylammonium (used to designate \( CH_3NH_2 \) and \( CH_3NH_3^+ \)) in the absence of \( K^+ \), whereas AmtB\(^{H318D}\) does not (14). A third mutant, AmtB\(^{H168D,H318E}\), that exhibits neither ammonium nor methylammonium transport activity even at low \( K^+ \) (Fig. 3 and Ref. 14) was also examined. Studies conducted on cells expressing these variants showed that the growth defect was eliminated if \( Na^+ \) replaced most of the \( K^+ \) in the culture medium (1 mM \( K^+ \) supplied to avoid \( K^+ \)-limiting growth conditions) (Fig. 2B).

**AmtB Twin-histidine Variants Conduct \( K^+ \)—**Having established a link between the external \( K^+ \) concentration and the growth defect observed in certain twin-histidine mutants, we next looked to see whether this defect resulted from AmtB-mediated inward \( K^+ \) leakage. A strain lacking the three major \( K^+ \) import systems (Kdp, Trk, and Kup) was constructed for this work. In the absence of these transport systems *E. coli* possesses only a residual \( K^+ \) uptake activity and requires an elevated concentration of this cation for growth (39, 40). We found that the kdp trk kup triple mutant failed to grow appreciably on media containing less than \( \sim 50 \text{mM} \) \( K^+ \), and expression of wild-type AmtB had no effect on this growth phenotype (Fig. 4; compare amtB-null and wild-type strains). Expression of AmtB\(^{H168D}\), AmtB\(^{H318D}\), or AmtB\(^{H168D,H318E}\), on the other hand, reduced the \( K^+ \) requirement to \( \sim 10, 5, \) and 1 mM, respectively.

The ability of AmtB to conduct \( K^+ \) was also assayed directly using ICP-AES. Initial experiments indicated that cytoplasmic \( K^+ \) pools of kdp trk kup triple mutants expressing a twin-histidine variant protein, in particular AmtB\(^{H185D}\) or AmtB\(^{H168D,H318E}\), were not only higher than their wild-type AmtB-expressing and amtB-null counterparts, but also larger than that of a wild-type AmtB-expressing strain carrying the Kdp, Trk, and Kup systems (Fig. 5). We next depleted each of these strains of their \( K^+ \) to study the net uptake of this cation. As expected, the \( K^+ \) transport rate of a kdp trk kup triple mutant expressing wild-type AmtB was no better than that of an otherwise isogenic amtB-null strain (Fig. 6A). Only a slight improvement (<2-fold) in \( K^+ \) uptake was observed when AmtB\(^{H168D}\) was expressed in this background. The initial \( K^+ \) transport rates of wild-type AmtB and AmtB\(^{H168D}\) were linearly proportional to the external \( K^+ \) concentration over the range tested (up to 50 mM) and markedly lower than the combined activities of Trk and Kup (Kdp not expressed under the conditions (100 mM \( K^+ \) in the growth medium) used for this assay; Refs. 39, 40).
Amt Channel Substrate Specificity

FIGURE 6. Kinetics of AmtB-mediated K⁺ transport. A, initial (1 min) rates of AmtB-mediated K⁺ uptake were measured in K⁺-depleted kdp trk kup cells. Strain genotypes were wild-type (○), amt-null (■), H168D (△), H318D (■), and H168D,H318E (●). The K⁺ uptake activity of the prototrophic E. coli K-12 parental strain NCM3722 (31) is shown (solid red squares). B, initial (1 min) rates of K⁺ uptake were measured in K⁺-depleted kdp trk kup glnK cells expressing AmtBH₃₁₈D (○) and AmtBH₁₆₈D,H₃₁₈E (■). Values reported in A and B are means ± S.D. (error bars) for at least three independent experiments. Kinetic constants for K⁺ transport determined from the data shown in A are reported under "Results" as means ± S.D. Half-saturation constant values and maximal transport rates determined from the data shown in B were 26 ± 9.1 nmol/ml per A₆₀₀ per min for AmtBH₃₁₈D and 60 ± 21 nmol/ml per A₆₀₀ per min for AmtBH₁₆₈D,H₃₁₈E.

