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REVIEW

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Metabolism of chloride in halophilic prokaryotes

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Abstract While much understanding has been achieved on the intracellular sodium and potassium concentrations of halophilic and halotolerant microorganisms and on their regulation, we know little on the metabolism of anions. Archaea of the family *Halobacteriaceae* contain molar concentrations of chloride, which is pumped into the cells by cotransport with sodium ions and/or using the light-driven primary chloride pump halorhodopsin. Most halophilic and halotolerant representatives of the bacterial domain contain low intracellular ion concentrations, with organic osmotic solutes providing osmotic balance. However, some species show a specific requirement for chloride. In *Halobacillus halophilus* certain functions, such as growth, endospore germination, motility and flagellar synthesis, and glycine betaine transport are chloride dependent. In this organism the expression of a large number of proteins is chloride regulated. Other moderately halophilic Bacteria such as *Halomonas elongata* do not show a specific demand for chloride. A very high requirement for chloride was demonstrated in two groups of Bacteria that accumulate inorganic salts intracellularly rather than using organic osmotic solutes: the anaerobic *Halanaerobiales* and the aerobic extremely halophilic *Salinibacter ruber*. It is thus becoming increasingly clear that chloride has specific functions in haloadaptation in different groups of halophilic microorganisms.

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Introduction

Halophilic and halotolerant microorganisms are generally grown in media based on NaCl as the principal salt. This is not surprising, as Na^+ and Cl^- are the major ions in seawater-derived and in thalassohaline (seawater-based) environments in which halophilic microorganisms thrive. Even in athalassohaline environments such as the Dead Sea and hypersaline soda lakes, Na^+ is a major component of the anion sum, and Cl^- is abundantly found as well.

A wealth of information has accumulated on the mechanisms used by different groups of halophilic prokaryotes to cope with high salt concentrations. Some groups accumulate inorganic ions (mainly K^+ and Cl^-) within the cell up to molar concentrations. Others exclude salts as much as possible from the cells, while providing the necessary osmotic balance by synthesizing or accumulating organic osmotic solutes. In all cases energy-dependent mechanisms exist in the cell membrane to regulate the ionic composition of the cytoplasm.

We now have a reasonably good understanding of the mechanisms used by different halophilic microorganisms for the removal of Na^+ from the cell and the accumulation of K^+. Studies on the requirement for other cations (Mg^{2+}, Ca^{2+}, Fe ions, etc.) have been performed as well (reviewed in Oren 2002). It is therefore surprising that so little is known on the specific requirement for anions for growth and for the operation of specific cellular functions. The use of chloride salts in physiological studies with halophilic microorganisms is generally taken for granted, and little thought is then devoted to the possibility that chloride may have more specific functions in the cell beyond serving as the counterion for the high concentrations of cations used in the growth media.

The first indications that chloride may have a more specific function came from studies by MacLeod and his coworkers in the 1950s. They isolated a number of marine bacteria which proved to be highly stimulated or even strictly dependent on the presence of chloride for growth (MacLeod and Onofrey 1956, 1957). Unfortunately no further physiological studies were performed with these isolates, and the strains have since been lost.

While our understanding of the specific effects of chloride still lags behind our knowledge on the importance of sodium and potassium ions in the physiology of different groups of halophilic microorganisms, observations of specific requirement for chloride for certain cellular functions have been accumulating in recent years. In this review we discuss several aspects of chloride metabolism in different groups of halophilic prokaryotes, archaeal as well as bacterial, in an attempt to assess the importance of this ubiquitously abundant anion in the life of salt-requiring microorganisms.

Methodological considerations

There is no simple method to reliably measure intracellular ionic concentrations, including those of chloride, within bacterial cells. Many of the controversies that exist in the literature on the true intracellular ionic concentrations of different microorganisms can be explained by methodological problems, especially issues related to the estimation of the true intracellular volume in cell pellets used for the analyses. An in-depth discussion of the problems involved is not within the scope of this review. More information can be found, for example, in reviews by Oren (2002) and Ventosa et al. (1998). As chloride is not as easy to assay as, for example, Na^+ and K^+, which can easily be measured by flame photometry, estimates for intracellular Cl^- concentrations are only seldom given in studies on the intracellular ionic concentrations in microorganisms. In those cases in which chloride was measured, titration was used to quantify its presence (see, for example, Christian and Waltho 1962; Kamekura and Kushner 1984; Oren 1986; Rengpipat et al. 1988). The possibility of using radiolabeled chloride ($^{36}Cl^-$) as a marker has seldom been exploited.

