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The Glucose Transport System of the Hyperthermophilic Anaerobic Bacterium *Thermotoga neapolitana*

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The glucose transport system of the extremely thermophilic anaerobic bacterium *Thermotoga neapolitana* was studied with the nonmetabolizable glucose analog 2-deoxy-D-glucose (2-DOG). *T. neapolitana* accumulated 2-DOG against a concentration gradient in an intracellular free sugar pool that was exchangeable with external D-glucose. This active transport of 2-DOG was dependent upon the presence of sodium ion and an external source of energy, such as pyruvate, and was inhibited by arsenate and gramicidin D. There was no phosphoenolpyruvate-dependent phosphorylation of glucose, 2-DOG, or fructose by cell extracts or toluene-treated cells, indicating the absence of a phosphoenolpyruvate:sugar phosphotransferase system. These data indicate that D-glucose is taken up by *T. neapolitana* via an active transport system that is energized by an ion gradient generated by ATP, derived from substrate-level phosphorylation.

Cytoplasmic membranes of bacteria serve many functions, including active transport of nutrients, maintenance of intracellular pH, and regulation of intracellular osmotic pressure. Accomplishing all of these goals becomes especially complicated for bacteria living at extremely high temperatures (hyperthermophiles), because passive ion permeability of the membrane increases with temperature (7). The mechanisms of survival at extreme temperatures range from the use of tetraether lipids (6) to apparent utilization of ADP-dependent kinases (14).

Thus far, amino acid transport in two moderately thermophilic anaerobic bacteria has been found to be driven by transmembrane gradients of Na^+ ions (10, 30). One of these thermophiles, *Clostridium fervidus*, was found to completely depend upon a Na^+ gradient for its membrane energetics (29). However, mechanisms of carbohydrate transport in thermophiles, particularly in hyperthermophiles, remain unclear. In most studies conducted so far, only the overall uptake of radiolabeled metabolizable sugars was measured (5, 9, 25). This approach did not separate transport from metabolism and cellular incorporation, and thus it provided only limited information on the nature of the transport processes. In one case, in which a glucose analog was used (4), no active transport was found, and a facilitated diffusion mechanism was suggested.

Here, we describe a glucose transport system in the hyperthermophilic marine bacterium *Thermotoga neapolitana*. *T. neapolitana* is a strict anaerobe that grows by fermentation at temperatures up to 90°C and can use a number of carbohydrates as carbon and energy sources (11, 12). This organism has evolved relatively slowly since its divergence from other bacteria (1, 33), so its physiology and modes of sugar transport may be similar to those of the earliest bacteria.

MATERIALS AND METHODS

Growth of *T. neapolitana* and preparation of cell suspensions. *T. neapolitana* NS-E (12) was maintained as described earlier (31). Cells were grown at 77°C under strictly anaerobic conditions in seawater-based TB mineral medium (3), supplemented with 0.2% yeast extract, 0.4% glucose, and 0.1% starch. Typically, 10 ml of overnight culture was inoculated into 500 ml of fresh medium in Wheaton medium bottles, grown for 6 to 8 h, and then cooled down to 4°C. The cells were harvested by centrifugation at 5°C in bottles with rubber-sealed caps and washed twice with NaTB (the salt portion of the TB medium), containing the following (per liter): NaCl, 17.55 g; KCl, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NH_4Cl, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; K_2HPO_4, 0.05 g; HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 4.8 g; cysteine HCl, 0.5 g, and resazurin, 1 mg (pH 7.5). The cells were finally resuspended in NaTB medium to 4 to 6 mg of protein per ml and dispensed in 2.5-ml portions into 5-ml capped serum bottles. All of the resuspension and dispensing procedures were performed at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) under a N_2-H_2 (90:10) atmosphere.

In experiments to determine the effect of Na^+ on transport, cells were washed and resuspended in Na^+-free KTB medium, containing 0.3 M KCl instead of NaCl. The energy source in these experiments was a pyruvic acid solution neutralized with KOH. The concentration of Na^+ was varied by resuspension of the cells in 5 ml of NaTB or KTB media, as specified.

