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The MotA Protein of E. coli Is a Proton-Conducting Component of the Flagellar Motor

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Summary

A number of mutants of *motA*, a gene necessary for flagellar rotation in E. coli, were isolated and characterized. Many mutations were dominant, owing to competition between functional and nonfunctional MotA for a limited number of sites on the flagellar motor. A new class of mutant was discovered in which flagellar torque is normal at low speeds but reduced at high speeds. Hydrogen isotope effects on these mutants indicate that MotA catalyzes proton transfer. We confirmed an earlier observation that overproduction of MotA leads to accumulation of the protein in the cytoplasmic membrane and to significant decreases in growth rate. When nonfunctional mutant variants of MotA were overproduced instead, they accumulated in the cytoplasmic membrane, but growth was not impaired. These results also suggest that MotA conducts protons. This was confirmed by measuring the proton permeabilities of vesicles containing wild-type or mutant MotA proteins.

Introduction

Cells of Escherichia coli and many other bacteria swim by rotating helical propellers (Berg and Anderson, 1973; Silverman and Simon, 1974; Berg, 1975), each driven by a reversible rotary motor embedded in the cell wall and cytoplasmic membrane (DePamphilis and Adler, 1971b; Coulton and Murray, 1978). The motor is a chemomechanical transducer: the energy for rotation comes from the flow of protons down a transmembrane electrochemical gradient (Larsen et al., 1974a; Belyakova et al., 1976; Manson et al., 1977; Matsuura et al., 1977). The propeller/motor organelle, called the flagellum, is a moderately complex assembly containing some 20 different kinds of polypeptides and requiring about 40 different genes for its assembly and function (for review see Macnab, 1987). Purified flagella consist of a long helical filament, a short hook, and a basal structure of four rings (two in gram-positive bacteria) mounted on a rod (DePamphilis and Adler, 1971a; Dimmitt and Simon, 1971; Aizawa et al., 1985). The functions of some of these components are known or may be guessed at from morphology: the distal rod is a drive shaft, the two outermost rings serve as a bushing that permits the rod to pass through the peptidoglycan layer and outer membrane, the hook is a flexible coupling, and the filament converts rotary motion into thrust. The polypeptides that comprise many of these components, together with the genes that encode them, have been identified (Ridgway et al., 1977; Aizawa et al., 1985; Homma et al., 1987a, 1987b; Jones et al., 1988; Homma et al., 1988; Malekooti et al., 1989; Kihara et al., 1989).

Several polypeptides known to be necessary for torque generation or for control of the sense of rotation are not found in purified flagella, but appear to be part of the intact motor (Enomoto, 1966; Silverman et al., 1976; Hilmen and Simon, 1976; Ridgway et al., 1977; Dean et al., 1983; Yamaguchi et al., 1986a, 1986b; Khan et al., 1988). Two of these, encoded by the *motA* and *motB* genes, are not required for the assembly of the flagellum, but can activate preexisting basal structures, restoring function in a series of discrete steps (Block and Berg, 1984; Blair and Berg, 1988). This led us to suggest that the motor contains several independent torque generators composed, at least in part, of MotA and MotB. By counting the number of steps in restoration experiments that go to completion or by comparing the magnitude of the elementary torque increment with the torque of fully functional motors, we estimated that the flagellar motor contains eight such generators (Blair and Berg, 1988).

Khan et al. (1988) saw rings of between 10 and 12 particles in freeze-fracture electron micrographs of cytoplasmic membranes of wild-type E. coli; however, these rings were not seen if either MotA or MotB was absent. Similar rings, containing between 14 and 16 particles, were seen in a motile Streptococcus. Coulton and Murray (1978) saw rings also containing between 14 and 16 particles surrounding annular depressions in membranes at the poles of Aquaspirillum serpens. It appears likely that these particles encircle the basal bodies of intact flagella and that they contain the Mot proteins. We have suggested (Blair and Berg, 1988) that the number of particles in a fully functional E. coli motor might be as high as 16, corresponding to two particles per torque generator.

Chun and Parkinson (1988) examined the membrane disposition of MotB and concluded that it traverses the cytoplasmic membrane once near its amino terminus, with its bulk situated in the periplasm. This conclusion is consistent with the nucleotide sequence of the *motB* gene (Stader et al., 1986), which encodes a polypeptide containing one strongly hydrophobic segment near the amino terminus. Based on this topological information, Chun and Parkinson have suggested that MotB might be a linker that connects the torque-generating machinery of the motor to the cell wall. A detailed study of MotA topology has not yet been made, but the amino acid sequence of MotA, deduced from the nucleotide sequence of the *motA* structural gene (Dean et al., 1984), contains four hydrophobic sequences that are probably transmembrane α helices. Thus, the MotA protein might be a transmembrane proton conductor. Such a function would be consistent with its transmembrane disposition and with the paralyzed phenotype of *motA* alleles.

To learn more about the function of MotA, we treated a plasmid carrying *motA* with hydroxylamine, isolated a number of mutant alleles, and characterized their pheno-

Table 1. Strains and Plasmids

Strain or Plasmid	Relevant Genotype
RP437	wild type for motility and chemotaxis
MS5037	*motA*
pLW3	P*trp-motA*$^+$, Ap^R
pSYC62	P*mocha-motB*$^+$, Cm^R
pDFB36	P*lac-motA*$^+$, $lacI^Q$, Ap^R
pDFB46	P*mocha-motA*$^+$, Cm^R

types in some detail. If the MotA protein is a component of the intact flagellar motor, dominant mutations in *motA* should exist encoding proteins that are nonfunctional but whose structure permits incorporation into the motor. Many dominant alleles of *motA* were found, and a variety of experiments were undertaken to test the idea that dominance reflects a competition between functional and defective copies of MotA for a limited number of sites on the motor.

A new type of *motA* mutant was isolated, characterized by slow swimming. Comparisons of the torques generated by the motors of these mutants when operating at low or high speed and measurements of deuterium isotope effects led to the conclusion that MotA catalyzes proton transfer.