X-ray microanalysis in the electron microscope provides a different approach to the estimation of intracellular concentrations of chloride and other ions. Here the ion content and cell volume are estimated in single cells collected on an electron microscope grid (Oren et al. 1997, 2002). Stressful handling of the sample is minimal. However, flattening of the cells during sample preparation may distort cell shape and cause erroneous calculations of the true cell volume.

When trying to assess specific effects of chloride on halophilic microorganisms suspended in solutions of high ionic strength, the nature of the alternative anions to be used to replace chloride should be carefully considered. Salts such as $NaNO_3$ and Na_2SO_4 are seemingly attractive alternatives in view of their high solubility and low price. However, both are "salting-in" salts with a considerable chaotropic action. Therefore any change in behavior of cellular systems in solutions in which high concentrations of chloride have been replaced by equivalently high concentrations of nitrate or sulfate may well be due to toxic effects of the alternative anions rather than to a specific requirement for chloride. Gluconate and glutamate as anions are less inhibitory, but unfortunately the solubility of their sodium salts is rather limited (about 1.8 M and 2.5 M, respectively). As a result, they cannot be used in experiments requiring salt concentrations approaching NaCl saturation, such as for example for some of the halophilic Archaea and for the extremely halophilic Bacterium *Salinibacter ruber* (Antón et al. 2002).

Chloride concentrations, chloride transport, and chloride requirement in the halophilic Archaea (family *Halobacteriaceae*)

There have been only few attempts to directly measure intracellular chloride concentrations within cells of halophilic Archaea. In all cases, concentrations reported were in the molar range, comparable to the medium chloride concentration. Thus, 3.61 M Cl^- was measured in cells of *Halobacterium salinarum* and 3.66 M Cl^- in *Halococcus morrhuae*, both grown in 4 M NaCl (Christian and Waltho 1962). In *Haloarcula marismortui* cells grown in 3.9 molal NaCl, apparent intracellular Cl^- concentrations were between 2.3 and 4.2 molal, depending on the physiological state of the cells (Ginzburg et al. 1970).

The finding of molar intracellular chloride concentrations in *Halobacterium* and related organisms signifies that the distribution of chloride is not in equilibrium because of the existence of an inside-negative membrane potential. It should therefore be expected that electrical potential-driven passive chloride movement would lead to a loss of chloride from the cells. Active uptake of Cl^- at the expense of energy is therefore essential for the cells to increase their volume during growth and cell division (Lanyi 1986; Schobert and Lanyi 1982). Two systems for active uptake of Cl^- have been identified in the *Halobacteriaceae*. One is a light-independent transport system in which inward transport of Cl^- is coupled with the influx of Na^+ ions (Duschl and Wagner 1986). Relatively little is known about this chloride pump. The second, much better characterized transport system is the light-driven chloride pump halorhodopsin.

Halorhodopsin is a member of the retinal proteins, of which four representatives have been characterized in the halophilic Archaea, the others being the outward primary proton pump bacteriorhodopsin and two

sensory rhodopsins. All consist of seven transmembrane helixes, in which the seventh helix has a retinal group covalently attached through the formation of a Schiff base between the aldehyde function of the chromophore and the ϵ-amino group of a lysine. Halorhodopsin was first recognized as a light-driven outward chloride pump in 1982 (Schobert and Lanyi 1982), although the presence of a second retinal ion pump in *H. salinarum* had been suggested earlier, when it was postulated to be an outward sodium pump instead (see, for example, Lindley and MacDonald 1979; Matsuno-Yagi and Mukohata 1977, 1980). The final proof that halorhodopsin is indeed a chloride pump came from a reconstitution experiment of the purified protein in an artificial membrane system (Bamberg et al. 1984).

Halorhodopsin is probably widely distributed among the members of the *Halobacteriaceae*, but no systematic survey of its occurrence has been performed as yet. Halorhodopsin of the alkaliphilic *Natronomonas pharaonis* has been studied in detail (Lanyi et al. 1990), and the pigment was also detected in several other haloalkaliphilic Archaea (Bivin and Stoeckenius 1986). The literature on halorhodopsin and its properties is very extensive. More in-depth information on this light-driven chloride pump can be found in specialized reviews (for example, Lanyi 1986, 1990; Oesterhelt 1995).

