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Sodium Ion Cycle in Bacterial Pathogens: Evidence from Cross-Genome Comparisons

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INTRODUCTION

Most bacteria rely on proton motive force as a source of energy for a variety of cellular processes. Usually, an H^+ cycle includes generation of the transmembrane electrochemical gradient of H^+ (proton motive force) by primary transport systems (H^+ pumps) and its use for ATP synthesis, solute transport, motility, reverse electron transport, etc. (reviewed, for example, in references 76, 128, 185, and 186). A substantial body of evidence indicates, however, that certain extremophilic, particularly alkalophilic and thermophilic, bacteria can use Na^+ as a coupling ion in an Na^+ cycle instead of, or in addition to, the H^+ cycle (47, 183–185, 188). As in the H^+ cycle, a fully operational Na^+ cycle would include a primary Na^+ pump that directly couples Na^+ translocation to a chemical reaction, an Na^+-transporting membrane ATP synthetase, a number of Na^+-dependent membrane transporters, and an Na^+-dependent flagellar motor. While certain Na^+-dependent functions, such as Na^+-dependent uptake of melibiose, proline, and glutamate, have been observed in many bacteria, including *Escherichia coli* and *Bacillus subtilis* (23, 37, 128, 161, 219), the ion gradients that served as energy sources for these transports have been generated by primary H^+ pumps and converted to Na^+ gradients by Na^+/H^+ antiporters (Fig. 1). As a result, until very recently the Na^+ cycle has been suspected in many different bacteria but experimentally verified in only precious few of them, such as *Vibrio alginolyticus, Propionigenium modestum*, and *Clostridium fervidus* (38, 39, 45, 188). Based on their Na^+ requirement for growth and Na^+-dependent respiration, Na^+ cycling has been proposed in a number of marine bacteria (148, 209; reviewed in reference 109). Here, by analyzing bacterial genomic sequences, including the recently published complete genomes of *Vibrio cholerae* (83), *Pseudomonas aeruginosa* (194), and *Pasteurella multocida* (131), we show that the Na^+ cycle may be common among human and animal

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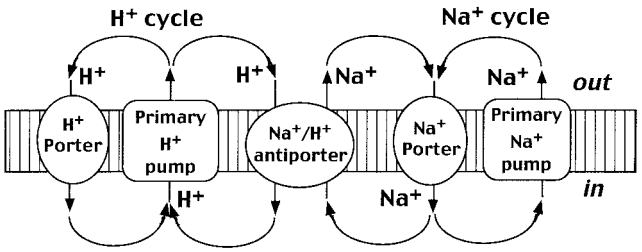

FIG. 1. Proton and sodium ion cycles in bacterial energetics. "Primary pump" indicates any proton or sodium motive force generator (e.g., respiratory ion pump, membrane ATPase, or a Na^+-transporting dicarboxylate decarboxylase). "H^+ (or Na^+) porter" indicates consumers of proton (or sodium) motive force (symporters, flagellar motor, etc.). The actual presence of partial components of both cycles in the membrane of each particular bacterial species may vary, depending on the physiological state of the cell. Na^+/H^+ antiporters convert proton motive force into sodium motive force and vice versa, playing an important role in cell homeostasis.

pathogens and we discuss its potential role in their virulence. Due to its emphasis on genome analysis, this review does not aim to cover detailed biochemical properties of the Na^+-dependent systems, which have been extensively reviewed previously (48, 50). Properties of membrane transporters, including Na^+-dependent ones, have been reviewed by Saier and coworkers (156, 169–171); recently, an analysis of the distribution of various transporters in the first 18 sequenced microbial genomes has been published (155). An extensive review of the type III protein secretion systems in various bacterial pathogens (93) included brief characterizations of the pathogens involved, some of which are subjects of this review.

PRIMARY Na^+ PUMPS

Na^+-Transporting Dicarboxylate Decarboxylases

The first evidence of an Na^+ cycle in bacteria came from the discovery that decarboxylation of oxaloacetate in the anaerobic bacterium *Propionigenium modestum* was Na^+ dependent and was coupled to the extrusion of Na^+ ions from the cytoplasm into the medium (41). In this way, the cells were able to conserve part of the free energy released during the exergonic decarboxylation reaction

$$^{-}OOC\text{-}CH_2\text{-}CO\text{-}COO^{-} + H^{+} \rightarrow CO_2 + CH_3\text{-}CO\text{-}COO^{-},$$

$$\Delta G^{o'} \cong -20\ kJ/mol$$

in the form of a transmembrane gradient of Na^+ ions (50). Further studies of oxaloacetate decarboxylase and similar biotin-dependent membrane-bound decarboxylases have shown that active export of Na^+ ions can also be driven by decarboxylation of malonate, methylmalonyl coenzyme A (methylmalonyl-CoA), and glutaconyl-CoA. These energy-conserving "dicarboxylate decarboxylases," functioning as primary Na^+ pumps, have been found in a number of bacteria that grow anaerobically on saturated dicarboxylic acids, such as *Klebsiella aerogenes, Veillonella alcalescens, Propionigenium modestum, Malonomonas rubra, Salmonella enterica* serovar Typhimurium, and *Acidaminococcus fermentans* (18, 41; reviewed in references 43, 44, 47, and 48). Na^+ gradients, generated by these enzymes, could be used for ATP synthesis and active transport (42, 160). Na^+ gradient-driven ATP synthesis, referred to as decarboxylation phosphorylation, is the only ATP-generating mechanism in *P. modestum* and *M. rubra* (49, 85). Genetic and enzymological analysis showed that the Na^+-transporting oxaloacetate decarboxylase enzyme consists of just three subunits, alpha, beta, and gamma, encoded in the *oadGAB* operon (51, 120). Malonate, methylmalonyl-CoA, and glutaconyl-CoA decarboxylases have a more complex organization but also contain alpha and beta subunits, homologous to the alpha and beta subunits, respectively, of oxaloacetate decarboxylase (12, 17, 18, 91, 92). Inspection of complete microbial genomes finds conserved *oadAB* operons (or, in some cases, separate *oadA* and *oadB* genes) in a number of phylogenetically distant (Fig. 2) bacteria, from the anaerobic hyperthermophile *Thermotoga maritima* to such human patho-

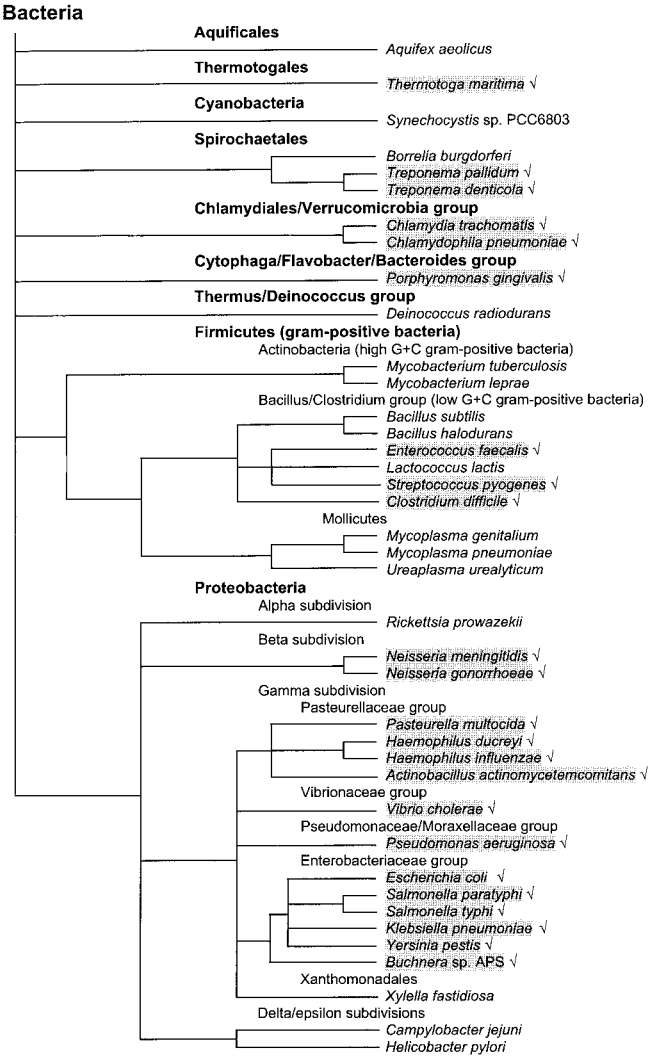

FIG. 2. Phylogenetic distribution of the bacterial pathogens that use the Na^+ cycle. The dendrogram shows the taxonomic positions of the organisms with completely sequenced genomes and several pathogens discussed in the text, according to the NCBI Taxonomy database (http://www.ncbi/nlm.nih.gov/Taxonomy) (218). The branches indicate taxonomic relations only; their lengths do not necessarily reflect evolutionary distances. The main bacterial phyla are shown in boldface. Bacterial species that appear to utilize the Na^+ cycle are shaded.

gens as *Salmonella enterica* serovar Typhi, and *Treponema pallidum* (Table 1). One could argue whether these data should be interpreted as evidence either of the ancient origin of this enzyme or of its propensity to be horizontally transferred among different bacterial phyla. The latter possibility seems quite plausible, since acquisition of the *oadGAB* operon would provide the bacterium with the ability to generate membrane potential at the expense of a fairly simple chemical reaction, which should be of selective advantage under some conditions. In any case, Na^+ gradient generation by decarboxylase-coupled ion transfer appears to occur in a limited number of (mostly) anaerobic bacteria, making it an exception rather than a rule in microbial world.