AmtBH₃₁₈D and AmtBH₁₆₈D,H₃₁₈E, on the other hand, exhibited considerable K⁺ uptake activity. These two twin-histidine variants were found to have 5–10-fold higher half-saturation constant values (4.9 ± 0.93 mM and 11 ± 1.4 mM for AmtBH₃₁₈D and AmtBH₁₆₈D,H₃₁₈E, respectively) and elevated maximal transport rates (250 ± 6.0 nmol/ml per A₆₀₀ per min and 270 ± 22 nmol/ml per A₆₀₀ per min for AmtBH₃₁₈D and AmtBH₁₆₈D,H₃₁₈E, respectively) relative to the aggregate kinetic properties of the Trk and Kup systems (half-saturation constant of 1.1 ± 0.07 mM; maximal transport rate of 210 ± 2.1 nmol/ml per A₆₀₀ per min; also see Ref. 40). Given that a 1 ml E. coli culture of A₆₀₀ = 1 has a total cell volume of ~3.6 μl (42), we calculate that AmtBH₃₁₈D and AmtBH₁₆₈D,H₃₁₈E concentrate K⁺ 3–10-fold during the first min of the transport reaction when K⁺ is present at 0.5–10 mM in the external medium.

Inhibition of AmtB-mediated K⁺ Transport—We used AmtBH₃₁₈D and AmtBH₁₆₈D,H₃₁₈E as vehicles to analyze the energy requirements of AmtB-mediated K⁺ conduction and the effect ammonium has on this transport reaction. The rate of K⁺ uptake by these two proteins was reduced 90–95% when the protonophore CCCP was present at a concentration of 10 μM (Fig. 7A), indicating that K⁺ uptake by AmtB was dependent on the proton motive force, presumably the membrane potential component, across the cytoplasmic membrane. In support of this conclusion is the finding that neither twin-histidine variant conducted K⁺ after treatment with 2,4-dinitrophenol, a process that both depletes the cytoplasmic K⁺ pool and discharges the proton motive force (39, 40), without subsequent addition of an energy source. Competition tests showed that K⁺ transport by these mutant proteins was also reduced in the presence of ammonium (Fig. 7B). The inhibitory effect was far greater for AmtBH₃₁₈D (Kᵢ = 4.9 ± 1.9 μM), which conducts ammonium, than for AmtBH₁₆₈D,H₃₁₈E (Kᵢ = 0.77 ± 0.37 μM), which lacks ammonium uptake activity. Because the GlnK protein quickly inactivates AmtB in the presence of micromolar quantities of external ammonium (43), the impact this compound had on AmtB-mediated K⁺ transport was also examined in a kdp trk kup glnK strain background. Half-saturation constant values for AmtBH₃₁₈D and AmtBH₁₆₈D,H₃₁₈E K⁺ uptake were increased ~5-fold when GlnK was not present (Fig. 6B). Similar-sized decreases in ammonium inhibition of K⁺ transport by AmtBH₃₁₈D (Kᵢ = 22 ± 5.3 μM) and AmtBH₁₆₈D,H₃₁₈E (Kᵢ = 2.9 ± 1.6 μM) were observed in the absence of the GlnK protein (Fig. 7B). These results suggest that the uptake of K⁺ is influenced by AmtB-GlnK interaction and, when taken together with the observation that AmtBH₃₁₈D K⁺ conduction was strongly inhibited by ammonium, provides support for the idea that K⁺ and ammonium share the same transport pathway through AmtB.

DISCUSSION

Controversy remains over the form of ammonium carried by members of the Amt family. Due to the strongly hydrophobic nature of the conduction pore it was proposed that these proteins facilitated diffusion of NH₃ (7, 11). A study of AmtB function in a reconstituted system provided evidence for such a transport mechanism (7), but the findings of that work have been criticized for being interpreted incorrectly and could not be corroborated (4, 44). Other observations question the plausibility of the facilitated NH₃ diffusion model. Quantitative analyses of ammonium flux have shown that this transport mechanism would not sustain the rates of growth observed under the low external ammonium conditions at which Amt proteins operate (15, 44). Also, given that these proteins are active when the internal to external ammonium concentration ratio is >1 (15), facilitated diffusion would lead to the export rather than import of ammonium without the involvement of a linked energy source. The alternative view of ammonium transport holds that Amt channels actively conduct NH₄⁺ or cotransport NH₃ and H⁺. In support of these mechanisms, all plant...
members of the Amt family functionally characterized to date, save for those from the distantly related AMT2 subfamily, have been found to mediate electrogentic ammonium uptake (4, 17, 18, 30, 45, 46). Moreover, Amt proteins from a number of other organisms, including E. coli, concentrate methylammonium in a membrane potential-dependent manner (3, 13, 16, 20). That certain AmtB twin-histidine variants conduct K⁺ against its concentration gradient (Fig. 6) is compatible with these active transport mechanisms. In addition, because K⁺ cannot separate from its charge, our results imply that ammonium migrates through the AmtB pore as either NH₄⁺ or as a closely associated NH₃/H⁺ pairing (see below). This is in contrast to a net NH₃⁺ uptake mechanism that proceeds via a symport reaction in which NH₃ passes through the pore alone while a H⁺ follows a separate unidentified transport pathway.