The photocycle of halorhodopsin is very similar to that of the proton pump bacteriorhodopsin, and includes isomerization of the all-*trans* form of the retinal group to the 13-*cis* form (Lanyi 1984; Schobert et al. 1983). The five intermediates of the photocycle (HR_{578}, HR_{600}, HR_{520}, HR_{640}, and HR_{565}) have been characterized using flash photolysis and fast difference spectra measurements (Tittor et al. 1987). The structure of the protein is now known in great detail thanks to electron crystallographic studies of two-dimensional crystals (resolution 5 Å; Kunji et al. 2000), and X-ray diffraction (resolution 1.8 Å; Kolbe et al. 2000). Two arginine residues (R-108 and R-200) form the anion-binding sites that donate and accept chloride ions during transport through the membrane.

There is little information on the specific chloride requirement for different cellular functions in the *Halobacteriaceae*. There may be certain enzymes that specifically require chloride for activation. We observed that the NADP-dependent isocitrate dehydrogenase activity of *H. salinarum* R1 has a huge demand for Cl^-, which cannot be replaced by gluconate. When 75% of the Cl^- in the assay mixture was replaced by gluconate (1.44 M total anion concentration), less than 20% of the activity remained, and replacement of all the chloride by gluconate led to complete loss of activity. On the other hand, the NAD-dependent malate dehydrogenase and the NAD-dependent glutamate dehydrogenase were better stimulated by 1.4–1.5 M sodium gluconate than by the same concentration of NaCl (Oren and Mana unpublished results).

Chloride concentrations, chloride transport, and chloride requirement in the halophilic Bacteria

Chloride requirement in the halophilic Bacteria

Some halophilic representatives of the bacterial domain show a specific requirement for chloride for growth. Examples are the aerobic Gram-positive *Halobacillus halophilus* (Roessler and Müller 1998), the extremely halophilic red bacterium *S. ruber* (*Cytophaga/Flavobacterium/Bacteroides* group), and the anaerobic fermentative *Halanaerobium praevalens* (see below). However, not all halophilic or highly halotolerant representatives of the domain Bacteria require chloride for growth. Well-studied organisms such as *Halomonas elongata* and *Salinivibrio costicola* are capable of sustained growth in media prepared with sodium gluconate instead of sodium chloride. It is unknown whether there still is a requirement for trace amounts of chloride, such as may be provided by the yeast extract and as contaminants in other medium components.

Intracellular chloride concentrations in different aerobic halophilic Bacteria

Intracellular chloride concentrations have only been determined in a few halophilic representatives of the bacterial domain. In some cases the apparent intracellular Cl^- concentrations are very low indeed. Examples are the value of 0.055 M Cl^- within *Halomonas halodenitrificans* cells grown in medium containing 1 M Cl^- (Christian and Waltho 1962) and 0.14 M Cl^- within stationary growth phase cells of *S. costicola*, also in medium with 1 M Cl^- (Kushner 1989; Shindler et al. 1977). When grown in 3 M NaCl, the apparent intracellular chloride concentration of *S. costicola* increased to 1.5 M (Kamekura and Kushner 1984). We measured even lower values using the X-ray microprobe in the electron microscope (0.28 ± 0.14 M Cl^- in cells grown in 3.4 M NaCl). In *H. elongata* grown in 3.4 M NaCl this value was 0.38 ± 0.11 (Oren et al. 2002). Relatively high values (0.71, 0.98, and 0.70 M Cl^-) were reported within cells of "*Pseudomonas halosaccharolytica*" grown in 1, 2, and 3 M NaCl, respectively (Masui and Wada 1973). High apparent intracellular chloride concentrations of 2.7 M were found in the gram-positive halophile *Bacillus haloalkaliphilus* when grown in medium containing 3.4 M Cl^- (Weisser and Trüper 1985). There is also a report on the intracellular Cl^- concentration of the halophilic unicellular cyanobacterium *Aphanothece halophytica*: values increased from 35 to 150 mM when the medium NaCl concentration was increased from 0.5 to 2 M (Incharoensakdi and Takabe 1988).