Na^+-Transporting NADH Dehydrogenase

Shortly after the discovery of the Na^+-transporting oxaloacetate decarboxylase, a respiratory Na^+ pump, the Na^+-translocating NADH:ubiquinone oxidoreductase (NQR), was reported in a marine bacterium, *Vibrio alginolyticus* (202, 204). Similar Na^+-transporting respiratory pumps have since been found in other *Vibrio* spp., *Alcaligenes* spp., *Bacillus* spp., and even *Escherichia coli* (8, 110, 206). In contrast to dicarboxylate decarboxylases, which appear to function mostly in anaerobes, NQR is the dedicated Na^+ pump in aerobic bacteria (see references 44 and 48 for reviews). After the genes encoding the *V. alginolyticus* pump were cloned and sequenced (10, 79, 80), homologous *nqrABCDEF* operons were found in *Haemophilus influenzae, Vibrio cholerae*, and *Vibrio harveyi* (77, 82, 228). The availability of complete microbial genome sequences allowed the identification of homologous genes encoding the NQR in a wide variety of bacteria, from *E. coli* to *Chlamydia trachomatis* (190, 192, 228) (Table 1). Remarkably, this enzyme is encoded even in the genome of the aphid symbiont *Buchnera* sp. strain APS, the second smallest of all known bacterial genomes (179). Our analysis of unfinished genome sequences, available through the web sites of the Sanger Centre (http://www.sanger.ac.uk). The Institute for Genomic Research (TIGR) (http://www.tigr.org), and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST), showed that the *nqr* operon is widely distributed in bacteria, including such important pathogens as *Neisseria gonorrhoeae, Pasteurella multocida, Porphyromonas gingivalis*, and *Yersinia pestis* (Table 1). The deduced protein products encoded by these operons display a significant degree of sequence conservation (typically, more than 25% identity). Among the sequenced genomes, there is no deviation from the *V. alginolyticus* gene order in *V. cholerae, H. influenzae, Neisseria meningitidis*, and *Pseudomonas aeruginosa* (Table 1). In *Thermotoga maritima*, the gene order is the same, but the *nqrF* gene, encoding the beta subunit of the enzyme, is replaced by a gene encoding a different Fe-S center-containing protein (referred to as *nqrG* in Table 1). In *E. coli* and *Buchnera* sp., *nqrE* and *nqrG* precede the other four genes instead of following them. This gene order is the same as in the previously described *mfABCDEF* (*Rhodobacter* nitrogen fixation) operon from *Rhodobacter capsulatus* (173). Indeed, sequence comparisons show that the *mfA* gene is homologous to *nqrE*, the *mfCDEF* genes are homologous, to *nqrABCD*, and *mfB* corresponds to *nqrG*. This observation shows that the terminal acceptor of electrons from quinone reduction, catalyzed by NQR, does not necessarily need to be oxygen; it can be nitrate or even 2,4-dinitrophenol (168). In addition, NQR can catalyze reverse electron transport from quinol to NAD^+ (see below). This would explain the presence of the NQR in *Thermotoga maritima*, an obligate anaerobe (141). Similar *nqrEGABCD* operons (in addition to *nqrABCDEF* operons) are present in the genomes of *V. cholerae, H. influenzae*, and *P. aeruginosa* (Table 1); the reason why these organisms should have two copies of the *nqr* genes is unclear. Finally, in *Chlamydia trachomatis* and *C. pneumoniae*, the *nqrA* and *nqrF* genes are located separately from the *nqrBCDE* operon (Table 1).

Na^+-Transporting ATPases

There appear to be two major classes of ATP-dependent primary Na^+ pumps that are capable of generating Na^+ gradients at the expense of ATP hydrolysis. One of them is an ABC (ATP binding cassette)-type transporter, NatAB, recently characterized in *Bacillus subtilis* (26). Similar Na^+-transporting ATPases are encoded in *B. firmus* (215) and in the genomes of *Deinococcus radiodurans, Thermotoga maritima, Clostridium difficile*, and *Legionella pneumophila*. However, the primary (if not the only) function of this Na^+ pump appears to be in prevention of Na^+ toxicity, that is, accumulation of Na^+ in the cytoplasm at the levels that would impair the normal cell functions (26, 215). Short of that, ATP expenditure for Na^+ export would be just too costly for cellular metabolism. Indeed, since the intracellular concentration of H^+ ions is approximately 10^6-fold lower than the concentration of Na^+ ions (10^{-7} to 10^{-8} M versus 10^{-1} to 10^{-2} M), it takes many fewer ATP molecules to create a 10^3-fold gradient of H^+ ions than of Na^+ ions, even taking into account the buffering capacity of the cytoplasm (182). As a result, for ATP-dependent uptake of nutrients, bacterial cells use ABC-type transporters rather than mediating it by ATP-dependent generation of the Na^+ gradient (155).

Na^+-transporting ATPases of the second class are simply F_0F_1-type and archaeal/vacuolar-type (V-type) ATP synthetases working in the reverse direction. Surprisingly, Na^+-transporting F_0F_1-type ATP synthetases are remarkably similar to the H^+-transporting ones (48). In fact, the cation specificity of an F_0F_1-type ATP synthetase can be switched just by several amino acid changes in the a or c subunits of its membrane component (101, 227). The same appears to be true for V-type ATPases that are also found in Na^+-transporting and H^+-transporting variants (89). It is clear that these enzymes are capable of Na^+ extrusion (89, 102, 103, 142). However, due to the huge energy costs of this process (see above), it would seem unlikely to be their function under natural conditions. Indeed, expression of the Na^+-transporting V-type ATPase of *Enterococcus hirae* is induced only by high pH or by high levels of intracellular Na^+ (94, 136). It therefore appears that bacterial cells spend ATP on Na^+ excretion only under extreme conditions, when it is necessary for their survival.

Recently, a P-type Na^+-transporting ATPase has been found in the facultatively anaerobic alkalophilic gram-positive bacterium *Exiguobacterium aurantiacum* (207). Previously, P-type ATPases in bacteria were not known to transport Na^+, as opposed to the eukaryotic Na^+/K^+ ATPase. If true, this would be an interesting example of the diversity of Na^+-transporting

TABLE 1. Distribution of primary Na^+ pumps in bacteria

Organism[a]	NQR		Oxaloacetate and malonate decarboxylases[d]		Sequencing center or reference[e]
	Gene order[b]	Gene name[c]	*oadGAB* gene name	*mdcABCDE* gene name	
Complete genomes					
Escherichia coli	*nqrEGABCD*	*ydgLMNOPQ*	—	—	13
Haemophilus influenzae	*nqrABCDEF nqrEGABCD*	HI0164–HI0171, HI1683–HI1688	—	—	59
Neisseria meningitidis	*nqrABCDEF*	NMB0569–NMB0564	—	—	153, 199
Treponema pallidum	—	—	TP0055–TP0057	—	217
Chlamydia trachomatis	*nqrBCDE, NqrA, NqrF*	CT278–CT281, CT634, CT0740	—	—	190
Chlamydia pneumoniae	*nqrBCDE, nqrA, NqrF*	CP*n*0427–CPn0430 CP*n*0743, CPn0883	—	—	104
Thermotoga maritima	*nqrABCDEG*	TM0244–TM0249	TM0128, TM0880	—	141
Vibrio cholerae	*nqrABCDEF nqrEGABCD*	VC2295–VC2290 VC1017–VC1012	VC0549–VC0551 VC0794–VC0792	—	83
Pseudomonas aeruginosa	*nqrABCDEF nqrEGABCD*	PA2999–PA2994 PA3489–PA3494	—	PA0208–PA0212	194
Pasteurella multocida	*nqrABCDEF nqrEGABCD*	PM1328–PM1333 PM0387–PM0382	PM1421–PM1423	—	131
Buchnera sp. strain APS	*nqrEGABCD*	BU113–BU118	—	—	179
Unfinished genomes					
Actinobacillus actinomycetemcomitans	*nqrABCDEF*	ND	*oadGAB*	ND	Oklahoma University
Clostridium difficile	*nqrABCDEG*	ND	ND	ND	Sanger Centre
Enterococcus faecalis	ND	ND	*oadA, oadB*	ND	TIGR
Haemophilus ducreyi	*nqrABCDEF*	ND	*oadGAB*	ND	University of Washington
Klebsiella pneumoniae	*nqrEGABCD*	ND	*oadGAB*	*mdcABCDE*	Washington University (120, 176)
Neisseria gonorrhoeae	*nqrABCDEF*	ND	ND	ND	Oklahoma University
Porphyromonas gingivalis	*nqrABCDEF*	ND	*oadA, oadB*	ND	TIGR, Forsyth Institute
Salmonella enterica serovar Paratyphi	*nqrEGABCD*	ND	*oadGAB*	ND	Washington University (221)
Salmonella enterica serovar Typhi	*nqrEGABCD*	ND	*oadGAB*	ND	Sanger Centre
Streptococcus pyogenes	ND	ND	*oadA, oadB*	ND	Oklahoma University Sanger Centre
Treponema denticola	*nqrABCDE*	ND	ND	ND	TIGR
Yersinia pestis	*nqrEGABCD*	ND	ND	ND	Sanger Centre

[a] Only pathogenic bacteria that encode a primary Na^+ pump (NQR or a dicarboxylate decarboxylase) are listed. Other bacteria with completely sequenced genomes, such as *Aquifex aeolicus, Bacillus subtilis, B. halodurans, Campylobacter jejuni, Deinococcus radiodurans, Helicobacter pylori, Lactococcus lactis, Mycobacterium tuberculosis, Mycobacterium leprae, Mycoplasma genitalium, Mycoplasma pneumoniae, Rickettsia prowazekii, Synechocystis* sp. strain PCC6803, *Ureaplasma urealyticum*, and *Xylella fastidiosa* do not appear to encode either of these two primary Na^+ pumps and are therefore presumed devoid of a functional Na^+ cycle, although most of them encode Na^+/H^+ antiporters and probably Na^+-dependent transporters. *Borrelia burgdorferi* encodes a homolog of NqrA and NqrB (BB0072) but does not encode other NQR subunits. The list of bacteria with unfinished genomes includes only organisms where a primary Na^+ pump could be unequivocally identified from the available sequence data. Both lists are expected to expand as new genomic sequences become available.