If MotA is a proton conductor that functions as such when dissociated from the motor, large amounts in the cytoplasmic membrane should cause proton leakage and retard cell growth. Wilson and Macnab (1988) examined the effects of overexpressing the *motA* gene and found large quantities of MotA in the cytoplasmic membrane (approximately 50 times the wild-type level). Growth was slowed by a factor of more than 2, but the effect was considered to be minor enough to argue against proton conduction. We have carried out analogous measurements using nonfunctional, dominant alleles of *motA*. When overexpressed, the mutant MotA proteins were found in the cytoplasmic membrane in amounts comparable to the wild-type protein, but none of them caused a comparable decrease in growth rate. The effects of overproduction of the wild-type protein could be mimicked by growth in the presence of a proton ionophore. These experiments suggest that the growth impairment is, in fact, caused by proton conduction through MotA. We compared the permeabilities of membranes containing the wild-type or mutant MotA protein by measuring the movement of protons into vesicles energized with a potassium diffusion potential. Membranes containing the wild-type protein were significantly more permeable to protons.

Results

Isolation of Mutants

A strain defective in *motA* (MS5037; see Table 1) was transformed with a plasmid encoding *Plac-motA* and resistance to ampicillin (pDFB36) that had been mutagenized by treatment with hydroxylamine. Approximately 1% of the ampicillin-resistant clones showed impaired swarming in semisolid agar. An initial screen, in which transformants isolated on solid agar were transferred to semisolid plates, turned up 38 such mutants. A more efficient screen, in which transformants were selected directly on semisolid agar, turned up 129 more. Of these 167 isolates, 97 failed to swarm on semisolid agar and were completely immotile (or only writhed) when grown on tryptone broth (both media containing ampicillin and isopropyl-β-D-thiogalactopyranoside [IPTG]). Most of the other 70 isolates swarmed much more slowly than the wild type and only writhed when cultured in tryptone broth. Approximately 20 isolates swarmed at an appreciable fraction of the wild-type rate and could swim, although more slowly than the wild type, when cultured in tryptone broth. We will refer to the latter isolates as "slow" mutants. Examples of wild-type, immotile-mutant, and slow-mutant swarms are shown in Figure 1.

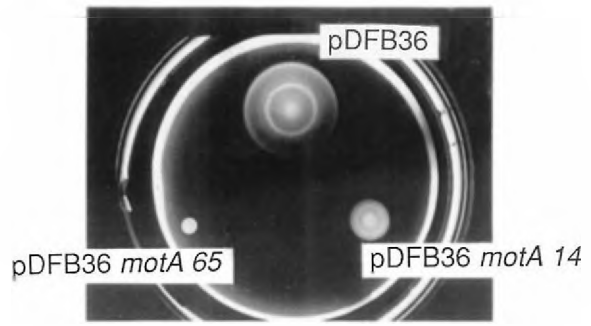

Figure 1. Swarming Phenotypes of Wild-type Cells and *motA* Mutants

Alleles 14 and 65, which are representatives of the slow and the completely immotile phenotypes, respectively, were obtained by hydroxylamine mutagenesis of pDFB36 and selection of transformants of MS5037 by the second method described in the text. The swarm plate shown contained 50 μg/ml ampicillin and 1 mM IPTG. The plate was photographed after ~6 hr at 32°C.

To verify that the mutations causing impaired motility were carried on pDFB36, the plasmids were purified from each of the 167 mutant isolates and reintroduced into freshly cultured MS5037. The mutant phenotype was reproduced in every case, confirming its association with the plasmid. Nucleotide sequencing (data not shown) showed that all of the alleles characterized here were missense mutations, of the kind expected for mutagenesis by hydroxylamine (C→T or G→A).

Dominance of *motA* Alleles

If the MotA polypeptide functions as a subunit in any larger aggregate, then dominant alleles of *motA* are likely to exist. To test this idea, we transformed each of the mutant plasmids into the wild-type strain RP437. The motility of each resulting strain was assayed, and each mutation was scored as recessive, partially dominant, or strongly dominant, based on its ability to impair motility in the wild-type background. Approximately 34% of the *motA* mutations were partially dominant and 18% strongly dominant. In the simplest interpretation, the strongly dominant mutations encode proteins that are nonfunctional but whose affinity for the motor is comparable to that of the wild type.

Table 2. Torques of Strains Coexpressing Wild-Type and Mutant MotA

Strain	MotA Complement	Relative Torque
RP437[pLW3]	~3× wild type	1.00
RP437[pLW3.4]	1× wild type, ~2× mutant 4	0.35 ± 0.06
RP437[pLW3.73]	1× wild type, ~2× mutant 73	0.34 ± 0.06

Torques of 20–25 cells of each strain were measured. The uncertainty given is the standard error of the mean.

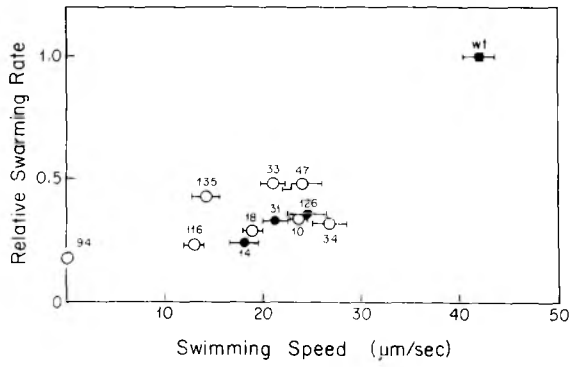

Figure 2. Relative Swarming Rate As a Function of Swimming Speed of Some Slow *motA* Mutants

Filled circles represent recessive alleles, open circles represent dominant alleles, "wt" is wild type. Error bars represent the standard error of the mean swimming speed for 20–25 cells. The swarming rates were determined twice, and the average plotted; the two determinations typically differed by less than 15%. Swarming rates were normalized to that of the wild type, which was 2.8 ± 0.03 mm/hr (mean ± SEM for 37 determinations). Experiments were done at 32°C.

To test this idea more quantitatively, two of the strongly dominant mutations were transferred onto pLW3, a plasmid originally constructed for the overexpression of *motA*. Using immunoblots, Wilson and Macnab (1988) showed that this plasmid directs the expression of MotA at approximately twice the wild-type level when cells are grown in the presence of the *trp* operon corepressor tryptophan. Thus, wild-type cells harboring *motA* mutant alleles on this plasmid should express wild-type levels of normal MotA and roughly twice this amount of the mutant protein when expression from the plasmid is inhibited by tryptophan. When tethered, cells of these strains generated an average torque about one-third that of a control strain expressing only wild-type MotA (Table 2). This supports the hypothesis that functional and defective MotA monomers compete with comparable success for a limited number of sites on the motor.