Transport measurements. Transport of 2-deoxy-D-[1-^{14}C]glucose ([^{14}C]2-DOG) was measured in cell suspensions contained in capped serum bottles kept at 65°C under a flow of nitrogen, essentially as described by Kashket (13). All additions and removal of samples were made through the rubber caps with syringes flushed with nitrogen. Before each experiment, both the cell suspensions and the transport substrate, [^{14}C]2-DOG (0.2 mCi/mmol; DuPont NEN Research Products, Boston, Mass.), dissolved in NaTB medium, were made anaerobic by being flushed with oxygen-free N_2. Our experience was that flushing of the gas space above the cell suspensions with N_2 was essential, and any exposure to oxygen led to loss of transport capacity. Cells were preincubated at 65°C for 15 min in the presence of 50 mM pyruvate before addition of [^{14}C]2-DOG, unless indicated otherwise. Inhibitors, when used, were added 5 min prior to [^{14}C]2-DOG addition. Samples of cell suspension (0.25 or 0.5 ml) were removed at appropriate times with nitrogen-flushed syringes and transferred into 1.5-ml microcentrifuge tubes containing a silicone oil mixture (0.3 ml of a 75:25 mix of Dow Corning fluids 550 and 556 covered with 0.2 ml of mineral oil) and immediately centrifuged (16,000 × *g*, 2 to 3 min) in an Eppendorf microcentrifuge. After careful removal of supernatants by aspiration, cell pellets were lysed in 0.2 ml of 0.2% Triton X-100. For determination of total radioactivity, the lysates were mixed with liquid scintillation fluid (Optifluor; Packard) and counted in a Beckman LS 3801 spectrometer. For determination of intracellular pools of free or phosphorylated 2-DOG, the lysates were cleared of cell debris by centrifugation and applied to columns (Bio-Rad Econocolumns; 0.8 by 4 cm) containing Bio-Rad AG1-X2 anion-exchange resin (analytical grade, 100/200 mesh, chloride form). Free sugars were eluted first with 20 ml of deionized water, and anionic sugar phosphates or sugar acids were subsequently eluted with two 3-ml portions of 1 M LiCl. This procedure allowed recovery of more than 95% of the total radioactivity applied to the columns.

The volume of intracellular water was measured as the difference between total space occupied by the permeant species ^3H_2O and the space occupied by a nonpermeant species, [^{3}H]polyethylene glycol (both DuPont NEN Research Products) (13). The aqueous space was found to be 1.9 μl of H_2O per mg of protein.

To determine the protein content of cell suspensions, samples of suspensions were centrifuged, cell pellets were resuspended in 0.2 ml of 0.2 M NaOH

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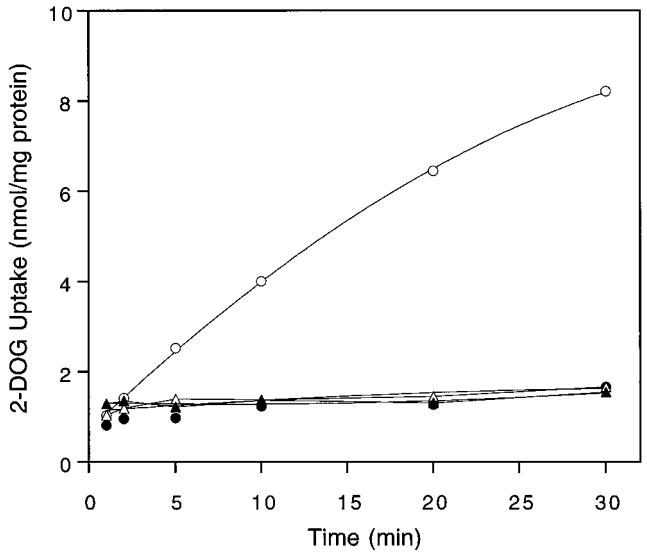

FIG. 1. Effect of energy source and energy inhibitors on the uptake of [^{14}C]2-DOG by *T. neapolitana*. Cells were incubated with 0.2 mM [^{14}C]2-DOG (0.2 mCi/mmol) added at time zero with no further addition (●), with 50 mM pyruvate added at −15 min (○), or with 50 mM pyruvate added at −15 min and 100 mM sodium arsenate (△) or 10 μM gramicidin D (▲) added at −5 min.

containing 1% sodium dodecyl sulfate and lysed by boiling for 10 min, and then protein was assayed by the method of Lowry et al. (17).