[b] Gene and operon assignments were made on the basis of BLAST searches (1) against finished and unfinished microbial genome databases at NCBI (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html) and TIGR (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?) using the sequences of protein products of the *nqrABCDEF* operon from *V. alginolyticus* (138), *oadGAB* operon from *K. pneumoniae* (120, 176), and *mdcABCDE* operon from *K. pneumoniae* (88), respectively, as queries. Gene assignments for the complete genomes were also compared against the COG database (197). Two *nqr* operons were detected in *H. influenzae, V. cholerae, P. aeruginosa*, and *P. multocida*, and two *oadGAB* operons were found in *V. cholerae*; the VC0793 gene is apparently disrupted by a frameshift mutation (83). The dash and ND (not detected) indicate the absence of the corresponding gene(s) in complete and unfinished genomes, respectively.

[c] The gene names are those from the complete genome sequences; they can be used to retrieve corresponding DNA sequences from GenBank or the deduced proteins from the NCBI protein database.

[d] Due to the high level of sequence similarity between the corresponding subunits of oxaloacetate, malonate, methylmalonyl-CoA, and glutaconyl-CoA decarboxylases (12, 91, 120, 221), gene assignments were made based solely on the operon structure; the exact substrate specificity of each of these enzymes remains to be determined.

[e] The WWW sites of the unfinished genome sequencing projects, listed in the table, are as follows: *A. actinomycetemcomitans, N. gonorrhoeae*, and *S. pyogenes*, http://www.genome.ou.edu; *C. difficile, S. enterica* serovar Typhi, *S. pyogenes*, and *Y. pestis*, http://www.sanger.ac.uk/Projects/Microbes; *E. faecalis* and *T. denticola*, http://www.tigr.org/tdb/mdb/mdb.html; *H. ducreyi*, http://www.htsc.washington.edu/hducreyi/info/index.cfm; *K. pneumoniae* and *S. enterica* serovar Paratyphi, http://genome.wustl.edu/gsc/Projects/bacteria.shtml; and *P. gingivalis*, http://www.pgingivalis.org. See http://www.niaid.nih.gov/factsheets/seqmicrobes.htm for more details.

ATPases in bacteria. A mutation in the P-type K^+-transporting ATPase KdpFABC of *E. coli* has recently been shown to result in low-level Na^+ transport (174).

Na^+-Transporting Terminal Oxidases

There have been reports that the cytochrome *d* terminal oxidase of *E. coli* is not an H^+ pump (162) but a Na^+ pump (6, 8, 15, 135). An Na^+-transporting terminal oxidase has also been found in a representative of the genus *Bacillus* (8, 115, 178), later identified as *Bacillus halodurans* (71). Those reports still remain unconfirmed, even though cytochrome *d*-type terminal oxidases are encoded in genomes of many organisms, including such Na^+ cycle-dependent ones as *E. coli, P. aeruginosa, V. cholerae, H. influenzae, C. trachomatis*, and *C. pneumoniae* (Table 2). On the other hand, very similar cytochrome *d*-type oxidases are encoded in the genomes of *B. subtilis, Synechocystis* spp., *Campylobacter jejuni*, and *Rickettsia prowazekii*, which do not seem to encode any (other) Na^+ pumps (Table 1) or require Na^+ for growth. It is possible also that cytochrome *d*-type oxidases do not pump either Na^+ or H^+ and charge the membrane solely by consuming H^+ ions from the cytoplasm to produce H_2O (162). Due to this uncertainty, we do not count cytochrome *d*-type enzymes as primary Na^+ pumps (Table 1) but, rather, tentatively consider them to be H^+ pumps (Table 2).

Because the cytochrome *bo*-type type terminal oxidase of *E. coli* has been directly demonstrated to be an H^+ pump (162, 163), similar enzymes in other bacteria are generally assumed to be specific to H^+ ions. However, in *Vitreoscilla*, a beta-subdivision proteobacterium that belongs to the *Neisseriaceae* family, cytochrome *o* has been repeatedly shown to function as a primary Na^+ pump (55, 56, 108, 152). Because the sequence of this Na^+-transporting cytochrome *o* is still not available, it is difficult to judge how unusual it is and whether other bacteria might also be able to utilize cytochrome *o* complexes as Na^+ pumps. It is remarkable that *Neisseria gonorrhoeae* and *N. meningitidis*, closely related to *Vitreoscilla* spp., do not encode cytochrome *o* complexes; instead, their terminal oxidases are of the cbb_3 type (Table 2).

Cytochrome *c* oxidases of the cbb_3- type are found primarily in microaerophiles, such as *Neisseria* spp., *Helicobacter pylori*, and *C. jejuni* (129, 187). This enzyme complex translocates H^+ ions across the membrane (33, 164, 205). The possibility that this complex could (also) pump Na^+ ions has not been investigated.

Na^+-Transporting Methyltransferase

Yet another type of primary Na^+ pump, found in methanogenic archaea, couples Na^+ export to methyl group transfer from tetrahydromethanopterin to CoM (see reference 35 for a recent review). No such enzyme has been reported in any bacteria.

UTILIZATION OF Na^+ GRADIENTS

Once the chemical energy is transformed into the electrochemical energy of the Na^+ gradient, it can be used to drive ATP synthesis, Na^+-dependent transports, and Na^+-dependent motility.

Na^+-Dependent ATP Synthesis

In contrast to ATP-dependent Na^+ transport, which has been demonstrated in a number of organisms (40, 118), Na^+-dependent ATP synthesis requires a relatively large Na^+ gradient and probably occurs only in a few bacteria. Such a reaction was first reported in *Propionigenium modestum*, one of the few organisms that appear to be exclusively dependent on the Na^+ cycle (46, 85). Shortly thereafter, ATP synthesis in response to an artificially imposed Na^+ gradient was demonstrated in *V. alginolyticus* and *E. coli* (7, 39). In each of these bacteria, Na^+-dependent ATP synthesis was catalyzed by a typical F_0F_1-type ATPase originally thought to be an exclusively H^+-transporting enzyme. It transpired that the cation specificity of this enzyme was not absolute and could be changed by mutations (100, 119, 227). As a result, there is currently no clear way to predict the cation specificity of a given F_0F_1-type ATPase from its sequence, even taking into account the latest data identifying some potentially important residues (53, 100, 101). One could safely assume, though, that under conditions of low proton motive force and high sodium motive force a normally H^+-transporting F_0F_1-type ATPase may function as an Na^+-transporting ATP synthetase. Hence, the ability to generate an Na^+ gradient through any primary Na^+ pump could be considered an important trait, helping the organism to synthesize ATP and ultimately survive under certain unfavorable conditions.

An analysis of ATP synthesis in microorganisms that encode an archaeal/vacuolar type (V-type) ATPase is similarly unable to determine the selectivity of the enzyme toward Na^+ and H^+ ions. It is particularly interesting to compare the two spirochetes *Treponema pallidum* and *Borrelia burgdorferi*, the causative agents of syphilis and Lyme disease, respectively, which both have V-type ATPases encoded by similarly organized operons (66). While *T. pallidum* has a primary Na^+ pump of the oxaloacetate decarboxylase type (Tables 1 and 2) and conceivably could utilize an Na^+ gradient for ATP synthesis, *B. burgdorferi* does not encode any known Na^+ pumps and appears to rely solely on the H^+ cycle.

Na^+-Dependent Symports

Obligately parasitic bacteria generally have smaller genomes than free-living ones and may depend on their hosts for essential nutrients such as amino acids, nucleobases, and cofactors (vitamins) (66, 113, 114). These nutrients are transported into the cell at the expense of energy that comes in the form of either ATP (ABC-type transport systems) or proton (and/or sodium) motive force (secondary transport). The diversity of bacterial transport systems encoded in complete microbial genomes varies widely but generally correlates with the genome size (155, 156). It has long been known that Na^+ symports are the principal, and sometimes the only, form of secondary transporters in alkalophilic and thermophilic bacteria (84, 189). The presence of primary Na^+ pumps in many pathogens (Table 1) indicates that they, too, could use sodium motive force for solute uptake. Indeed, most of them encode Na^+-dependent symporters for alanine, proline, and several other amino acids (Table 2).