Nonfunctional copies of MotA might also create an impediment to motor rotation. For example, if MotA catalyzed a reaction step obligatorily coupled to motor rotation, a defective MotA molecule that could not catalyze this step but was properly integrated would stop the motor. A tethered cell of the wild-type strain harboring such a *motA* allele on a plasmid would either spin at wild-type speed (because the motor that was tethered contained only wild-type protein) or it would not spin at all (because that motor contained defective copies of MotA). Cells of six such strains were tethered and their rotation rates analyzed. The average rotation rate varied among the strains, but in all cases many cells spun, generating torques substantially smaller than the wild type. (Alleles tested: 4, 5, 7, 11, 12, and 73.)

Nonfunctional copies of MotA might also impair motility by binding to and sequestering other components essential for motility, rather than by binding to the motor itself. The most obvious candidate for such an essential factor is MotB, since it shares with MotA the capacity to restore function when added last to an otherwise complete complement of motor parts. When introduced on a second plasmid (pSYC62; Table 1), multiple copies of *motB* linked to its native promoter did not measurably improve the motility of wild-type cells harboring any one of five different dominant *motA* mutations on pDFB36. (*motA* alleles tested: 5, 7, 11, 12, and 19.) When additional copies of wild-type *motA* were introduced instead (using pDFB46), the motility of all five strains dramatically improved.

Slow-Swimming *motA* Alleles

As noted above, approximately 20 mutants (slow mutants) swarmed at an appreciable fraction of the wild-type rate and could swim in broth, although more slowly than the wild type. This is shown for 11 alleles in Figure 2. The swarming rates and swimming speeds of the slow mutants were roughly correlated. Thus, the slower swarming can be ascribed to slower swimming, rather than to any switching defect that would cause these mutants to be reclassified as *che* alleles. To make certain that the slow-swimming phenotype was not a trivial consequence of a reduction in the number of flagellar motors, filaments were visualized by a wet-mount staining method (Heimbrook et al., 1986). All of the slow mutants had a normal complement of filaments (mean ± SD 4.2 ± 0.6 filaments per cell for 15 different slow mutants, as compared with 4.5 ± 0.7 for the wild type). Some of the slow *motA* alleles were dominant, while others were recessive (as indicated by the open and filled circles, respectively, in Figures 2–4). Evidently, some of the slow alleles encode proteins that associate with the motor, but less strongly than the wild-type protein.

Nine of the slow-swimming mutants were tethered and their mean torques determined. Most of the mutants had torques similar to the wild type (Figure 3). Those with significantly diminished torque were among the slowest swimmers. One mutant (allele 94) that writhed but did not swim was examined; it produced torque approximately one-half that of the wild type. All the mutants showed reversals in motor direction. Some mutants (alleles 41, 66, and 99; not shown in Figure 2) swarmed more slowly than the wild type, yet swam at speeds similar to the wild type when cultured for several hours in tryptone broth. These mutants were inoculated onto swarm agar plates (together with wild-type controls), and samples were picked from the swarm edges for microscopic examination. Cells taken from swarms of the mutants swam more slowly than

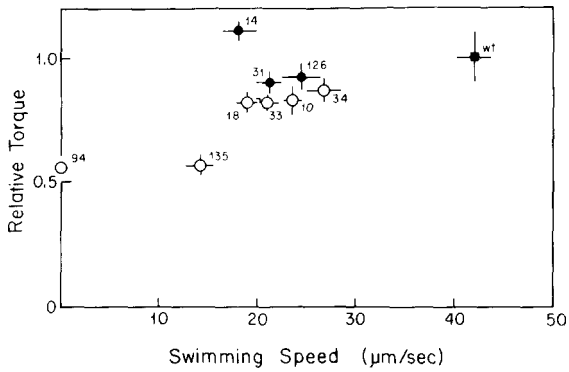

Figure 3. Relative Torque of Tethered Cells As a Function of Swimming Speed of Some Slow *motA* Mutants

Error bars represent the standard error of the mean for 20–25 cells. Torques were normalized to that of the wild type, which was 15.3 ± 1.6 × 10^{-12} dyn-cm (mean ± SD for three determinations, each with 20 cells). Symbols are as described in the legend to Figure 2. Experiments were done at 32°C.

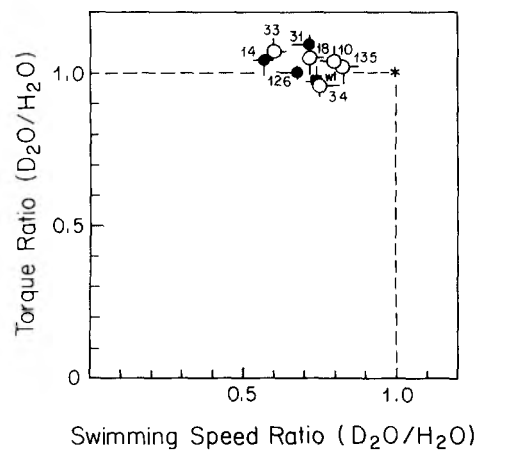

Figure 4. Deuterium Solvent Isotope Effect in Slow *motA* Mutants and the Wild Type

Torques of tethered cells were compared in medium containing 0 or 99% D_2O; speeds of swimming cells were compared in 0 or 90% D_2O. Symbols are as described in the legend to Figure 2, except that swimming speed measurements were for between 20 and 97 cells, and torque measurements were for approximately 10 cells. Experiments were done at 32°C.

cells from swarms of the wild type (speeds in μm per second ± SEM for 20–25 cells: allele 41, 14.1 ± 1; allele 66, 12.9 ± 1; allele 99, 15.4 ± 1.5; wild type, 21.1 ± 1.1). Evidently, the slower swarming rates of these mutants are due to impaired swimming in swarm plates, which is not observed in cells cultured in broth for measurements of swimming speed. Nucleotide sequencing (data not shown) revealed that these mutants encode the wild-type MotA protein; therefore, it is likely that the mutation is in *Plac* or *lacI*^{Q} and affects the level of expression of the *motA* gene.