In work presented here, we have described a set of AmtB twin-histidine variants that have altered substrate specificity and now conduct K⁺. The spectrum of compounds (methylammonium, ammonium, and K⁺) carried by each mutant depends on the position and number of acidic residues present in the conduction pore. Thus, AmtBHI68D transports all three compounds, AmtB$^{\text{T1}1\text{S}18\text{E}}$ conducts both ammonium and K⁺ but no longer exhibits methylammonium uptake activity, and AmtB$^{\text{H}1\text{S}168\text{D},\text{T}318\text{E}}$ carries only K⁺ (Figs. 3, 4, and 6; and Ref. 14). These findings lead us to conclude that the His$^{168}$/His$^{318}$ twin-histidine element serves as a substrate selectivity filter that prevents K⁺ transport. How might this element enable members of the Amt family to discriminate between K⁺ and ammonium? If, as our results predict, ammonium crosses the phenyl ring conduction and enters the conduction pore as NH₃⁺ its charge will need to be masked to migrate across this hydrophobic environment. By accepting a H⁺ from NH₃⁺ and only transferring it back just prior to substrate release into the cytoplasm, a transport mechanism shown to be plausible in a recent simulation study (47), the twin-histidine element allows the H⁺ to remain delocalized as it moves in parallel with NH₃ through the conduction pore. The transport of K⁺ is prohibited because, unlike NH₄⁺ and CH₃NH₃⁺, this cation is incapable of separating from its charge. Certain acidic amino acid substitutions of the twin-histidine element reduce the need for such charge separation by increasing pore hydrophicity and, as a consequence, allow K⁺ to be carried. It is not clear why these mutations would also cause progressive decreases in CH₃NH₃⁺ and then NH₃⁺ transport. However, the underlying trend suggests that this behavior results from the differences in the manner by which the introduced acidic residues handle H⁺ and K⁺ as well as changes to the hydrophobic character of the pore.

The strong preference of Amt family members for ammonium over K⁺ (3, 17, 18, 29, 30, 48) implies that this selectivity is physiologically important. Our current analysis highlights why these proteins do not carry K⁺. The inward leakage of this cation through AmtB, although able to substitute for the K⁺ uptake activities of the Trk and Kup systems, is associated with a large energy cost (Fig. 2A and C). We suggest that this cost is incurred by the action of two processes. First, K⁺ movement across the cytoplasmic membrane is known to play an important role in regulating pH homeostasis (39, 49, 50). Given the elevated K⁺ pools found in cells expressing AmtB twin-histidine variants, substantial energy reserves would likely be used to preserve normal intracellular pH. Similarly, energy would be required to maintain the membrane potential that would otherwise be dissipated by illicit AmtB-mediated K⁺ conduction. These energy expenditures can prove problematic because members of the Amt family oftentimes need to operate in nutrient-poor environments having high K⁺ levels. For instance, the two Amt proteins of Nitrosopumilus maritimus, a member of a group of ammonium-oxidizing marine archaea that are key intermediates in the global nitrogen cycle, are likely active in the open ocean where ammonium and K⁺ concentrations are <1 μM and ~10 mM, respectively (51–53). Growth and survival under such energy- and nitrogen-limited conditions would be severely compromised if Amt proteins did not discriminate against K⁺.