While substrate specificity of many permeases encoded in microbial genomes is not known, it can often be predicted, at

TABLE 2. Pathogenic bacteria that utilize the Na^+ cycle

Organism[a]	Disease(s) caused	Primary Na^+-pump types[a]	Primary H^+ pump types[b]	Na^+/H^+ antiporter types[c]	Na^+-dependent transports[c]	Comments
Treponema pallidum	Syphilis	DD	None	None	Ala, P_i	*T. pallidum* appears to rely exclusively on the Na^+ cycle, while *T. denticola* is more flexible
Treponema denticola	Necrotizing gingivitis	NQR	TH	NhaC	Ala, Glu, P_i, citrate	
Chlamydia trachomatis	Trachoma, vaginitis	NQR	CYD	NhaD	Ala	The presence of a single Na^+ pump and just one Na^+/H^+ antiporter suggests that Na^+ circulation is critical for chlamydial physiology
Chlamydia pneumoniae	Bronchitis, pneumonia	NQR	CYD	NhaD	Ala, Pro	
Porphyromonas gingivalis	Adult periodontitis	NQR, DD	TH, CYD	NhaA NhaP	Pro	Na^+ gradient may help *P. gingivalis* to survive pH swings and high Ca^{2+} levels
Enterococcus faecalis	Endocarditis; wound and urinary tract infections	DD	CYD	NhaC NhaP	Ser, citrate	*oadB* and *oadA* genes form an operon with *citCDEFG*, indicating a possibility of the Na^+/citrate cycle in both *E. faecalis* and *S. pyogenes*
Streptococcus pyogenes	Pharyngitis, rheumatic fever	DD	ND	ND	Ala, Ser, P_i, citrate	
Clostridium difficile	Pseudomembranous colitis	NQR	ND	NhaC	Ala, Glu, Pro, P_i	
Neisseria meningitidis	Meningitidis	NQR	NDH, TH, CBB	NhaC	Ala, Glu, Pro, Ser	Both species of *Neisseria* have versatile membrane energetics; the Na^+ cycle is unlikely to be crucial for their survival
Neisseria gonorrhoeae	Gonorrhea	NQR	NDH, TH, CBB	NhaC NhaP	Ala, Glu, Pro, Ser	
Pasteurella multocida	Fowl cholera in poultry	NQR, DD	TH, CYD	NhaA NhaB NhaC NhaP	Ala, Pro, drugs	
Haemophilus ducreyi	Chancroid	NQR, DD	TH, CYD	NhaA NhaB	Ala, Glu, Pro, Ser, drugs	
Haemophilus influenzae	Pneumonia, otitis media	NQR	TH, CYD	NhaA NhaB NhaC	Ala, Glu, Pro, Ser, drugs	
Actinobacillus actinomycetemcomitans	Juvenile periodontitis	NQR	TH, CYD	NhaB NhaC	Glu, Pro, drugs	
Vibrio cholerae	Cholera	NQR, DD	TH, CBB, CYD	NhaA NhaB NhaC NhaD NhaP Mnh	Ala, Glu, Pro, Ser, P_i, citrate, drugs	Na^+ gradient plays an important role in the motility and virulence of *V. cholerae*
Pseudomonas aeruginosa	Lung and skin infection	NQR, DD	NDH, CYO, CBB, TH, CYD	NhaB NhaP	Ala, Glu, Pro, Ser, P_i, drugs	
Escherichia coli	Enteritidis, urinary tract infections	NQR	NDH, CYO, TH, CYD	NhaA NhaB NhaP	Ala, Glu, Pro, Ser, P_i, drugs	*E. coli* might use the Na^+ cycle for survival at alkaline pH
Salmonella enterica serovar Paratyphi	Paratyphoid fever	NQR, DD	NDH, CYO, TH, CYD	NhaA NhaB NhaC NhaP	Ala, Glu, Pro, Ser, P_i, citrate, drugs	*K. pneumoniae, S. enterica* serovar Typhi and Paratyphi are extremely versatile pathogens; they might use the Na^+ cycle for energy buffering and for survival at high pH; they are all capable of Na^+-dependent citrate fermentation that uses NQR for NAD^+ reduction
Salmonella enterica serovar Typhi	Typhoid fever	NQR, DD	NDH, CYO, TH, CYD	NhaA NhaB NhaC NhaP	Ala, Glu, Pro, Ser, P_i, citrate, drugs	
Klebsiella pneumoniae	Pneumonia	NQR, DD	NDH, TH, CYD	NhaA NhaB NhaP	Ala, Glu, Pro, Ser, P_i, citrate, drugs	
Yersinia pestis	Plague	NQR	NDH, CYO, TH, CYD	NhaA NhaB NhaC NhaP	Glu, Pro, Ser, drugs	

[a] Only pathogenic bacteria that have a primary Na^+ pump of the NQR or DD (dicarboxylate decarboxylase) type (see Table 1) are listed. The list is ordered according to the position of the respective microorganism on the 16S rRNA-based phylogenetic tree (Fig. 2).

[b] NDH, H^+-translocating NADH:ubiquinone oxidoreductase; CYO, cytochrome *o*-type terminal oxidase; CBB, cbb_3-type terminal oxidase; TH, pyrimidine nucleotide transhydrogenase; CYD, cytochrome *bd*-type terminal oxidase (see the text for a discussion of the cation specificity of cytochrome *o*-type and cytochrome *d*-type terminal oxidases). The presence of each type of terminal oxidase has been determined on the basis of TBLASTN (1) searches against the databases of complete and unfinished genomes, maintained at NCBI and TIGR, using the following *E. coli* proteins as queries: NDH, NuoA, NuoB, and NuoCD (216); CYO, CyoA, CyoB, and CyoC (27); TH, PntA and PntB (28); CYD, CydA and CydB (70). For CBB, the CcoN and CcoO proteins of *Rhodobacter capsulatus* (201) were used as queries. Functional assignments of the database hits obtained in these searches were checked by comparing each of them to the COG database, as described earlier (140, 197). ND, not detected (in the unfinished genome).

[c] The distribution of the Na^+/H^+ antiporters and Na^+ symporters has been established on the basis of TBLASTN (1) searches, using the following proteins as queries: NhaA, NHAA_ECOLI (106); NhaB, NHAB_ECOLI (158); NhaC, NHAC_BACFI (97); NhaD, gil3123728 (146); NhaP, gil3327262 (210); Mnh, MnhABCDEFG, GenBank accession number AB015981 (87); Ala, ALCP_BACP3 (105); Glu, GLTS_ECOLI (34); Pro, PUTP_ECOLI (137); P_i, NPT2_HUMAN (127); Ser, P42602 (147); citrate, CitS of *Klebsiella pneumoniae* (211); drugs, NorM of *Vibrio parahaemolyticus* (134). Functional assignments of the database hits obtained in these searches were checked by comparing each of them to the COG database, as described previously (140, 197). The list of (predicted) Na^+-dependent transporters is not meant to be complete; other Na^+-dependent transporters are likely to be encoded in the genomes of these bacteria.

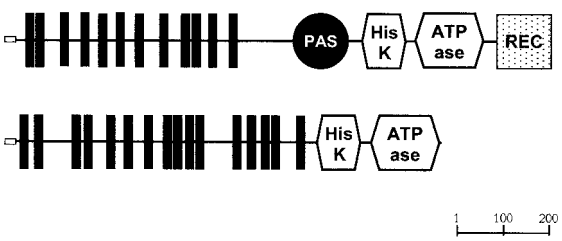

FIG. 3. Domain organization of a new type of sensor histidine kinases. SMART (175) diagrams of the common domain structure *V. cholerae* protein VC0303 and *P. aeruginosa* proteins PA3271 and PA4725 (A) and *R. prowazekii* protein RP465 (B). (A) The small open box on the left indicates the likely signal peptide, predicted by the SignalP program (144). The vertical boxes indicate 12 transmembrane helices of the solute/sodium symporter family (TC 2.A.21) transporter, predicted by the TopPred program (29). The circle indicates the PAS domain (198); the two hexagons indicate the phosphoacceptor and ATPase domains, respectively, of the histidine kinase; and the dotted square on the right indicates the CheY-type receiver domain (193). (B) *R. prowazekii* protein RP465 contains 16 predicted transmembrane helices and both domains of a histidine kinase but lacks PAS and CheY-type domains. The ruler indicates the length of the protein in amino acid residues.

least in general terms, using the protein family assignment based on the recently developed transporter classification (TC) (see reference 170 for a review). Certain transporter families include both Na^+-dependent and H^+-dependent members. For example, the branched-chain amino acid/cation symporter family (LIVCS; TC 2.A.26) includes both an Na^+-dependent transporter, BraB, from *Pseudomonas aeruginosa* and an H^+-dependent transporter, BrnQ, from *Lactobacillus delbruckii* (90, 195). However, analysis of permeases that belong to conserved families of transporters shows that in most cases, the cation specificity stays the same throughout the family (166). Reizer et al. have identified 11 conserved protein families of (mostly) Na^+/solute symporters that comprised the sodium solute symporter superfamily (166). So, although the exact substrate specificity of most permeases encoded in microbial genomes is still obscure, the pathogens that utilize the Na^+ cycle (Table 2) seem to encode a significant share of permeases that belong to Na^+-dependent transporter families (155).

One such conserved family, the solute/sodium symporter family (SSS; TC 2.A.21) unifies the experimentally characterized Na^+/proline and Na^+/pantothenate permeases PutP and PanF from *E. coli* (165) with the Na^+/glucose symporter SglT from *V. parahaemolyticus* (170). Homologous transporters are encoded in the genomes of *P. gingivalis, C. pneumoniae, C. difficile, N. meningitidis, N. gonorrhoeae, P. multocida, H. influenzae, H. ducreyi, A. actinomycetemcomitans, K. pneumoniae, P. aeruginosa, S. enterica* serovars Typhi and Paratyphi, *V. cholerae*, and *Y. pestis* (Table 2). All these proteins are likely to function as Na^+-dependent symporters, although some of them contain additional domains similar to sensory kinase components of signal transduction systems and may be involved in signal transduction. For instance, in *Rickettsia prowazekii* protein RP465, *V. cholerae* protein VC0303, and *P. aeruginosa* proteins PA3271 and PA4725, putative members of the solute/sodium symporter family are fused to PhoR-like sensory kinase domains (Fig. 3). The signal transduced by these proteins, if any, has not been identified.