Deuterium Isotope Effects

Previously, we have contrasted the operating characteristics of the flagellar motor at high and low speeds (Lowe et al., 1987) and suggested that the upper limit on the speed is due, in part, to one or more proton transfer reactions (Meister et al., 1987; Lowe et al., 1987). This suggestion was based on the observation that cells of an artificially energized Streptococcus swam more slowly when D_2O was substituted for H_2O (Meister et al., 1987), while the mean torque of tethered cells was unaffected (Khan and Berg, 1983). Analogous measurements of wild-type E. coli and ten slow-swimming *motA* alleles are summarized in Figure 4. All of the *motA* mutants produced approximately equal torques in H_2O and D_2O. However, their swimming speeds, already slower than that of the wild type, were significantly reduced in D_2O, suggesting that the kinetic limitation on motor rotation in these mutants involves proton transfer. D_2O is approximately 20% more viscous than H_2O, but the associated small increase in load is not expected to affect swimming speeds (Lowe et al., 1987). This expectation was verified by comparing the swimming speeds of seven mutants (alleles 10, 14, 18, 31, 33, 126, and 131) in motility medium with their speeds in motility medium containing 1% Ficoll, whose viscosity at 32°C is equal to that of 90% D_2O. The increased viscosity had no measurable effect on the swimming speeds (mean speed ratios ± SD for the seven strains: D_2O/H_2O = 0.64 ± 0.09; 1% Ficoll/H_2O = 0.97 ± 0.20; 20–25 cells of each strain measured in each medium). The pK_as of several amino acid side chains are known to be increased somewhat in D_2O relative to H_2O (Fersht, 1985). To verify that the observed D_2O effect is not an artifact of what is effectively a shift in pH, the pH dependence of swimming speed of the wild type was examined. No significant pH dependence was observed in the range between pH 6.0 and 7.6 (mean speeds ± SEM in μm per second for 20–25 cells, and corresponding pH values: 36.5 ± 1.5, pH 5.95; 41.2 ± 1.7 , pH 6.36; 37.9 ± 1.5, pH 6.78; 38.1 ± 1.9, pH 7.20; 36.9 ± 1.5, pH 7.6).

Overexpression of Wild-Type and Mutant MotA Protein

Wilson and Macnab (1988) constructed the plasmid pLW3, described above, to direct high-level expression of MotA. When cells containing this plasmid were cultured in the presence of the *trp* operon inducer indoleacrylic acid (IAA), the MotA protein was found in the cytoplasmic membrane in steadily increasing amounts, approaching 50 times the normal level after 20 hr. A more than 2-fold decrease in growth rate also was observed, which was not seen in a control strain harboring a plasmid that lacked the *motA* gene. MotA might be a proton conductor and so cause sufficient membrane leakage to retard growth when overexpressed. However, it is also possible that the synthesis and membrane insertion of large amounts of the MotA protein slows growth by causing more general membrane defects or by adversely affecting other essential membrane components via mechanisms unrelated to its ordinary function in the flagellar motor. To assess better the relation between growth impairment by MotA and its function in motor rotation, we transferred five of the nonfunctional, dominant *motA* alleles from pDFB36 onto

Table 3. Growth Rate of Strains Overexpressing Wild-Type or Mutant MotA

Strain	*motA*	Induction with IAA	Growth Rate (Generation per Hour)	Relative Amount of MotA in Inner Membrane
RP437[pLW3]	wild type	–	1.86 ± 0.03	
		+	.99 ± 0.14	1.00
RP437[pLW3.4]	mutant	–	1.73 ± 0.04	
		+	1.69 ± 0.04	0.94
RP437[pLW3.7]	mutant	–	1.73 ± 0.05	
		+	1.67 ± 0.02	1.05
RP437[pLW3.58]	mutant	–	1.72 ± 0.01	
		+	1.63 ± 0.03	0.87
RP437[pLW3.73]	mutant	–	1.86 ± 0.03	
		+	1.71 ± 0.03	0.84
RP437[pLW3.114]	mutant	–	1.95 ± 0.06	
		+	1.75 ± 0.02	0.84
RP437[pDB47]	deleted	–	1.69 ± 0.07	
		+	1.63 ± 0.03	0

Growth rates were determined in triplicate; the reported uncertainty is the standard deviation. The MotA quantification was carried out with cells from induced cultures and is the average of two determinations that differed by an average 7% and by no more than 20%. All of the mutant *motA* alleles tested here were dominant.

pLW3 and used the resulting plasmids to direct the overexpression of mutant MotA protein. In all five cases, the effect of the mutant protein on growth rate was insignificant compared with that seen with the wild-type protein (Table 3). To verify that the mutant proteins were still found in the cytoplasmic membrane, membrane fractions were purified and analyzed by SDS–PAGE. The electrophoretic mobilities of the five mutant proteins varied somewhat relative to the wild type, but all were found in the inner membrane fraction in amounts comparable to the wild-type protein (Figure 5). Apart from the differing MotA mobilities, other changes were noted in these gels: two bands, at approximately 70 kd and 50 kd, were significantly decreased in intensity in the strain overexpressing wild-type MotA, but not in the *motA* deletion strain or the strains overexpressing mutant protein.

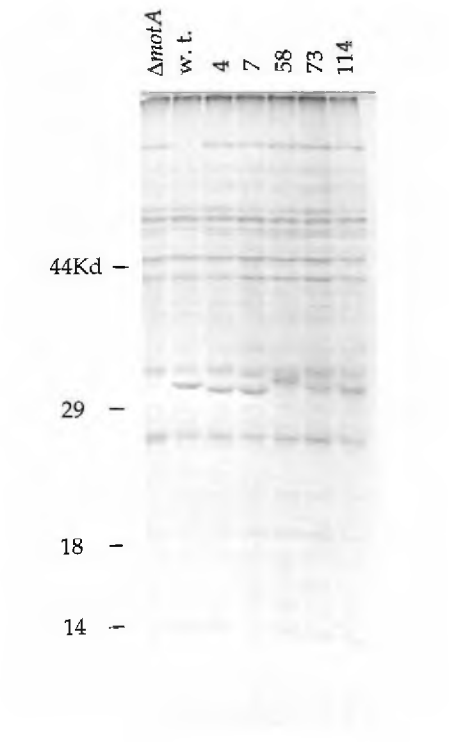

Figure 5. SDS–Polyacrylamide Gel of Inner Membrane Fractions from a Strain Deleted for *motA* and from Strains Overexpressing Wild-Type and Mutant MotA Proteins

The MotA bands are in the 30–35 kd range. Each lane contains 10 μg of inner membrane protein. The MotA band was much less intense in outer membrane samples (not shown). Two different gels were stained with Coommassie brilliant blue and scanned to quantitate the MotA band (Table 3).