Halobacillus halophilus as a model for the study of chloride dependence in halophilic representatives of the domain Bacteria

The aerobic, endospore-forming, gram-positive bacterium *H. halophilus* was isolated from salt marsh sediments at the North Sea coast of Germany and originally described as *Sporosarcina halophila* (Claus et al. 1983). Based on 16S rRNA homologies, it was later reclassified as *Halobacillus halophilus* (Spring et al. 1996). Growth of *H. halophilus* is strictly salt dependent and optimal at a concentration of 0.5–2.0 M NaCl illustrating the adaptation to the salt marshes with their fluctuating salt concentrations which can be as high as 30%. Growth of *H. halophilus* is strictly dependent on Cl^-. No growth is observed at Cl^- concentrations of 0.2 M, optimal growth occurs at 0.8–1.0 M Cl^-. *Halobacillus halophilus* is the first bacterium for which a specific chloride dependence has been demonstrated (Roessler and Müller 1998). It should be noted that Cl^- can be substituted by bromide and, after some adaptation time, also by nitrate, but the final optical densities are lower in nitrate-containing media. However, this is of no physiological significance since nitrate and bromide concentrations in the ecosystem are far below those values required for optimal activity. In addition to growth, germination of endospores as well as flagella production and motility were identified to be chloride dependent (Dohrmann and Müller 1999; Roessler et al. 2000).

Halobacillus halophilus uses various organic molecules in different concentrations as compatible solutes to counterbalance the external salt concentration (Severin 1993). Therefore, the role of Cl^- is not as an internal osmolyte. What could then be the function of Cl^-? The very different functions of Cl^- point to a more global, regulatory role such as in gene activation. To test this, the effect of Cl^- on the production of flagella was determined recently on a molecular level. Therefore, flagellin, the major component of the flagellum, was purified and antibodies were raised and used to monitor the cellular flagellin content. Western blot analyses revealed that the production of flagellin in *H. halophilus* was impaired in NO_3^--containing media (Roessler and Müller 2002). However, addition of Cl^- restored the cellular flagellin pool in a concentration-dependent manner. Optimal flagellin production was achieved at 0.8–1.0 M Cl^-. In addition, the transcription of *fliC*, the gene encoding flagellin, was shown to be stimulated by Cl^- but the extent of stimulation was less compared to the effect of Cl^- on protein production (Roessler and Müller 2002). These experiments demonstrated that Cl^- acts on flagellin synthesis on both the transcriptional and translational level, but the effect on translation is much more pronounced. In addition, this was the first time that a Cl^- dependence of gene expression and protein production was shown in a moderate halophile. Two-dimensional gel analyses of protein patterns of cells grown in the presence of Cl^- or NO_3^- revealed five more proteins specifically produced in Cl^- grown cells of *H. halophilus* (Roessler and Müller 2002). These were identified as homologs from *Bacillus subtilis* proteins involved in stress protection. These experiments gave clear evidence that Cl^- is a novel environmental signal of a global regulatory network in *H. halophilus*.

What could be the function of the Cl^--dependent regulatory network? At least one function must be essential to growth since growth of *H. halophilus* is strictly Cl^- dependent. One has to keep in mind that one essential function of moderate halophiles is to sense external salinity and to respond to it on a transcriptional, translational, and enzyme activity level to adjust the intracellular pool size of the compatible solutes. The nature of the signal sensed is still unknown but it was hypothesized that Cl^- is used as a signal molecule for external salinity in *H. halophilus* (Roessler and Müller 2002). This is corroborated by the finding that transport of the compatible solute glycine betaine is strictly Cl^- dependent. In addition, glycine betaine transport after an osmotic upshock was impaired in the absence of Cl^- but restored by addition of Cl^- (Roessler and Müller 2001). Unfortunately, the transporter(s) catalyzing Cl^--dependent glycine betaine transport have not been identified yet, but these experiments clearly revealed the first Cl^--dependent glycine betaine transport system in prokaryotes. Experiments are under way to define the role of Cl^- in the accumulation of compatible solutes in *H. halophilus*.

A Cl^--dependent signal transduction chain does not necessarily require transport of Cl^- into the cell but could involve membrane-bound sensors. Although this question has not been settled, it was shown that the intracellular Cl^- (Cl^-_i) concentration increases with the external Cl^- (Cl^-_e) concentration (Roessler and Müller 1998). At suboptimal Cl^-_e concentrations Cl^-_i is ten times lower than Cl^-_e, at 0.5 M Cl^-_e the Cl^-_i concentration was 0.08 M, and in the range of 0.8–2.0 M the Cl_{-e}/Cl^-_i gradient decreased to a nearly constant value of 1.5–2. Thermodynamic calculations indicate an active transport of Cl^- which could argue for an intracellular receptor. However, additional experiments are required to define a potential Cl^- receptor.