Another conserved family of Na^+-dependent transporters, identified by Reizer et al. (166), includes experimentally characterized alanine and glycine transporters from the marine bacterium *Alteromonas haloplanktis* and the thermophilic bacterium PS3 (105). Proteins belonging to this family (AGCS; TC 2.A.25) and probably involved in the uptake of alanine and/or glycine are found in many bacteria, including most human pathogens (Table 2).

Glutamate, aspartate, serine, and threonine also can be taken up by bacterial cells by an Na^+ symport mechanism. The Na^+/glutamate symporter GltS from *E. coli* remains the only characterized member of the glutamate/sodium symporter family (ESS; TC 2.A.27). Members of this family in other bacteria can be assumed to have the same narrow substrate specificity (Table 2). The Na^+/serine-threonine symporter, SstT, from *E. coli* belongs to the diverse family of transporters (DAACS; TC 2.A.23) that also includes Na^+- and H^+-dependent symporters for glutamate and dicarboxylic intermediates of the Krebs cycle. Members of this family are widely represented in bacterial genomes (156). Unfortunately, their exact substrate and cation specificity is still difficult to establish based solely on sequence comparisons.

Members of the citrate/cation symporter family (CCS; TC 2.A.24) are involved in Na^+-dependent uptake of such 2-hydroxycarboxylates as citrate, malate, and lactate (211, 212). Some of these permeases reportedly can also transport citrate or malate in symport with H^+ ions (130). Actually, the true substrate of the Na^+/citrate symporter CitS, best studied in *Klebsiella pneumoniae*, appears to be the protonated (divalent) form of citrate, transported in symport with two Na^+ ions. Thus, technically, CitS is an $H^+/2Na^+$/citrate symporter (52, 211). In *K. pneumoniae, S. enterica* serovar Typhimurium, and several other bacteria, CitS catalyzes the first stage of anaerobic citrate fermentation pathway. In this remarkable Na^+-dependent pathway, citrate is first transported into the cell at the expense of the Na^+ gradient (Fig. 4) and then split into acetate and oxaloacetate by citrate lyase (16). Decarboxylation of oxaloacetate into pyruvate by the Na^+-transporting oxaloacetate decarboxylase restores the Na^+ gradient and produces pyruvate, which is further metabolized into acetate with acetyl-CoA and acetyl phosphate as intermediates (16). The last stage of this pathway, catalyzed by acetate kinase, yields ATP and thus results in energy conservation. Based on the presence of genes encoding both CitS-type carrier and oxaloacetate decarboxylase (Table 2), such a pathway can be assumed to function in *Treponema denticola, V. cholerae, S. enterica* serovars Typhi and Paratyphi, and, possibly, *Streptococcus pyogenes*. Not surprisingly, in *K. pneumoniae, V. cholerae*, and *S. enterica* serovar Typhi, the genes for the Na^+/citrate symporter and oxaloacetate decarboxylase are located within a single operon (16) (Table 1).

Na^+-Dependent Drug Efflux

Drug resistance of human pathogens is a growing problem, threatening to negate the success of the antibacterial efforts of the last 50 years and make humans once again vulnerable to a siew of infectious diseases. Comparative genomics is being widely used for identification of potential drug targets (2, 65, 172). The recurring problem in drug design, however, is the presence of multidrug efflux pumps that excrete a wide variety

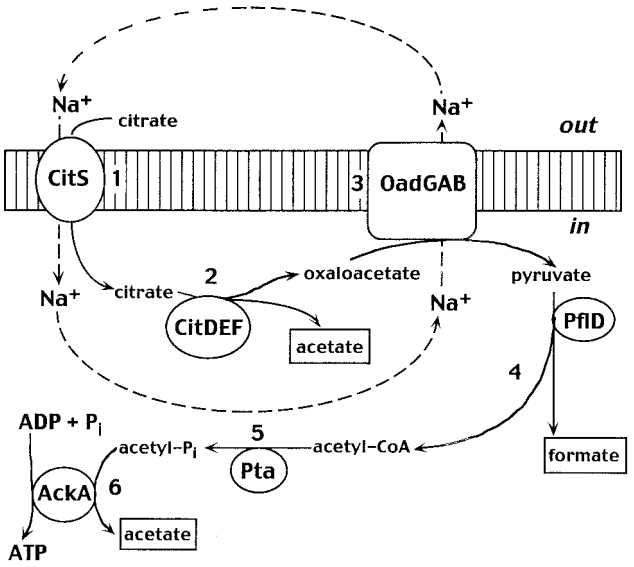

FIG. 4. Scheme of the Na^+-dependent citrate fermentation pathway. Citrate is transported into the cell in symport with Na^+ ions by CitS (step 1) and split into acetate and oxaloacetate by citrate lyase CitDEF (step 2). Decarboxylation of oxaloacetate into pyruvate by Na^+-transporting oxaloacetate decarboxylase, OadGAB (step 3), restores the Na^+ gradient and produces pyruvate. Pyruvate-formate lyase PflD splits pyruvate into formate and acetyl-CoA (step 4), which is further converted into acetylphosphate by phosphotransacetylase Pta (step 5). Dephosphorylation of acetylphosphate by acetate kinase AckA (step 6) yields ATP, resulting in energy conservation. The enzymes are indicated by their standard gene names, where available. The fermentation end products are boxed. See reference 16 for more details.

of compounds, decreasing their cellular concentrations below the required MICs (122, 123, 154). Indeed, preventing drug efflux significantly increases the efficacy of even standard antibiotics, such as streptomycin and tetracycline (121, 191). Most of the known multidrug efflux pumps belong to one of three groups of secondary transporters, typically energized by the protonmotive force: the major facilitator superfamily (MFS; TC 2.A.1), the small multidrug resistance family (SMR; TC 2.A.7.1), and the resistance/nodulation/cell division family (RND; TC 2.A.6) (154). Several other groups of multidrug transporters belong to the ATP-dependent ABC superfamily (ABC; TC 3.A.1). Recently, a multidrug transporter, NorM, from *V. parahaemolyticus* that caused Na^+-dependent efflux of norfloxacin has been described (133, 134). NorM turned out to be a representative of a new large family of multidrug efflux pumps (20, 134), termed the multiantimicrobial extrusion family (MATE; TC 2.A.66). Members of this family are encoded in almost every sequenced genome, including nearly identical NorM proteins in *V. cholerae, E. coli, H. influenzae*, and *P. aeruginosa*. Close homologs of NorM are also found in *S. enterica* serovars Typhi and Paratyphi, *K. pneumoniae, A. actinomycetemcomitans, P. multocida, H. ducreyi*, and *Y. pestis* (Table 2). While it is still too early to speculate, which members of this family of transporters are Na^+ dependent and which, if any, are H^+ dependent, there is little doubt that the sodium motive force can serve as an energy source for drug efflux pumps in a number of bacterial pathogens.

Reverse Electron Transport

The presence of *nqr* genes in the genomes of anaerobic microorganisms, including the obligately anaerobic hyperthermophile *Thermotoga maritima* (Table 1), indicates that NQR can also work in the reverse direction, using the energy of the Na^+ gradient to reduce NAD^+ to NADH. Indeed, Na^+-dependent NAD^+ reduction by formate has been experimentally demonstrated in *K. pneumoniae* grown anaerobically on citrate (157). This reaction was sensitive to the quinone analog 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), a well-characterized inhibitor of NQR. For *K. pneumoniae*, Na^+-dependent reverse electron transport could help to produce reducing equivalents, needed for assimilation of the formate formed in the anaerobic citrate fermentation pathway (see above) (Fig. 4).

Na^+-Dependent Motility

Motility is widely believed to be an important virulence factor in bacterial pathogens, since it allows the bacterium to penetrate different host tissues and/or helps it to attach to the surface of epithelial cells (86, 107, 132, 222). Indeed, loss of motility has been reported to correlate with significant decrease of virulence in host-parasite models for *H. pylori, V. cholerae, Y. enterocolitica*, and other bacteria (72, 98, 150, 225).

Many bacterial pathogens that depend on an Na^+ cycle (Table 2) are motile. Their genomes contain the full set of ca. 30 genes that are required for the formation of a functional flagellum (126). For several of them, motility has been directly shown to be Na^+ dependent (5, 14, 78, 111). In *V. parahaemolyticus* and *V. alginolyticus*, lateral and polar flagella are reportedly powered by proton motive force and sodium motive force, respectively (5, 14, 111). Some components of the Na^+-dependent motor can be functionally replaced by homologous components of the H^+-dependent motor (3, 68). Uncovering the details of the organization of the Na^+-dependent motors of *V. cholerae* and related bacteria is quite important, because it could clarify the contribution of motility to their pathogenicity.

Is the Na^+ Gradient Involved in Toxin Export?