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REFERENCES

1. Walter, A., and Gutknecht, J. (1986) Permeability of small nonelectrolytes through lipid bilayer membranes. J. Membr. Biol. 90, 207–217
2. Andrade, S. L., and Einsle, O. (2007) The Amt/Mep/Rh family of ammonium transport proteins. Mol. Membr. Biol. 24, 357–365
3. Fong, R. N., Kim, K. S., Yoshihara, C., Inwood, W. B., and Kustu, S. (2007) The W148L substitution in the Escherichia coli ammonium channel AmtB increases flux and indicates that the substrate is an ion. Proc. Natl. Acad. Sci. U.S.A. 104, 18707–18711
4. Javelle, A., Lupo, D., Li, X. D., Merrick, M., Chami, M., Ripoche, P., and Winkler, F. K. (2007) Structural and mechanistic aspects of Amt/Rh proteins. J. Struct. Biol. 159, 243–252
5. Ludewig, U. (2006) Ion transport versus gas conduction: function of Amt/Rh-type proteins. Transfus. Clin. Biol. 13, 111–116
6. Bostick, D. L., and Brooks, C. L. (2007) Deprotonation by dehydration: the origin of ammonium sensing in the AmtB channel. PLoS Comput. Biol. 3, e22
7. Khademi, S., O’Connell, J., 3rd, Remis, J., Robles-Colmenares, Y., Miercke, L. J., and Stroud, R. M. (2004) Mechanism of ammonia transport by Amt/Mep/Rh: structure of AmtB at 1.35 Å. Science 305, 1578–1594
8. Lin, Y., Cao, Z., and Mo, Y. (2009) Functional role of Asp160 and the deprotonation mechanism of ammonium in the Escherichia coli ammonium channel protein AmtB. J. Phys. Chem. B 113, 4922–4929
9. Nygaard, T. P., Rovira, C., Peters, G. H., and Jensen, M. Ø. (2006) Ammonium transport versus gas conduction: function of AmtB/Rh-type proteins. Transfus. Clin. Biol. 13, 111–116
10. Khademi, S., O’Connell, J., 3rd, Remis, J., Robles-Colmenares, Y., Miercke, L. J., and Stroud, R. M. (2004) Mechanism of ammonia transport by Amt/Mep/Rh: structure of AmtB at 1.35 Å. Science 305, 1578–1594
11. Lin, Y., Cao, Z., and Mo, Y. (2009) Functional role of Asp160 and the deprotonation mechanism of ammonium in the Escherichia coli ammonium channel protein AmtB. J. Phys. Chem. B 113, 4922–4929
12. Nygaard, T. P., Rovira, C., Peters, G. H., and Jensen, M. Ø. (2006) Ammonium transport versus gas conduction: function of AmtB/Rh-type proteins. Transfus. Clin. Biol. 13, 111–116
13. Khademi, S., O’Connell, J., 3rd, Remis, J., Robles-Colmenares, Y., Miercke, L. J., and Stroud, R. M. (2004) Mechanism of ammonia transport by Amt/Mep/Rh: structure of AmtB at 1.35 Å. Science 305, 1578–1594
14. Lin, Y., Cao, Z., and Mo, Y. (2009) Functional role of Asp160 and the deprotonation mechanism of ammonium in the Escherichia coli ammonium channel protein AmtB. J. Phys. Chem. B 113, 4922–4929
15. Yang, H., Xu, Y., Zhu, W., Chen, K., and Jiang, H. (2007) Detailed mechanism of ammonia transport based on the crystal structure of AmtB of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 104, 17090–17095
16. Bockstaela, M., Andrè, B., and Marini, A. M. (2008) Distinct transport mechanisms in yeast ammonium transport/sensor proteins of the Mep/Amt/Rh family and impact on filamentation. J. Biol. Chem. 283, 21362–21370
17. Boussiba, S., Dilling, W., and Gibson, J. (1984) Methylammonium transport in Anacystis nidulans R-2. J. Bacteriol. 160, 204–210
18. Hall, I. A., and Kustu, S. (2011) The pivotal twin histidines and aromatic
Ammonium transport by the AMT/Rh homolog TaAMT1;1 is stimulated by acidic pH. *Pflugers Arch.* **458**, 733–743

Ullmann, R. T., Andrade, S. L., and Ullmann, G. M. (2012) Thermodynamics of transport through the ammonium transporter Amt-1 investigated with free energy calculations. *J. Phys. Chem. B* **116**, 9690–9703

Walter, B., Küsters, M., Ansorge, D., Krämer, R., and Burkowski, A. (2008) Dissection of ammonium uptake systems in *Corynebacterium glutamicum*: mechanism of action and energetics of AmtA and AmtB. *J. Bacteriol.* **190**, 2611–2614