Assay of sugar phosphorylation by cell extracts and toluene-treated cells. Cells grown as described above were washed and resuspended in a buffer solution containing 50 mM Tris-HCl, 50 mM K^+-HEPES, 25 mM NaF, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 2 mM sodium dithionite (pH 8.0 at 25°C). Cell extracts were prepared by intermittent sonication of cell suspensions kept in an ice bath under a nitrogen atmosphere (five 1-min bursts with 1-min cooling periods) followed by centrifugation at 16,000 × *g* for 15 min to remove cell debris. Toluenized (decryptified) cells were prepared with 0.1% (vol/vol) toluene according to the method of Kornberg and Reeves (15). Phosphorylation was measured at 77°C for 10 to 30 min essentially as described previously (18). The standard assay mixture contained 1 mM ^{14}C-labeled sugar substrate (0.2 to 0.5 mCi/mmol), 10 mM phosphoryl donor, cell extract or toluenized cells, and the suspension buffer specified above in a total volume of 0.1 ml. Reactions were initiated by the addition of appropriate phosphoryl donors and were stopped by dilution of the reaction mixtures with 1 ml of ice-cold water. The diluted reaction mixtures were transferred to Bio-Rad AG1-X2 (chloride form) anion-exchange columns and analyzed for sugar phosphates as described above.

RESULTS

Cells of *T. neapolitana* were able to accumulate 2-DOG against a concentration gradient, provided an external source of energy was supplied. Figure 1 shows that cells incubated in the presence of pyruvate were able to effect an intracellular concentration of 2-DOG that was approximately 25-fold that of the external medium, on the basis of an intracellular water content of 1.9 μl per mg of protein. There was essentially no accumulation of 2-DOG without addition of pyruvate, indicating that *T. neapolitana* cells harvested and washed free of external nutrients are in an energy-depleted state. Pyruvate yields intracellular ATP by the combined actions of pyruvate: ferredoxin oxidoreductase, phosphotransacetylase, and acetate kinase, a key enzyme system found in *Thermotoga maritima* (2, 22). The energy dependence of this concentrative uptake of 2-DOG is further demonstrated by its abolition by sodium arsenate, which inhibits ATP synthesis, and the ionophore gramicidin D.

Anion-exchange chromatographic analysis of lysates of cells that had taken up [^{14}C]2-DOG showed that more than 90% of the total intracellular radioactivity was nonanionic, indicating that there was limited phosphorylation of 2-DOG and that intracellular 2-DOG was predominantly in a free and unaltered state (Fig. 2). Furthermore, addition of excess D-glucose to cells that had been allowed to accumulate [^{14}C]2-DOG for 30 min provoked a rapid efflux of radioactivity (Fig. 2), showing that the intracellular pool of [^{14}C]2-DOG was mobile and rapidly exchangeable with an external homologous species. Figure 2 also shows that addition of excess D-glucose before the commencement of [^{14}C]2-DOG uptake resulted in strong inhibition of 2-DOG uptake, indicating further that D-glucose and 2-DOG are transported via a common carrier and that 2-DOG is a suitable nonmetabolizable analog for the study of D-glucose transport.