In spite of their slower growth, cultures of cells overexpressing MotA reach a very high density, comparable to cultures of wild-type cells. Is this consistent with growth impairment caused by proton leakage and an associated decrease in available energy? This question was addressed by culturing cells of the wild type in the presence of various concentrations of 2,4-dinitrophenol (DNP), a known proton ionophore. The growth rates of these cultures decreased monotonically as the DNP concentration was increased from 0 to 1.25 mM (Figure 6). The growth impairment caused by MotA overexpression was comparable to that caused by 0.75 mM DNP; cultures in 0.75 mM DNP reached densities approximately 80% that of cultures containing no DNP. To assess the effect of DNP on the cytoplasmic membrane proteins, membranes were prepared from a culture of wild-type cells in 0.75 mM DNP and analyzed by SDS–PAGE. DNP had an effect similar to that seen when wild-type MotA was overexpressed; i.e., the same two bands were significantly diminished in intensity (data not shown).

Proton Permeabilities of Vesicles Containing Wild-Type or Mutant MotA Protein

Membrane vesicles were prepared from strains overexpressing either wild-type or mutant MotA protein, loaded

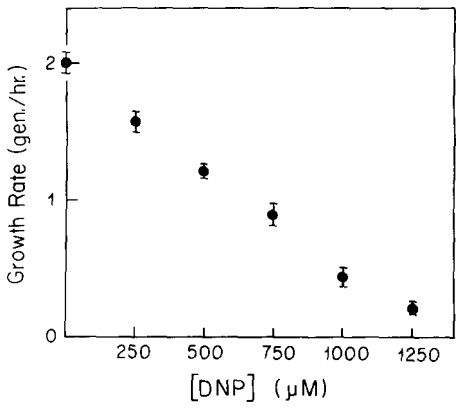

Figure 6. Effect of the Proton Ionophore DNP on the Growth Rate of the Wild-Type Strain RP437

Cells were cultured in LB at 34°C. A linear least-squares fit to a plot of $\ln(OD_{600})$ was used to estimate the growth rate. The error bars represent the standard deviation for four determinations.

with potassium phosphate at a high concentration (about 0.2 M; Hirata et al., 1974), and suspended in a medium containing potassium phosphate at a low concentration (0.25 mM). When the potassium ionophore valinomycin is added to this suspension, an electrical potential gradient is generated across the vesicle membranes by outward diffusion of potassium. As a result, positively charged species, including protons, are driven in. If the external medium is weakly buffered, proton uptake can be monitored by changes in pH. The results of such an experiment with vesicles containing wild-type MotA protein or either of two mutant variants are shown in Figure 7. The initial proton flux was approximately 4-fold larger with vesicles containing the wild-type protein, directly demonstrating a proton-conducting activity of the MotA protein. The total proton uptake also was larger, which might suggest that the vesicles containing mutant MotA protein were less efficiently loaded with potassium. This possibility was ruled out by measuring the additional influx caused by the addition of the proton ionophore carbonylcyanide m-chlorophenylhydrazone (CCCP; data not shown). The total proton influx induced by valinomycin and CCCP was comparable in the three samples (70 neq/mg protein for the wild type, 68 neq/mg for allele 4, and 51 neq/mg for allele 7). A similar correlation between the rate and extent of proton uptake driven by a diffusion potential has been seen with F_0, the proton channel of the ATP synthase (Fillingame, 1981; see Discussion). The experiment shown in Figure 7 was carried out at 17°C. For both the wild type and allele 4, the process was accelerated by about a factor of 2.5 at 27°C (data not shown). Pretreatment with N,N′-dicyclohexylcarbodiimide (DCCD), which should completely inhibit proton flow through the ATP synthase, caused a ~5-fold decrease in the proton flux in both the wild type and allele 4—the flux was still much larger with the vesicles from the wild type.

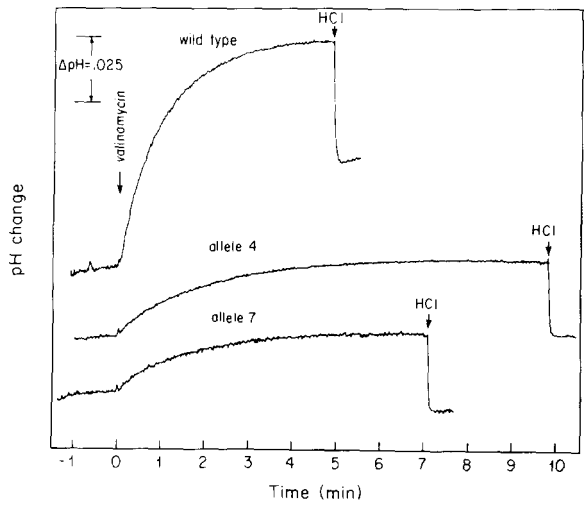

Figure 7. Proton Flux into Vesicles Containing Wild-Type or Mutant MotA Protein

Vesicles were prepared and loaded with K^+ as described in the text. After ~3 min equilibration at 17°C, valinomycin was added to generate a potassium diffusion potential, and the resulting pH changes were recorded. At the times indicated by arrows, 50 neq HCl was added to determine buffering capacities. The initial proton fluxes (estimated from the initial slopes of the curves) were approximately 2 neq per second for the wild type, and 0.5 neq per second for the mutants. The total content of MotA, determined as described in the text, was 13 μg for the wild type, 20 μg for allele 4, and 29 μg for allele 7. The curves have been shifted to facilitate comparison; the initial pH values of the samples were 6.88, 6.94, and 6.84 for the wild type, allele 4, and allele 7, respectively.

Discussion

Dominance of *motA* Alleles

A large fraction of the *motA* mutants were dominant, implying that MotA functions as part of a larger assembly. Evidence from several sources (Khan et al., 1988; Blair and Berg, 1988; Wilson and Macnab, 1988) suggests that MotA functions in physical association with the motor. The dominant character of many *motA* mutations furnishes additional evidence for this association: dominance would simply reflect displacement of functional MotA from the motor by nonfunctional MotA. Dominance often occurs because a protein functions as a dimer or larger multimer; in this case the mutant protein can inactivate the wild-type protein via a direct interaction. Our results do not imply any such direct interaction between MotA molecules. If anything, the results in Table 2 suggest that MotA functions as a monomer, since the observed torque is proportional to the fraction of MotA protein that is wild type, rather than to a higher power of this fraction. Further substantiation of this suggestion would require closer examination of several more alleles. The recessive character of some of the mutations could be due to a decreased affinity for the motor of the MotA proteins they encode, instability and consequent degradation of the proteins they encode, or defects within the promoter rather than the *motA* structural gene.