Andrade, S. L., Dickmanns, A., Ficner, R., and Einsle, O. (2005) Crystal structure of the archaeal ammonium transporter AmtB from *Arachaeoglobus fulgidus*. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 14994–14999

Blakey, D., Leech, A., Thomas, G. H., Coutts, G., Findlay, K., and Merrick, M. (2002) Purification of the *Escherichia coli* ammonium transporter AmtB reveals a trimeric stoichiometry. *Biochem. J.* **364**, 527–535

Gruswitz, F., Chaudhary, S., Ho, J. D., Schlessinger, A., Pezeshki, B., Ho, C. M., Sali, A., Westhoff, C. M., and Stroud, R. M. (2010) Function of human Rh based on structure of RhCG at 2.1 Å. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 9638–9643

Li, X., Jayachandran, S., Nguyen, H. H., and Chan, M. K. (2007) Structure of the *Nitrosomonas europea* Rh protein. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 19279–19284

Lupo, D., Li, X. D., Durand, A., Tomizaki, T., Cherif-Zahar, B., Matass, G., Merrick, M., and Winkler, F. K. (2007) The 1.3-Å resolution structure of *Nitrosomonas europaea* Rh50 and mechanistic implications for NH4 transport by Rhesus family proteins. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 19303–19308

Gruswitz, F., O’Connell, J., 3rd, and Stroud, R. M. (2007) Inhibitory complex of the transmembrane ammonia channel, AmtB, and the cytosolic regulatory protein, GlkN, at 1.96 Å. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 42–47

Petrek, M., Oteypka, M., Banáš, P., Kosinová, P., Koca, J., and Damborský, J. (2006) CAVER: a new tool to explore routes from protein clefts, pockets and cavities. * BMC Bioinformatics* **7**, 316

Javelle, A., Lupo, D., Zheng, L., Li, X. D., Winkler, F. K., and Merrick, M. (2006) An unusual twin-β-lobed cavities which serve as the sole source of carbon or nitrogen for *Salmo nella typhimurium* LT-2. *J. Bacteriol.* **190**, 736–747

Bender, R. A., and Magasanik, B. (1977) Regulatory mutations in the *Klebsiella aerogenes* structural gene for glutamine synthetase. *J. Bacteriol.* **130**, 102–105

Sopuene, E., van Heeswijk, W. C., Plumbridge, J., Stewart, V., Bertenthal, D., Lee, H., Prasad, G., Palj, O., Charrennoppakul, P., and Kustu, S. (2003) Physiological studies of *Escherichia coli* strain MG1655: growth defects and apparent cross-regulation of gene expression. *J. Bacteriol.* **185**, 5611–5626

Inwood, W. B., Hall, J. A., Kim, K. S., Demirkhanyan, L., Wemmer, D., Zgurskaya, H., and Kustu, S. (2009) Epistatic effects of the protease/chaperone HsB on some damaged forms of the *Escherichia coli* ammonium channel AmtB. *Genetics* **183**, 1327–1340

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008

Gutnick, D., Calvo, J. M., Klopotowski, T., and Ames, B. N. (1969) Compounds which serve as the sole source of carbon or nitrogen for *Salmo nella typhimurium* LT-2. *J. Bacteriol.* **100**, 215–219

Booth, I. R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* **49**, 359–378

Booth, I. R. (1999) The regulation of intracellular pH in bacteria. *Novartis Found. Symp.* **221**, 19–28; discussions 28–37

Capone, D. G. (2000) in *Microbial Ecology of the Oceans* (Kirchman, D. L., ed) pp. 455–493, John Wiley & Sons, New York

Miller, F. J. (2006) *Chemical Oceanography*, pp. 55–62, 3rd Ed., CRC Press, Boca Raton, FL

Walker, C. B., de la Torre, J. R., Klotz, M. G., Urakawa, H., Pinel, N., Arp, D. J., Brochier-Armanet, C., Chain, P. S., Chain, P. P., Gollabirg, A., Hemp, J., Hügler, M., Karr, E. A., König, A., König, M., Karr, E. A., Könneke, M., Shin, M., Lawton, T. J., Lowe, T., Martens-Habbena, W., Sayavedra-Soto, L. A., Lang, D., Sievert, S. M., Rosenzweig, A. C., Manning, G., and Stahl, D. A. (2010) *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 8818–8823

channel AmtB.