While the low intracellular concentration of anionic 2-DOG did not suggest the operation of a phosphorylative group translocation system, such as the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS), assays for this system were carried out with cell extracts and toluenized cells to determine whether this system might be operative for the natural substrate D-glucose or for D-fructose, the most common sugar PTS substrate (19, 20). The results are shown in Table 1. There was strong ATP-dependent glucose phosphorylation in the cell extract but no significant PEP-dependent phosphorylation of glucose. There was also no PEP-dependent phosphorylation of 2-DOG, and the low ATP-dependent phosphorylation of this analog by the cell extract correlated with the small intracellular pool of phosphorylated 2-DOG that was detected in the whole-cell transport experiments described above (Fig. 2). PEP-dependent phosphorylation of fructose was also much less than the ATP-dependent phosphorylation, and not significantly more than that in the absence of any added phosphoryl donor.

The possibility that the lack of PEP-dependent PTS activity was due to instability of PEP at 77°C was ruled out by a positive control experiment in which the PEP-containing reaction mixture was kept at 77°C for 30 min and then was transferred to 37°C. Subsequent addition of a cell extract of *Enterococcus faecalis*, which has a PEP:glucose PTS, resulted in a high level of glucose phosphorylation activity compared with that of a similarly treated reaction mixture that lacked PEP (not shown). This showed that the previously heated PEP-containing reaction mixture was able to support the PTS activity of an organism known to possess this system.

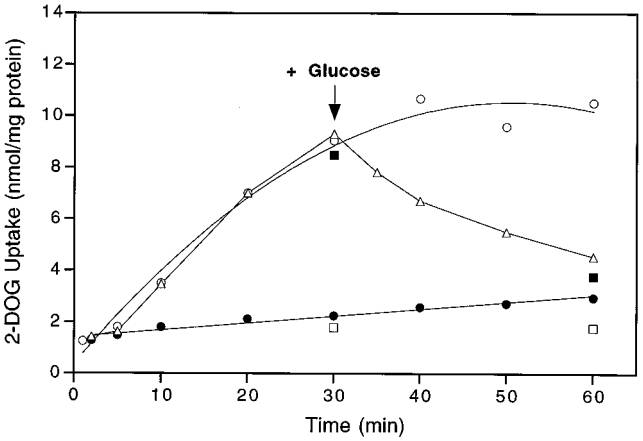

FIG. 2. Effect of glucose on [^{14}C]2-DOG uptake by *T. neapolitana*. Cells were incubated in the presence of 0.2 mM [^{14}C]2-DOG (0.2 mCi/mmol) and 50 mM pyruvate, with no further addition (○) or with 20 mM D-glucose added at −2 min (●) or +30 min (△). Intracellular levels of free [^{14}C]2-DOG (■) and anionic, phosphorylated [^{14}C]2-DOG (□) at the time of glucose addition (30 min) and 30 min after glucose addition (60 min) are shown.

TABLE 1. PEP- or ATP-dependent phosphorylation of D-glucose, 2-DOG, or D-fructose by cell extracts or toluene-treated *T. neapolitana* cells

Sugar	Sugar phosphate formed (nmol/min/mg of protein) with phosphoryl donor:		
	None	PEP	ATP
Cell extracts			
D-Glucose	3.1	3.2	117.0
2-DOG	1.6	1.8	16.9
D-Fructose[a]	4.8	5.1	36.5
Toluene-treated cells			
D-Glucose	3.7	3.7	9.0
D-Fructose[a]	1.7	2.2	12.5

[a] Fructose phosphorylation was assayed with cells grown on 0.4% fructose instead of glucose.

Glucose phosphorylation by the cell extract also took place in the presence of ADP, but not AMP, carbamyl phosphate, acetyl phosphate, or pyrophosphate (not shown).

Cells that were washed twice and resuspended in TB medium in which NaCl was replaced by KCl showed essentially no capacity to transport 2-DOG. This transport capability was restored by provision of Na^+ in a concentration-dependent manner, with full activity at 100 mM Na^+ when the total NaCl-KCl concentration was 300 mM (Fig. 3). Thus, the ability of *T. neapolitana* to concentrate 2-DOG intracellularly was clearly sodium dependent.