If nonfunctional MotA protein encoded by dominant *motA* alleles is incorporated into the motor, it could in principle hamper or block rotation driven by functional copies

of MotA. For instance, in a tightly coupled mechanism in which motor rotation is obligatorily linked to proton transfer, a single clogged proton conductor would lock the motor. Since this was not seen, it appears likely that rotation unlinked to proton transfer is possible, at least at slow speeds.

Slow-Swimming *motA* Alleles

The slow mutants as well as the wild type generated the same torque when tethered in D_2O and H_2O. Thus, at the slow rotation speeds characteristic of tethered cells, proton transfer is not rate limiting, as has been noted for an artificially energized Streptococcus (Khan and Berg, 1983; Meister and Berg, 1987). Furthermore, since the torque is expected to depend on protonmotive force (Manson et al., 1977, 1980), the protonmotive force is not significantly decreased in D_2O. However, all of the slow mutants as well as the wild type swam significantly more slowly in D_2O than in H_2O. It is known that the motor spins much more rapidly in swimming cells than it does in tethered cells and that in swimming cells its torque is much smaller (Lowe et al., 1987). The simplest interpretation for the deuterium isotope effect is that the process limiting motor speed in swimming cells involves one or more proton transfers (Meister et al., 1987). D_2O might cause minor conformational changes in motor components; however, deuterium isotope effects of the magnitude observed here are ordinarily interpreted as arising from an increase in the activation barrier of a reaction step that involves partial breakage of a bond to hydrogen, owing to the decreased zero point energy of D relative to H (Gandour and Schowen, 1978). In the slow mutants, the rate of proton transfer could be decreased by mechanisms that increase hydrogen ion dissociation in the activated state, leading to a greater isotope effect than in the wild type (e.g., allele 14) or by mechanisms that decrease or do not affect the degree of dissociation, thus leading to an unaltered or even a smaller isotope effect (e.g., allele 135). We conclude that MotA catalyzes one or more proton transfer reactions in the motor. A less likely alternative is that any of several different alterations in MotA affect the rate of proton transfer catalyzed by another protein with which it interacts.

The rates of internal processes do not determine the torque of motors driving tethered cells. However, the torque would be decreased if the efficiency of coupling between proton transfer and motor rotation were decreased by alterations to the torque-generating machinery. Efficiency would be decreased by increasing the rates of wasteful processes (e.g., proton transfers unlinked to rotation) relative to energy-conserving processes. Most of the slow alleles of *motA* do not have this kind of effect, since the rotation of tethered cells was normal. Allele 135 may be an exception; its behavior will be considered again in another report in the light of sequencing results (unpublished data).

A swimming cell is propelled by several flagella working together in a bundle, which is stabilized by hydrodynamic and other mechanical forces (Anderson, 1975; Macnab, 1977). It is expected that the bundle will not be stable below some threshold speed of rotation, imposing a lower limit on the mean swimming speed that can be observed in strains whose motors rotate slowly (Shioi et al., 1980). Presumably, the motors of allele 94 rotate at a speed below this threshold (Figure 3).

Growth Impairment by MotA

We have confirmed the growth impairment caused by high levels of MotA, first observed by Wilson and Macnab (1988). They concluded that the effect was not drastic enough to be the result of proton leakage through MotA. However, we have shown that increasing concentrations of a known proton ionophore (DNP) cause a graded decrease in growth rate, without greatly decreasing the final cell density reached by the culture. Mutant variants of MotA that do not function in torque generation also do not retard growth, even when they are inserted into the cytoplasmic membrane in amounts comparable to the overexpressed wild-type MotA protein. This suggests that the protein function(s) required for motor rotation are the same as those that retard growth.

Assuming that respiration is rate limiting in rich cultures, as some available evidence suggests (Anderson and von Meyenburg, 1980), a 2-fold reduction in growth rate should correspond roughly to a 2-fold decrease in net respiration-linked proton translocation. One can estimate that the electron transport chains of a typical cell pump about 10^7 protons per second (Haddock and Jones, 1977; Anderson and von Meyenburg, 1980). If MotA slows growth 2-fold by conducting protons, it must permit the passage of roughly half of these protons, or 5×10^6 per second. At 2–3 hr after IAA induction, at which time growth is already slowed, each cell contains about 3600 copies of MotA (Wilson and Macnab, 1988). Thus, the growth impairment could be explained by the passage of ~1400 protons per second through each MotA monomer. If our previous estimates of proton stoichiometry are correct (Meister et al., 1987) and if the motor has eight channels (Blair and Berg, 1988), then the maximum proton flux through each channel is 10^4 per second. Considering that the overexpressed MotA protein cannot be involved in all of its normal associations with other components, and therefore may not adopt its optimal conformation, its conductance when free in the membrane appears to be a respectable fraction of that in the operational motor.

Proton Permeability of Vesicles Containing MotA

Vesicles containing wild-type MotA are more permeable to protons than vesicles that contain mutant variants of MotA (Figure 7). This observation is the most direct demonstration of a proton-conducting activity of the MotA protein. The initial proton flux in this experiment was approximately 2 neq of protons per second at 17°C, at a nominal diffusion potential of ~175 mV, close to the total pmf in a respiring cell. At 27°C, the time course of proton uptake was accelerated by about a factor of 2.5. At 34°C, measurements were difficult to make, but assuming Arrhenius behavior, the flux would be increased by an additional factor of about 2. By scanning a Coomassie-stained SDS–PAGE gel of the vesicle sample, the amount of MotA was estimated at approximately 13 μg, or 4.2×10^{-10} equiva-

lents. This would correspond to a conductivity of 25 H^+ per second per MotA molecule, substantially less than that required to explain the growth impairment discussed above. This difference could arise from a variety of factors, for example, loss of activity during vesicle preparation, loss of periplasmic molecules that facilitate proton flow to the MotA channels, or inhibition of flux by species present in the vesicle buffer. The correlation between rate and extent of proton uptake might be explained by a slow dissipation of the diffusion potential during the course of the measurements (Altendorf et al., 1974) or by a competing outward flow of protons, driven in the present experiments by electroneutral proton/phosphate symport.