Chloride in halophilic anaerobic members of the *Halanaerobiales*

The *Halanaerobiales* form an order consisting entirely of halophilic anaerobes. Most are fermenters, but some have a homoacetogenic metabolism. Phylogenetically they belong to the low G+C gram-positive branch of the domain Bacteria. In spite of their phylogenetic affiliation to the Bacteria, they share many physiological and biochemical properties with the halophilic aerobic Archaea. Thus, rather than accumulating organic osmotic solutes they use inorganic ions to

osmotically balance their cytoplasm with the outside medium (Oren 1986; Oren et al. 1997; Rengpipat et al. 1988). High chloride concentrations are required for growth. In experiments with *Halanaerobium praevalens* in which 60% of the NaCl in the medium was replaced by sodium gluconate or 70% of the NaCl was replaced by $NaNO_3$, while maintaining the total concentration at 1.8 M, growth ceased completely (Oren, Mana and Hasgall unpublished results).

The intracellular Cl^- concentration of *H. praevalens* cells grown in medium with 2.3 M Cl^- was estimated at 2.24 M, using bulk measurements in cell pellets (Oren 1986). Analyses of single cells with the X-ray microprobe in the electron microscope gave mean values of 1.2 and 3.3 M in cells grown in 2.3 and 3.1 M Cl^-, respectively (Oren et al. 1997). Similarly high values have been measured in *Halanaerobium acetethylicum* (1.2 and 2.5 M Cl^- intracellularly in cells grown in the presence of 1.4 and 2.7 M Cl^-, respectively) (Rengpipat et al. 1988).

Salinibacter ruber, a chloride-requiring, red, extremely halophilic member of the Bacteria

Another member of the bacterial domain that requires high chloride concentrations and that maintains high intracellular chloride concentrations is *S. ruber*. This rod-shaped red bacterium is probably a major component of the microbial community in NaCl-saturated saltern crystallizer ponds. It was recently isolated from a saltern in Spain (Antón et al. 2002).

The organism has a high salt requirement (optimum growth being achieved between 2.5 and 3.9 M NaCl, and a minimum of 1.7 M being needed for growth). Therefore a complete exchange of NaCl with sodium gluconate in the medium to test for chloride requirement is not feasible in view of its limited solubility. However, already when 20% of the chloride (3.6 M total) was replaced by gluconate, growth ceased completely. Growth was possible in medium containing 2.5 M NaCl but not in medium containing 1.875 M NaCl and 0.625 M sodium glutamate (Oren, Mana and Hasgall unpublished results).

Elemental analyses of single cells of *S. ruber* using the X-ray probe in the electron microscope showed high intracellular Cl^- concentrations, in addition to high concentrations of K^+ and Na^+. The mean apparent Cl^- concentration thus measured in cells grown in 3.3 M NaCl was about 0.9 M. In control experiments with *Halobacterium salinarum*, a similar discrepancy between the expected and the measured value was found (apparent intracellular Cl^- concentration 1.14 M in cells grown in 4.3 M NaCl), and the true intracellular concentration was probably higher than indicated. In addition, no organic osmotic solutes could be demonstrated in significant concentration within the cells (Oren et al. 2002).

Epilogue

The information accumulated thus far shows that chloride may have many specific functions in the life of halophilic microorganisms. The case of the moderately halophilic Bacteria shows that chloride is required by some, but not by all. If required, it is involved in a surprisingly large number of specific functions, and more such functions will probably be discovered in the future. The case of *H. halophilus* shows that chloride may even be a general regulator of cell metabolism on different levels.

Several groups of halophilic prokaryotes use inorganic salts for osmotic adaptation of the cells. These include the Archaea of the family *Halobacteriaceae*, the anaerobic Bacteria of the order *Halanaerobiales*, and the recently discovered *S. ruber*. In all three cases a specific requirement for chloride for growth has been demonstrated, and at least in some cases chloride is required for the activation of certain enzymes.

The specific role of chloride in the life of halophilic prokaryotes is only now becoming fully recognized. It may be expected that future studies, extending both the range of organisms used as experimental models and the range of activities tested, will show that chloride may have many more specific functions, beyond serving as the counterion of physiologically important cations such as sodium and potassium.

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