The contribution of the flagellar genes to virulence is not limited to the colonization stage of infection. The flagellar export apparatus, which is responsible for the secretion and assembly of a functional organelle, participates in the secretion of a variety of virulence factors, including certain bacterial toxins (reviewed in references 30, 62, and 93). This toxin export apparatus is referred to as a type III protein secretion system and participates, for example, in secretion of extracellular proteins by *Y. enterocolitica*, including the virulence-associated phospholipase YplA (226). Because the flagellar export system traverses the cytoplasmic membrane, the peptidoglycan layer, and the outer membrane, proteins exported via this system can be delivered directly to the exterior and may even penetrate the cytoplasm of the host cell (93). The energy for this complex process is apparently supplied in the form of ATP and is used by the ATPase FliI, which is homologous to the catalytic beta subunit of the F_0F_1-type H^+-ATPase (58, 93). Genome comparisons show that genes encoding type III protein secretion systems have a wider phylogenetic distribution than the rest of the flagellar genes; they can be found even in such nonmotile

organisms as *C. trachomatis* and *C. pneumoniae* (143, 190). It is important to note here that in a motile cell, a significant part of the flagellar apparatus ("rotor") rotates together with the flagellar filament relative to the remainder of the flagellar machinery ("stator") and the rest of the cell, providing torque that propels the bacterium (11, 126). Remarkably, homologs of the FliF protein that forms the two membrane rings (M and S) of the rotor, i.e., the flagellar motor switch protein FliG, located at the interface between the rotor and the stator, and the FliN protein, which forms the inner (cytoplasmic) ring of the rotor (149, 200, 208), appear to be involved in the functioning of the export machinery in several bacterial pathogens (93). In *Y. enterocolitica* and *Y. pestis*, these homologs of FliF, FliG, and FliN, referred to as YscJ, YscD, and YscQ, respectively (SctJ, SctD, and SctQ according to the new nomenclature suggested by Hueck), are encoded on large virulence plasmids that encode both components of the export machinery and secreted virulence proteins. These observations suggest that SctD, SctQ, and particularly SctJ might be able to rotate in the membrane. Unfortunately, the relation, if any, between the proper functioning of the flagellar export machinery and its rotation remains unknown. Early work on the role of proton motive force in the elongation of the flagellar filament suggested that flagellar rotation might be needed for flagellin export in *E. coli* (63). It is tempting to speculate that Na^+-dependent rotation of the basal body might be related to the secretion of virulence proteins, either promoting or impeding it. This idea, however, is far from having any experimental support.

INTERPLAY OF Na^+ AND H^+ CYCLES IN BACTERIAL PATHOGENS

The presence of genes encoding primary Na^+ pumps in a number of important human pathogens (Table 1) indicates that these bacteria rely on the Na^+ cycle for at least part of their energy metabolism. However, in addition to a primary Na^+ pump, it appears that most of them encode primary H^+ pumps (Table 2). Although one cannot be sure that every primary Na^+ pump and H^+ pump has been accounted for, we can judge whether a particular microorganism uses the Na^+ cycle based on the presence of any of the two proven primary Na^+ pumps, NQR and dicarboxylate decarboxylases. In any case, most genomes encode multiple Na^+/H^+ antiporters (Table 2), which should allow the generation of an H^+ gradient at the expense of an Na^+ gradient and vice versa (see Fig. 1). This (at least partial) interchangeability of proton motive force and sodium motive force is a striking feature of the bioenergetics of nearly all bacteria studied to date. In the following section we consider the possible role(s) of Na^+ cycle in the energy metabolism of several potentially Na^+-dependent bacterial pathogens in more detail.

Treponema pallidum and *T. denticola*

T. pallidum, the causative agent of syphilis, has remained quite an enigmatic organism even after its genome was completely sequenced (61, 217). It still cannot be continuously cultivated in vitro, and a syphilis vaccine remains elusive (181). An analysis of the genome sequence of *T. pallidum* showed that this organism encodes a very limited number of biosynthetic pathways for amino acids, nucleotides, and cofactors, which partly explains its complex growth requirements (61). A recent analysis of the protein set of *T. pallidum* revealed a number of organism-specific gene products whose exact biological role remains unclear (196, 217). An analysis of the energy metabolism of *T. pallidum* (Table 2) reveals a stunning picture. This organism does not appear to encode any primary H^+ pumps or Na^+/H^+ antiporters, and oxalate decarboxylase seems to be the only ionic pump encoded in its genome. The apparent absence of respiratory ionic pumps is quite unexpected, since *T. pallidum* is a microaerophile rather than an obligate anaerobe and should be routinely attacked by superoxide radicals generated by the host defense systems; it even has a dedicated superoxide reductase (125, 217). It seems likely that "decarboxylation phosphorylation," i.e., ATP synthesis at the expense of the Na^+ gradient generated by oxalate decarboxylation (48, 50), serves as a major energy source for *T. pallidum*. The only peculiarity of this process in *T. pallidum* is that its ATP synthetase is of the archaeal/vacuolar type, very similar to the Na^+-transporting V-type ATPase of *Enterococcus hirae* (66). As was noted above, solute uptake, energized by ion gradients that have been generated at the expense of ATP, is energetically costly. Indeed, *T. pallidum* mostly relies on ABC-type transporters for solute uptake (155). The apparent absence of Na^+/H^+ antiporters suggests that the *T. pallidum* cell must tightly balance its H^+ ion fluxes, i.e., the proton motive force-dependent solute uptake with proton motive force-generating efflux of fermentation products. Such a mechanism of proton motive force generation has been previously demonstrated in *Streptococcus cremoris* (112). Another possibility, of course, is that *T. pallidum* exclusively uses Na^+ as a coupling ion. A survey of the secondary transporters encoded in the *T. pallidum* genome (155) appears to support this possibility. Most of them either are Na^+ symporters or belong to the families of transporters that can be powered by either Na^+ or H^+ gradients. The former group includes predicted the Na^+/alanine symporters TP0414 and TP0998, the Na^+/branched-chain amino acid symporter TP0265, the Na^+/phosphate symporter TP0771, and the Ca^{2+}/Na^+ antiporter TP1034 (155, 197), (Table 2). Most other *T. pallidum* permeases have unknown specificity and can be characterized only using transporter protein family assignment (170). This group includes TP0023, a member of the neurotransmitter/sodium symporter family (TC 2.A.22); TP0106, a member of the betaine/carnitine/choline transporter family (TC 2.A.15); TP0555 and TP0934, members of the dicarboxylate-amino acid/cation symporter family (TC 2.A.23); and TP0901, a member of the multiantimicrobial extrusion family (TC 2.A.66). While the exact substrate and coupling-ion specificities of the transporters in this second group are still obscure, some or all of them use Na^+ gradient as the energy source.

The genome of *T. denticola*, a close relative of *T. pallidum*, is currently being sequenced at TIGR with support from the National Institute of Dental and Craniofacial Research (see http://www.nidr.nih.gov for more details). The currently sequenced part of *T. denticola* genome encodes an Na^+ pump, but here it is NQR, not an oxaloacetate decarboxylase, as in *T. pallidum* (Table 2). This contrast between the two spirochetes could be due to a higher availability of oxygen in the oral cavity, which is the ecological niche of *T. denticola*. The absence of the NQR in *T. pallidum* then probably reflects the loss

of *nqr* genes in the course of its adaptation to its own ecological niche. Indeed, it still encodes distant homologs of the NqrA and NqrB subunits in TP0152 and TP0151. In addition to NQR, *T. denticola* encodes $NAD^+/NADP^+$ transhydrogenase, a proton motive force generating-enzyme, an Na^+/H^+ antiporter, and several Na^+- or H^+-dependent transporters (Table 2). It is possible, of course, that NQR in *T. denticola* is functioning in the reverse direction and is used for Na^+-dependent NAD^+ reduction, as discussed above. Nevertheless, the fact that both *Treponema* spp. retain primary Na^+ pumps supports the idea that they are dependent on the Na^+ cycle for at least part of their membrane energetics.

Chlamydia trachomatis and *C. pneumoniae*

Both *C. trachomatis* and *C. pneumoniae* are extremely important pathogens. In addition to being the causative agent of trachoma, an eye infection that may lead to blindness, *C. trachomatis* is one of the most common pathogens of human genital tract (190). *C. pneumoniae* is a common cause of infections of the respiratory tract and can be found in many other organs; it appears that virtually every human is infected with *C. pneumoniae* at least once (69). Probably the most intriguing aspect of *C. pneumoniae* pathogenicity is its apparent involvement in the development of atherosclerosis (see references 22 and 69, and other reviews in the special issue of the *Journal of Infectious Diseases*, Vol. 181, Suppl. 3; June 2000). The enticing perspective of using antibiotics to prevent or treat coronary artery disease adds some urgency to the task of understanding the basics of chlamydial physiology. Recently, genome comparisons were used to identify the unusual DhnA-type fructose-1,6-bisphosphate aldolase as a potential target for a chlamydia-specific "magic bullet" (64). Genome analysis also shows that both *C. trachomatis* and *C. pneumoniae* encode a primary Na^+ pump, NQR (190) (Table 1). They have another potential ion pump in cytochrome *d*-type terminal oxidase, but, as discussed above, the mechanism and energy yield of a cytochrome *d* complex remains unclear. Like *Treponema* spp. chlamydias have an H^+- (or Na^+)-transporting V-type ATPase, which turns out to be common in bacteria (66). Another similarity between these two phylogenetically very distant groups of bacteria is in the organization of their transport systems, which is probably due to their common reliance on the Na^+ cycle. Like *T. pallidum*, each of the two chlamydias encodes two predicted Na^+/alanine symporters (CT409 and CT735 in *C. trachomatis*, CPn0876 and CPn0536 in *C. pneumoniae*), an Na^+/branched-chain amino acid symporter (CT554 and CPn0836), and an uncharacterized transporter of the neurotransmitter:sodium symporter family (CT231 and CPn0290) (155, 197). On the other hand, other chlamydial transporters, such as the phosphate permease PitA (CT692 and CPn0680), the glutamate transporter GltS (CT401 and CPn0528), ADP/ATP translocase (CT065, CT495, CPn0351, and CPn0614), amino acid-polyamine-organocation family (TC 2.A.3) transporters (CT374, CT216, CPn0282 and CPn1031), and several other predicted transporters are likely to be energized by an H^+ rather than an Na^+ gradient. Table 2 shows that generation of the proton motive force in chlamydiae could be accomplished through the action of either Na^+/H^+ antiporters of the NhaD type or cytochrome *d*-type terminal oxidases.