The flux was decreased but not blocked by DCCD; the relative decrease was similar in the wild type and in allele 4. The increase in flux with temperature also was similar in the wild type and the mutant. The simplest interpretation for these results is that the proton conductance in the vesicles from the mutant, which is about 6-fold less than that in the wild type for a given amount of MotA protein (cf. legend to Figure 7), is due to a residual activity of the mutant MotA protein and that both mutant and wild type are partially inhibited by DCCD. The MotA protein sequence contains at least one acidic amino acid in a moderately hydrophobic environment (Glu-33); mutational replacement of this residue causes a severe disruption of function (unpublished data).

The F_0 channels of E. coli have a similarly low conductance both in F_1-depleted vesicles (~18 H^+ per second per channel; Fillingame, 1981), or when reconstituted into liposomes (25 H^+ per second; Schneider and Altendorf, 1985), in spite of the fact that they must conduct protons much more rapidly in the ATP synthase F_1F_0. A reliable estimate of the conductivity of individual MotA channels must await closer analysis of the conduction properties of purified MotA.

Experimental Procedures

Media and Buffers

Cells were cultured in tryptone broth for motility assays or in LB for transformations and growth rate determinations (Maniatis et al., 1982). Ampicillin was used at 100 μg/ml in plates and at 50 μg/ml in liquid culture. IPTG was used at 1 mM. IAA was used at 100 μg/ml, introduced as a 10 mg/ml solution in ethanol. Tryptophan was used at 400 μg/ml. Motility medium contained 120 mM sodium lactate, 67 mM NaCl, 10 mM potassium phosphate (pH 7.0), 10^{-4} M EDTA, 10^{-6} M methionine.

Plasmids and Strains

Plasmids were constructed using the recombinant DNA procedures outlined in Maniatis et al. (1982). Transformations and DNA purifications were according to Maniatis et al. (1982) or Silhavy et al. (1984). The *motA* structural gene in pDFB36 is derived from pLW3 (Wilson and Macnab, 1988; the gift of L. Wilson and R. M. Macnab, Yale University). The E. coli lactose operon promoter in pDFB36 is derived from pUC9 (Vieira and Messing, 1982), and the *lacI*^{Q} gene is from pCR63 (the gift of C. Russell, via F. W. Dahlquist, University of Oregon). pSYC62 (the gift of S.-Y. Chun and J. S. Parkinson, University of Utah) encoded *motB* under its native promoter and chloramphenicol resistance. pDFB46, encoding *motA* under its native promoter and chloramphenicol resistance, was constructed by deleting a portion of the plasmid pJL13 (the gift of J. Liu and J. S. Parkinson). Selected *motA* alleles were transferred from pDFB36 onto pLW3 by purifying the BglII–MluI fragment from the mutant pDFB36 plasmid and ligating it into pLW3 that had been digested with MluI and BglII and treated with calf intestinal phosphatase. The alleles that mapped to this segment were determined by a mapping procedure like that of Mutoh et al. (1986). (The BglII–MluI fragment contains approximately 235 bp from the middle of the *motA* structural gene; for the nucleotide sequence see Dean et al., 1984.) The *motA* strain MS5037 was the gift of M. Simon (California Institute of Technology), and the wild-type strain RP437 was the gift of J. S. Parkinson.

Mutagenesis

Plasmid pDFB36 was treated with 0.4 M hydroxylamine in 0.05 M sodium phosphate (pH 6.0), 10^{-3} M EDTA for 60 or 120 min at 72°C (Humphreys et al., 1976). The plasmid was then diluted in 0.1 M NaCl, 0.01 M Tris (pH 8.0), 10^{-3} M EDTA and precipitated with ethanol twice before being used in transformation of the *motA* strain MS5037. Initially, transformants were selected on LB plates containing ampicillin and 1.5% agar and subsequently screened by picking them onto semisolid tryptone plates (agar concentration 0.35%) containing ampicillin and IPTG. The procedure was later made more efficient by plating the freshly transformed cells directly in semisolid agar containing ampicillin and IPTG, at a density that gave about 100 transformants per plate. Completely immotile transformants were readily identifiable as tight, dense colonies. Transformants that swarmed, but at a rate less than the wild type, were less obvious but could nevertheless be recognized by their comparatively dense swarms.

Dominance

To assay the dominance of the plasmid-borne *motA* alleles, each plasmid was purified and transformed into the wild-type strain RP437. The effect of inducing transcription of the mutated *motA* gene was assessed by culturing the transformants with or without IPTG. Each allele was scored as recessive (no difference between the induced and uninduced cultures), partially dominant (a significant impairment of motility in the induced culture), or strongly dominant (complete or nearly complete abolition of motility in the induced culture). Two investigators conducted the scoring independently; their results were the same for approximately 90% of the alleles. The disagreements involved the distinction between partial and strong dominance. The differences were resolved by adjustment of the scoring criteria. If the evidence for an allele's partial dominance was so weak as to leave any doubt, it was classified as recessive.

Torque

An overnight culture was diluted 100:1 in tryptone broth containing ampicillin and IPTG and incubated with shaking for 4.5 hr at 32°C. The cells were washed twice in motility medium, sheared, and tethered, as described previously (Block et al., 1982). Their behavior was observed in a flow cell (Berg and Block, 1984) at 32°C by phase-contrast microscopy and recorded on videotape. Rotation rates were measured during playback using a system that timed the intervals between successive crossings of a video cursor (Block and Berg, 1984). The length and radius of gyration of each cell body was estimated from the video images. These estimates were used in conjunction with the rotation rates to compute the torque generated by the motor driving each cell, using formulas given by Tirado and de la Torre (1979, 1980). Relative torques in H_2O and D_2O were estimated by shifting the cells from motility medium prepared in H_2O to motility medium prepared in D_2O (final D_2O concentration approximately 99%). Motor torque was assumed to be proportional to rotation rate and to viscosity, which is approximately 20% greater for D_2O than for H_2O at 32°C.