DISCUSSION

Extremely thermophilic bacteria and archaea appear to be the most slowly evolving organisms (1, 33). Studies of their biochemical functions have already revealed a number of unique features that provide a better understanding of the evolution of metabolic pathways (14, 21). As an initial approach to study the evolution of membrane transport systems, we have undertaken an investigation of carbohydrate transport in the phylogenetically deep-rooted bacterium *T. neapolitana.*

The results presented in this work demonstrate the presence of an active transport system for glucose in *T. neapolitana*. This system was found to transport the nonmetabolizable analog 2-DOG against a concentration gradient into an intracellular nonphosphorylated free sugar pool that could be exchanged for extracellular D-glucose. Energy for this concentrative uptake was provided by pyruvate, which generates ATP by substrate-level phosphorylation (2). Transport required Na^+ and was inhibited by sodium arsenate, which would inhibit ATP synthesis and lead to depletion of intracellular ATP, and by gramicidin D, an ionophore which would collapse an ion gradient that might be generated by ATP. The most reasonable interpretation of these data is that *T. neapolitana* actively transports glucose via an active transport system energized by an ion gradient, which is in turn generated from ATP derived from substrate-level phosphorylation in the fermentative metabolism of this organism. The ion gradient involves Na^+ in some way that needs to be determined by further study.

T. neapolitana is a marine organism, so the requirement for Na^+ in the active transport of 2-DOG that has been demonstrated here would not be unexpected. Participation of Na^+ in transport systems of extremophiles and marine organisms is common (for reviews, see references 16 and 26–28). The mode of participation varies in different organisms, however, depending on whether the bioenergetic cycles that power active transport involve Na^+ gradients only or involve both H^+ and Na^+ gradients, interconvertible by a Na^+/H^+ antiporter. It is of interest that the anaerobic thermophile *C. fervidus* has only a Na^+ cycle and no H^+ cycle, and it has been suggested that this system provides a selective advantage to a fermentative organism with limited capacity for energy generation because of greater passive transmembrane leakage of H^+ relative to Na^+ at elevated temperatures (16, 30). It will be of interest to determine whether a similar situation pertains in *Thermotoga* species and other fermentative hyperthermophiles.

An earlier preliminary screening did not reveal any PEP: sugar phosphotransferase activity in *Thermotoga* cells (19). Here, we looked more extensively for evidence of a possible PTS involvement, but found no PEP-dependent phosphorylation of glucose, 2-DOG, or fructose in toluenized cells or cell extracts. Thus, glucose apparently enters the cell unmodified and is then phosphorylated by glucokinase at the expense of ATP. ADP-dependent glucose phosphorylation was recently reported in the hyperthermophilic archaeon *Pyrococcus furiosus* (14). However, phosphorylation of glucose in *T. neapolitana* appears to be dependent on ATP; our observation that addition of ADP to cell extracts of *T. neapolitana* resulted in glucose phosphorylation is probably due to the highly active and thermostable adenylate kinase, recently discovered in a close relative, *T. maritima* (8). Indeed, we found that addition of ADP caused rapid ATP synthesis in *T. neapolitana* cell extracts. Further glucose fermentation would proceed via the Embden-Meyerhof-Parnas pathway (23).

It has been suggested that PTS-like systems could have evolved earlier than the systems of active solute transport (20). However, the PTS has been found so far only in groups of bacteria that have branched from the evolutionary tree much later than *Thermotoga* species (19). On the other hand, ion-coupled transport systems have been described in deeply rooted archaea (24, 27). These findings and the results of this work are consistent with the suggestion of Wilson and Lin (32) that early transport processes were linked to ion movements.

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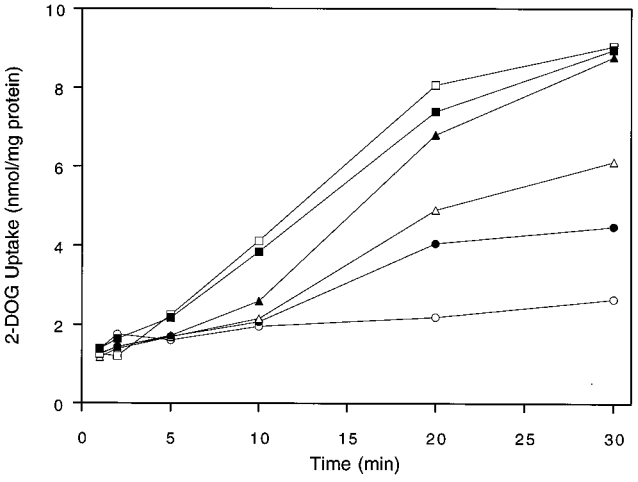