Porphyromonas gingivalis and *Actinobacillus actinomycetemcomitans*

P. gingivalis and *A. actinomycetemcomitans*, both of which are periodontitis-causing bacteria, encode both the primary Na^+ pump NQR and $NAD^+/NADP^+$ transhydrogenase, an H^+ pump (Table 2). The reason why these two residents of the oral cavity, where the pH almost never goes above 7.8 and is often much lower (117), would need a primary Na^+ pump is not quite clear. One possible explanation is that an Na^+ gradient might help in stabilizing the levels of the proton motive force in these bacteria, which could otherwise vary due to the huge swings of the pH in the oral cavity, caused by consumption of fluids of variable ion content. Such a "buffering" role of Na^+ gradient has been experimentally demonstrated in several diverse bacterial species (19). Another reason for the existence of a primary Na^+ pump in oral pathogens is that in combination with the Ca^{2+}/Na^+ antiporter, it could help in protecting the bacteria from excessive influx of Ca^{2+} ions from Ca^{2+}-saturated saliva (117). Finally, the most convincing explanation of the possible role of NQR in *P. gingivalis* and *A. actinomycetemcomitans* relies on the fact that periodontitis caused by these bacteria is often accompanied by gum bleeding. As a result, the salt content in the ecological niche occupied by these microorganisms approaches that of the blood plasma, i.e., is characterized by a relatively high concentration of Na^+ ions. Thus, NQR could be as important for such oral pathogens as *T. denticola, P. gingivalis*, and *A. actinomycetemcomitans* as it is for marine microorganisms. A possible consequence of this adaptation is that periodontal pathogens may be fit to survive in blood and cause bacteriemia. Indeed, *P. gingivalis* and *A. actinomycetemcomitans* have been recently detected in atherosclerotic plaques in the carotid artery (73).

Escherichia coli and *Haemophilus influenzae*

Early research of the membrane energetics of *E. coli* failed to demonstrate an Na^+ pump in this organism (203). Later studies, however, provided ample evidence for primary active Na^+ transport under conditions of low proton motive force (6, 7, 37). A Δ*nhaA* Δ*nhaB* mutant lacking two principal Na^+/H^+ antiporters retains the capacity to excrete Na^+ ions when incubated in the presence of high concentrations of K^+ (74). After the genome sequence of *H. influenzae* became available, it was found to contain an *nqr* operon very similar to the one in *V. alginolyticus* (Table 1). Soon thereafter, the presence of NQR in *H. influenzae* was demonstrated experimentally (82). Recent experiments with *E. coli* confirmed the presence of a primary Na^+ pump in this organism (192). In spite of all these findings, the *nqr* genes in the *E. coli* genome sequence remained unidentified until now (Table 1), probably due to their unusual order in the operon and the likely nonorthologous displacement of the beta subunit.

Vibrio cholerae

While *V. cholerae* clearly has multiple H^+ and Na^+ pumps and a number of Na^+/H^+ antiporters (Table 2), it relies on Na^+-dependent polar flagella for its motility (111). *V. cholerae* also provides the only well-documented case of the connection between transmembrane Na^+ circulation and the expression of

pathogenicity determinants (78). Dissipation of the sodium motive force by ionophores, *nqr* mutations, or NQR inhibitors in each case led to an increased expression of virulence-related genes encoding cholera toxin and toxin-coregulated pili (78). Changes in Na^+ circulation appeared to affect a set of regulatory membrane proteins, TcpP and TcpH, which, in turn, are required for the expression of ToxT, a transcriptional activator of the major virulence factors in *V. cholerae* (77, 78). At high NaCl concentrations in the growth medium, the TcpP/TcpH-mediated activation of *toxT* was diminished (78). Thus, at least in *V. cholerae*, a functional linkage between the virulence factor expression machinery and the Na^+ cycle has been shown experimentally. The reason for this connection is not clear, although it has been speculated that one of the functions of cholera toxin might be the generation of an Na^+-rich environment in the intestinal lumen to boost the efficiency of the Na^+ cycle in *V. cholerae* cells immersed in alkaline medium (9).

In many pathogens including *V. cholerae*, motility is considered a virulence factor (72, 150). However, the relationship between motility and virulence in *V. cholerae* is quite complicated, since the motility phenotype itself appears to affect the expression of virulence determinants. Indeed, some nonmotile mutants of *V. cholerae* showed increased *toxT* transcription and constitutive expression of cholera toxin and toxin-coregulated pili under alkaline conditions (67, 78). Deceleration of flagellar rotation by different means, such as high medium viscosity or inhibitory drugs, had a similar effect (78). On the other hand, cholera toxin and toxin-coregulated pilus expression was repressed in several spontaneous hypermotile mutants (67). A hypermotile phenotype was also observed in *toxR* mutants, which are defective in the regulatory protein ToxR, which also is required for expression of ToxT (67, 78). Thus, not only is the motility phenotype controlled by the ToxR regulon in this species, but also there appears to exist a specific signal transduction mechanism that monitors cell motility and conveys that information to the virulence regulatory cascade. However, the molecular mechanisms underlying these signal transduction events remain obscure. It could be noted in this regard that products of two ToxR-regulated genes, *tcpI* (VC0825) and *acfB* (VC0840), located on the pathogenicity island responsible for the expression of toxin-coregulated pili, are homologous to methyl-accepting chemotaxis receptors. Mutations in these two genes positively affect the motility of *V. cholerae* as assayed by swarm plate assays (57, 75). Further experiments are required to try to pinpoint the exact signals(s) that links the Na^+ cycle and expression of pathogenicity factors in *V. cholerae*. The likely candidates for such a signal are changes in the total level of sodium motive force (78) or one of its components, e.g., the membrane potential (36). It would be also extremely interesting to determine which of the *V. cholerae* proteins acts as the primary "bioenergetic" sensor. In summary, *V. cholerae* seems to be a useful system for studying the possible involvement of Na^+ cycle elements in the regulation of virulence.

Na^+ PUMPS AS DRUG TARGETS

The structures of some of the inhibitors of the Na^+ cycle discussed in this section are shown in Fig. 5.

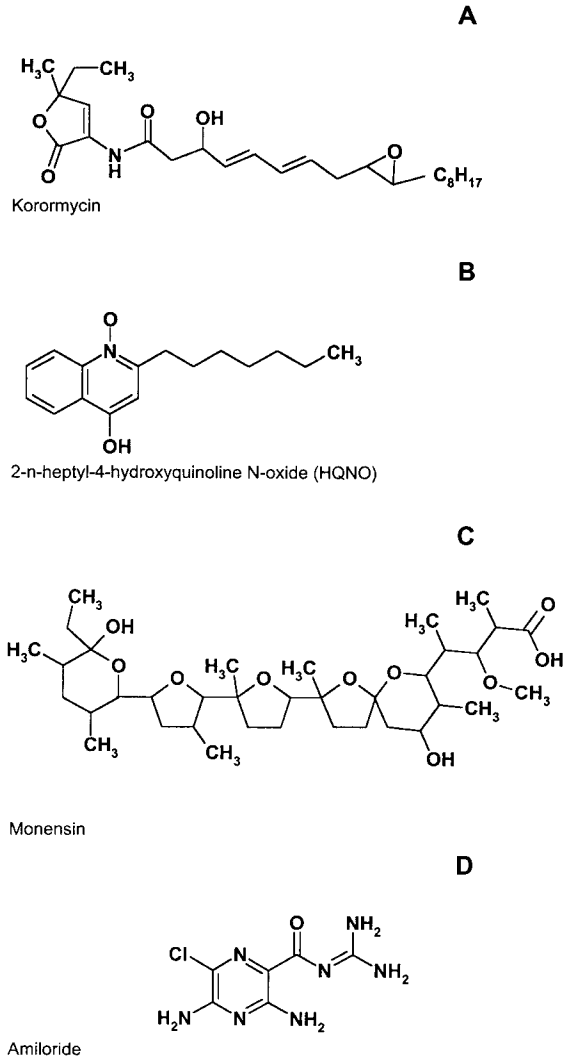

FIG. 5. Structures of some inhibitors of the Na^+ cycle. (A) Korormicin. (B) 2-*n*-Heptyl-4-hydroxyquinoline N-oxide (HQNO). (C) Monensin. (D) Amiloride.

Korormicin

The importance of the Na^+ cycle in the energy metabolism of certain human pathogens suggests that primary Na^+ pumps might hold promise as potential drug targets. Indeed, korormicin, a powerful inhibitor of NQR (223), was originally isolated as an antibiotic, secreted by a marine bacterium, *Pseudoalteromonas* sp. strain F-420 and demonstrating antibacterial activity against other marine bacteria (139, 224).

Korormicin is an extremely effective noncompetitive inhibitor ($K_i \approx 8 \times 10^{-11}$ M) of the interaction of NQR with its quinone substrate. As a result, korormicin was approximately 10^3-fold more active in inhibiting purified NQR than was 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), a traditional and well-studied inhibitor of NQR (223). Korormicin proved to be a specific inhibitor of NQR, since it had no effect on Na^+-independent NADH oxidase (NADH:menadione reductase) activity (223). At the cellular level, korormicin was active against a variety of gram-negative halophilic bacteria, includ-

ing *Vibrio alginolyticus, Shewanella putrefaciens*, and *Alteromonas macleodii* (223). These observations show that inhibiting NQR is lethal for at least some marine bacteria and that perhaps the same approach could work against human pathogens that depend on the Na^+ cycle. Other known inhibitors of the Na^+ cycle in bacteria include Li^+ and Ag^+ions, amiloride, and monensin, an artificial electroneutral Na^+/H^+ antiporter.