Swimming Speed

Cells were cultured in the same way as for torque measurements, except that the cell pellets were resuspended by gentle agitation on a test-tube carousel. More vigorous resuspension methods, such as agitation with a Pasteur pipette, caused a marked decrease in swimming speeds, presumably by shearing the flagellar filaments. The cells were mixed with 9 vol of motility medium containing L-aspartate and L-serine (final concentrations 5 mM), and the suspension was drawn into a flow cell at 32°C. Following stimulation with these attractants, the cells swam smoothly for several minutes (Larsen et al., 1974b; Berg and Tedesco, 1975), which facilitated the speed measurements. Cells swimming parallel to the coverslip were videotaped and their swimming speeds clocked during slow playback. Speeds were measured

either from trajectories traced on plastic screen overlays or by using the program MEASURE (Sheetz et al., 1986), which traces cell trajectories with the aid of a video cursor. The two methods gave essentially the same results. On-screen distances were calibrated using an objective micrometer. Measurements of swimming speed in D_2O were done in the same way, except that the motility medium was made up in D_2O (final D_2O concentration 90%). pD was computed from the relation pD = pH + 0.4 (Gandour and Schowen, 1978), where pH is measured with a glass electrode. In control experiments, the increased viscosity of D_2O relative to H_2O was simulated by adding Ficoll. The viscosity of 90% D_2O at 32°C was measured using a Cannon-Ubbelohde viscometer (Cannon Instrument Company no. 75-L321, viscometer constant 0.00813 centistokes per second) and found to be 1.18 times that of pure water. The viscosity of solutions of Ficoll in water were measured in the same way, and the concentration that gave a viscosity equal to that of 90% D_2O (1.0%) was determined by interpolation.

Swarm Rate

Swarm rates were determined in semisolid agar (0.35%) containing tryptone broth, ampicillin, and IPTG. Strains carrying mutant and wild-type plasmids were inoculated near opposite edges of the same plate. The distance of the outermost ring from the point of inoculation was measured at intervals of approximately 1 hr, and the best linear fit to a plot of distance vs. time was determined. To compensate for any small plate-to-plate variations, the swarm rate of each allele was normalized to that of the wild type on the same plate. Swarming rates were measured at 20°C, 32°C, and 37°C (data not shown), and these rates, together with the pattern of swarm rings, were used to determine which mutant isolates corresponded to different alleles and which were likely to be duplicates. The experiments described here were carried out with mutants that appeared to be unique on the basis of these criteria, and nucleotide sequencing confirmed this (unpublished data).

Flagellar Stain

Flagellar filaments were stained using the wet-mount procedure of Heimbrook et al. (1986). For each allele, the average number of flagella per cell was determined by counting the flagella on ten cells selected at random.

Growth Rate

Cells were cultured at 34°C to saturation, diluted ~50-fold, and recultured until they were in midexponential phase ($OD_{600} \approx 0.5$). Then they were diluted into a medium containing the inducer indoleacrylic acid (100 μg/ml; final ethanol concentration 1%), or into a medium containing just the ethanol, and shaken at 34°C. Growth was monitored by absorbance at 600 nm. At absorbances between 0.01 and 0.40 U, the growth was exponential, and rates were determined by a linear least-squares fit to a plot of $\ln(OD_{600})$ vs. time.

Preparation of Inner Membranes

Cells were cultured at 34°C in LB to saturation, diluted ~100-fold into fresh LB, and recultured to an OD_{600} of 0.1. IAA was then added, and growth continued overnight. Membranes were prepared by the method of Wilson and Macnab (1988). Inner membranes were separated from outer membranes by a modification of the sucrose gradient method of Osborn et al. (1972). The membranes were layered on top of step gradients prepared by adding 55% sucrose (1.5 ml), 47.5% sucrose (3 ml), and 30% sucrose (3 ml) to SW41 ultracentrifuge tubes. The tubes were spun for 2 hr at 38,000 rpm (rcf = 177,000 × g at r_{av}) at 4°C. Inner membranes migrated to the interface between the 30% and 47.5% layers; on SDS–PAGE gels, the pattern of proteins from this fraction resembled that of authentic inner membrane proteins (Wilson and Macnab, 1988). Outer membranes migrated to the interface between the 47.5% and 55% layers; the pattern of proteins from this fraction resembled that of authentic outer membrane proteins (Wilson and Macnab, 1988) with smaller contributions of inner membrane proteins. Most (~80%) of the MotA was found in the inner membrane fraction; partitioning between inner and outer membrane fractions was similar for the wild-type and the mutant proteins. SDS–PAGE was basically according to Laemmli (1970); gels contained 11.7% acrylamide, 0.32% bisacrylamide, 0.1% SDS. Inner membrane protein concentrations were determined by the bicinchoninic acid assay (Smith et al., 1985), and equal amounts of inner membrane protein were loaded onto each lane. Gels were stained with Coomassie brilliant blue, and bands were quantitated by scanning densitometry (Hoefer model GS300).

Vesicle Permeability Measurements

Membrane vesicles were prepared by the method of Kaback (1971), except that cells were cultured to saturation in LB, and Na^+ was substituted for K^+ in all buffers (following Hirata et al., 1974). Vesicles were loaded with K^+ by the method of Hirata et al. (1974), except that the loaded vesicles were pelleted and resuspended in the low K^+ external medium twice rather than once, and the external medium was 0.4 M sucrose, 10 mM magnesium sulfate, 25 mM choline chloride, 0.25 mM potassium phosphate (pH 7.0). The buffer used to load the vesicles contained 0.5 M potassium phosphate; Hirata et al. (1974) estimated that the internal potassium concentration in such vesicles was about 0.2 M. When the F_0 inhibitor DCCD was used, it was added to a final concentration of 50 μM during the potassium-loading step (which involved incubation for 20 min at 40°C). Experiments were carried out at 17°C or 27°C; at higher temperatures, the baseline flux prior to the addition of valinomycin was unacceptably large. The vesicle sample was stirred rapidly, and proton uptake was initiated by adding valinomycin to a final concentration of 0.9 μg/ml (from a 1 mg/ml solution in ethanol). The buffering power of the medium was measured by the addition of small aliquots of 10 mM HCl or NaOH. As a positive control for proton permeability, CCCP was added to a final concentration of 5.6 μM, from a 6.25 mM stock in ethanol. The pH of the solution was monitored with a Radiometer pH meter (PHM84 with combination electrode GK2321C) connected to a chart recorder (Linear Instruments 1201-9022). Following the permeability measurements, samples were assayed for protein content, electrophoresed, and scanned densitometrically for MotA, as described above for inner membrane samples. Known amounts of carbonic anhydrase (29 kd), loaded onto adjacent lanes, were used as standards.

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