FIG. 3. Effect of Na^+ on [^{14}C]2-DOG transport by *T. neapolitana*. Uptake of [^{14}C]2-DOG was measured in cells washed in Na^+-free KTB solution and resuspended in mixtures of NaTB and KTB solutions containing the following concentrations of NaCl or KCl: ○, 300 mM KCl, no NaCl; ●, 10 mM NaCl, 290 mM KCl; △, 25 mM NaCl, 275 mM KCl; ▲, 50 mM NaCl, 250 mM KCl; □, 100 mM NaCl, 200 mM KCl; ■, 200 mM NaCl, 100 mM KCl.

REFERENCES

1. **Achenbach-Richter, L., R. Gupta, K. O. Stetter, and C. R. Woese.** 1987. Were the original eubacteria thermophiles? Syst. Appl. Microbiol. **9:**34–39.
2. **Adams, M. W. W.** 1994. Biochemical diversity among sulfur-dependent, hyperthermophilic microorganisms. FEMS Microbiol. Rev. **15:**261–277.
3. **Childers, S. E., M. Vargas, and K. M. Noll.** 1992. Improved methods for cultivation of the extremely thermophilic bacterium *Thermotoga neapolitana*. Appl. Environ. Microbiol. **58:**3949–3953.
4. **Cook, G. M., P. H. Janssen, and H. W. Morgan.** 1993. Uncoupler-resistant glucose uptake by the thermophilic glycolytic anaerobe *Thermoanaerobacter thermosulfuricus* (*Clostridium thermohydrosulfuricum*). Appl. Environ. Microbiol. **59:**2984–2990.
5. **Cook, G. M., P. H. Janssen, J. B. Russell, and H. W. Morgan.** 1994. Dual mechanisms of xylose uptake in the thermophilic bacterium *Thermoanaerobacter thermohydrosulfuricus*. FEMS Microbiol. Lett. **116:**257–262.
6. **DeRosa, M., A. Gambacorta, and A. Gliozzi.** 1986. Structure, biosynthesis, and physicochemical properties of archaebacterial lipids. Microbiol. Rev. **50:**70–80.
7. **Elferink, M. G. L., J. G. De Wit, A. J. M. Driessen, and W. N. Konings.** 1994. Stability and proton-permeability of liposomes composed of archaeal tetraether lipids. Biochim. Biophys. Acta **1193:**247–254.
8. **Gilles, A.-M., P. Glaser, V. Perrier, A. Meier, R. Longin, M. Sebald, L. Maignan, E. Pistotnik, and O. Bârzu.** 1994. Zinc, a structural component of adenylate kinases from gram-positive bacteria. J. Bacteriol. **176:**520–523.
9. **Hernandez, P. E.** 1982. Transport of D-glucose in *Clostridium thermocellum* ATCC-27405. J. Gen. Appl. Microbiol. **28:**469–477.
10. **Heyne, R. I. R., W. De Vrij, W. Crielaard, and W. N. Konings.** 1991. Sodium ion-dependent amino acid transport in membrane vesicles of *Bacillus stearothermophilus*. J. Bacteriol. **173:**791–800.
11. **Huber, R., and K. O. Stetter.** 1992. The *Thermotogales*: hyperthermophilic and extremely thermophilic bacteria, p. 185–194. *In* J. K. Kristjansson (ed.), Thermophilic bacteria. CRC Press, Boca Raton, Fla.
12. **Jannasch, H. W., R. Huber, S. Belkin, and K. O. Stetter.** 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic eubacterial genus *Thermotoga*. Arch. Microbiol. **150:**103–104.
13. **Kashket, E. R.** 1981. Proton motive force in growing *Streptococcus lactis* and *Staphylococcus aureus* cells under aerobic and anaerobic conditions. J. Bacteriol. **146:**369–376.
14. **Kengen, S. W. M., F. A. M. de Bok, N. D. van Loo, C. Dijkema, A. J. M. Stams, and W. M. de Vos.** 1994. Evidence for the operation of a novel Embden-Meyerhof pathway that involves ADP-dependent kinases during sugar fermentation by *Pyrococcus furiosus*. J. Biol. Chem. **269:**17537–17541.