Ag^+

While antibacterial effects of silver salts were first noticed long ago (see reference 180 for a review), NQR has been recently recognized as one of the targets of Ag^+ ions. In two independent studies, nanomolar concentrations of Ag^+ ions were shown to inhibit energy-dependent Na^+ transport in inside-out vesicles of alkalophilic *Bacillus* sp. strain FTU and to inhibit purified NQR of *V. alginolyticus* (81, 177). Later, Ag^+ was shown to irreversibly bind to the beta subunit of NQR (NqrF or Nqr6), causing enzyme denaturation and the loss of its flavin adenine dirucleotide cofactor (139). Half-maximal inhibition of the enzyme activity was attained at concentrations between 0.5 and 2 nM Ag^+, making NQR one of the most vulnerable targets of Ag^+ ions.

Li^+

A number of Na^+-dependent bacterial permeases, including NhaA- and NhaB-type Na^+/H^+ antiporters, can use Li^+ instead of Na^+ (95, 146, 151). As a result, Li^+ is effectively exported from the cell and thereby decreases its toxicity. Thus, growth inhibition of wild-type *E. coli* required as high as 700 mM Li^+ in the medium, while a Δ*nhaA* Δ*nhaB* double mutant could not grow even in the presence of 30 mM Li^+ (95). The growth of *P. aeruginosa* is also Li^+ sensitive (96). NQR does not seem to use Li^+ as substrate, and some data suggest that Li^+ inhibits this enzyme (214). If so, Li^+ might have potential as drug against Na^+ cycle-dependent bacteria; however, it should be noted that Li^+ is mildly toxic for humans. It is currently used in the treatment of bipolar affective disorder and is known to affect thyroid function, leading to hypothyroidism and goiter.

Monensin

An artificial electroneutral Na^+/H^+ antiporter, monensin has been traditionally used as a nutritional additive (growth promoter) in cattle (54, 213). Monensin addition reduces amino acid fermentation and, hence, ammonia production in the rumen by disrupting the Na^+ cycle in ruminal *Peptostreptococcus* spp., which imports some amino acids in symport with Na^+ ions (24, 25). An Na^+-motive, biotin-dependent glutaconyl-CoA decarboxylase and an Na^+-motive membrane ATPase are apparently operative in this bacterium (24). Well-established activity of monensin against many anaerobic bacteria including *Clostridium perfringens, Streptococcus bovis*, and others (21, 167) suggests that it holds promise as a prototype for new antibacterial drugs.

Amiloride

The diuretic drug amiloride and its 5-aminoalkylated derivatives are potent inhibitors of mammalian Na^+/H^+ antiporters of the NHE family (60, 145). Amiloride was found to be ineffective against the NhaA-type Na^+/H^+ antiporter in *E. coli*, but it inhibited the NhaB-type antiporter with $K_{0.5}$ = 6.0 μM (159). Tenfold-higher concentrations of amiloride were reported to inhibit the NhaA antiporter of *V. parahaemolyticus* (116). However, high doses of amiloride should be used with caution, since at high concentrations it may act as a nonspecific uncoupler (32). Amiloride and some of its derivatives inhibit the Na^+-dependent motility of *V. parahaemolyticus, V. alginolyticus*, and *V. cholerae* (4, 99), which indicates that it could be used in preventing colonization.

NqrA AS A VACCINE CANDIDATE

Because NQR is a membrane protein, whose phylogenetic distribution is apparently limited to marine bacteria and certain human and animal pathogens (Table 2), its subunits could make interesting vaccine candidates. A study of *Actinobacillus pleuropneumoniae*, the causative agent of pleuropneumonia in swine, showed that the NqrA protein (referred to as AopA by the authors) was immunogenic in infected pigs (31). Even though NqrA is a cytoplasmic membrane protein that was not detected in the outer membrane in any significant amounts, the serum of convalescent-phase pigs infected with *A. pleuropneumoniae* was found to contain anti-NqrA antibodies. It is tempting to speculate that switching off the Na^+ pump of *A. pleuropneumoniae* might have helped those pigs to fight infection. The NqrA protein of *Porphyromonas gingivalis* has been patented in Australia as a "50 kD antigen PG1" (GenBank accession number AF144076), presumably due to its immunogenic properties. These observations, while still preliminary, suggest yet another direction of future studies of the role(s) of primary Na^+ pumps in bacterial infection.

CONCLUSIONS AND PERSPECTIVES

Although analysis of the role of Na^+ ions in bacterial virulence is still in its infancy, a few things are becoming increasingly clear. The presence of genes encoding primary Na^+ pumps in the genomes of a number of phylogenetically diverse pathogenic bacteria (Table 1) indicates that generation of the Na^+ gradient is an important part of their membrane energetics. It should be noted that such microorganisms as *Mycoplasma genitalium, M. pneumoniae, B. burgdorferi, H. pylori*, and *Mycobacterium tuberculosis* do not seem to encode any primary Na^+ pumps (see Table 1) and may not depend on Na^+ circulation. Most bacterial pathogens, however, encode both Na^+ and H^+ pumps and multiple Na^+/H^+ antiporters (Table 2) that ensure the maintenance of both Na^+ and H^+ gradients on their cytoplasmic membrane. It appears, therefore, that the sodium motive force supplements the proton motive force as an additional source of energy in these bacteria. In an extreme case, the syphilis spirochete *T. pallidum* appears to encode no primary H^+ pumps; it thus might exclusively depend on the sodium motive force, generated by Na^+-transporting oxaloacetate decarboxylase, for its energy metabolism. For several other important pathogens, including *C. trachomatis, C. pneumoniae*, and *H. influenzae*, NQR comprises the principal respiratory ionic pump; their genomes also encode pyrimidine nucleotide transhydrogenase and/or cytochrome *bd*-type terminal

oxidase (Table 2). Several pathogens, including *K. pneumoniae, V. cholerae*, and *S. enterica* serovar Typhi, are capable of anaerobic citrate fermentation, which includes Na^+ cycling across the cytoplasmic membrane.

One could think of several possible explanations for the widespread distribution of the elements of the Na^+ cycle among pathogenic bacteria. First, Na^+-based membrane energetics could improve the versatility of a pathogen by providing it with additional means of ATP synthesis, motility, and solute uptake. This would improve its chances for colonization of the host cells and survival in the host organisms, where defense mechanisms, such as generation of superoxide radicals impair the integrity of the bacterial membrane and decrease the levels of the proton motive force. Second, because Na^+ concentrations in most natural environments are almost 10^6-fold higher than H^+ concentrations, sodium motive force levels are unlikely to change as rapidly as proton motive force levels, making sodium motive force a much more reliable source of energy. Finally, the well-known similarity between the salt content of blood and seawater could create evolutionary pressure toward the development of similar adaptation mechanisms in human pathogens and marine microorganisms or, alternatively, acquisition of the corresponding genes through horizontal gene transfer.

The dualistic H^+- and Na^+-based character of membrane energetics in pathogenic bacteria propounds a number of intriguing questions. First, is the persistent appearance of elements of the Na^+ cycle in very different pathogens just a consequence of the adaptive advantage of having more than one chemiosmotic coupling ion, or is there a more profound, mechanistic link between the presence of an Na^+ cycle and virulence? And, if the latter is true, what is the exact mechanism linking Na^+ energetics to the regulatory events responsible for the expression of pathogenicity determinants? In other words, does a change in Na^+ homeostasis signal the pathogenic organism that it has reached its destination inside the host and that it is time to activate the expression of virulence factors? At least in the case of *V. cholerae*, the cells appear to respond to alterations in Na^+ circulation by modulating the expression of the main virulence regulon (78). The exact nature of the potential sensor and the mechanism of this regulation are, unfortunately, still unknown.

From the practical point of view, the peculiar character of the Na^+ cycle in bacterial pathogens makes its components attractive potential targets for the development of "smart" drugs and therapeutic strategies that would have minimal side effects at acceptable antimicrobial potency. A few examples considered in this review illustrate the potential of different chemicals acting as specific inhibitors of primary Na^+ pumps (korormicin and Ag^+), Na^+/H^+ antiporters (amiloride and its analogs), substrate analogs (Li^+), and Na^+-translocating ionophores (monensin). It should be stressed that for a large number of Na^+ transporters, there are no known specific inhibitors. However, our analysis shows that a variety of new potential drug targets could be pinpointed by screening complete and partially complete bacterial genomes. The putative system of the Na^+-dependent anaerobic fermentation of citrate in *T. denticola, V. cholerae, S. enterica* serovar Typhi, and some other pathogens may be mentioned as such a potential target. In addition, the immunogenic efficacy of NqrA demonstrates that Na^+ pumps, residing in the cytoplasmic membrane of gram-negative bacteria, are much more appealing targets for the development of effective vaccines than it would have seemed from their "nonsurface" localization. Although drugs targeted against the components of Na^+ cycle, such as NQR, would certainly have a limited antibacterial spectrum, they might be very helpful weapons against persistent infections caused by *Treponema* or *Chlamydia* species and potentially might even help in preventing coronary artery disease and atherosclerosis, to which chlamydias are now believed to contribute.

The presence of primary Na^+ pumps in modern archaeal and bacterial hyperthermophiles suggests that the Na^+ cycle was a primary mechanism of energy conservation in the common ancestor of these two branches of the Tree of Life (see references 124 and 220 for discussions). As is the case with free-living extremophiles (thermophiles, halophiles, and alkalophiles), human pathogens may rely on the Na^+ cycle to survive and grow in the hostile environment created by host defense mechanisms. It seems reasonable to expect that elucidation of the precise role of Na^+ circulation in pathogenic bacteria would open new avenues of research, which potentially could bring not only additional knowledge but also novel approaches to cure infectious diseases.

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