15. **Kornberg, H. L., and R. E. Reeves.** 1972. Inducible phosphoenolpyruvate-dependent phosphotransferase activities in *Escherichia coli*. Biochem. J. **128:**1339–1344.
16. **Lolkema, J. S., G. Speelmans, and W. N. Konings.** 1994. Na^+-coupled versus H^+-coupled energy transduction in bacteria. Biochim. Biophys. Acta **1187:**211–215.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
18. **Romano, A. H., G. Brino, A. Peterkofsky, and J. Reizer.** 1987. Regulation of β-galactoside transport and accumulation in heterofermentative lactic acid bacteria. J. Bacteriol. **169:**5589–5596.
19. **Romano, A. H., and M. H. Saier.** 1992. Evolution of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. I. Physiologic and organismic considerations, p. 143–170. *In* R. P. Mortlock (ed.), The evolution of metabolic function. CRC Press, Boca Raton, Fla.
20. **Saier, M. H.** 1985. Mechanisms and regulation of carbohydrate transport in bacteria. Academic Press, New York.
21. **Schäfer, T., and P. Schönheit.** 1992. Maltose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus*: evidence for the operation of a novel sugar fermentation pathway. Arch. Microbiol. **158:**188–202.
22. **Schäfer, T., and P. Schönheit.** 1993. Acetyl-CoA synthase (ADP-forming) in archaea, a novel enzyme involved in acetate formation and ATP synthesis. Arch. Microbiol. **159:**72–83.
23. **Schröder, C., M. Selig, and P. Schönheit.** 1994. Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*. Arch. Microbiol. **161:**460–470.
24. **Severina, L. O., N. V. Pimenov, and V. K. Plakunov.** 1991. Glucose transport in the extremely halophilic archaebacteria. Arch. Microbiol. **155:**131–136.
25. **Severina, L. O., N. V. Zhilina, and V. K. Plakunov.** 1991. Some characteristics of glucose transport systems in extreme thermoacidic bacteria belonging to the genus *Sulfurococcus*. Mikrobiologiya **60:**285–289.
26. **Skulachev, V. P.** 1989. Bacterial Na^+ energetics. FEBS Lett. **250:**106–114.
27. **Skulachev, V. P.** 1993. Bioenergetics of extreme thermophiles, p. 25–40. *In* M. Kates, D. J. Kushner, and A. T. Matheson (ed.), The biochemistry of archaea (archaebacteria). Elsevier, Amsterdam.
28. **Skulachev, V. P.** 1994. The latest news from the sodium world. Biochim. Biophys. Acta **1187:**216–221.
29. **Speelmans, G., B. Poolman, T. Abee, and W. N. Konings.** 1993. Energy transduction in the thermophilic anaerobic bacterium *Clostridium fervidus* is exclusively coupled to sodium ions. Proc. Natl. Acad. Sci. USA **90:**7975–7979.
30. **Speelmans, G., B. Poolman, and W. N. Konings.** 1993. Amino acid transport in the thermophilic anaerobe *Clostridium fervidus* is driven by an electrochemical sodium gradient. J. Bacteriol. **175:**2060–2066.
31. **Vargas, M., and K. M. Noll.** 1994. Isolation of auxotrophic and antimetabolite-resistant mutants of the hyperthermophilic bacterium *Thermotoga neapolitana*. Arch. Microbiol. **162:**357–361.
32. **Wilson, T. H., and E. C. C. Lin.** 1980. Evolution of membrane bioenergetics. J. Supramol. Struct. **13:**421–446.
33. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.