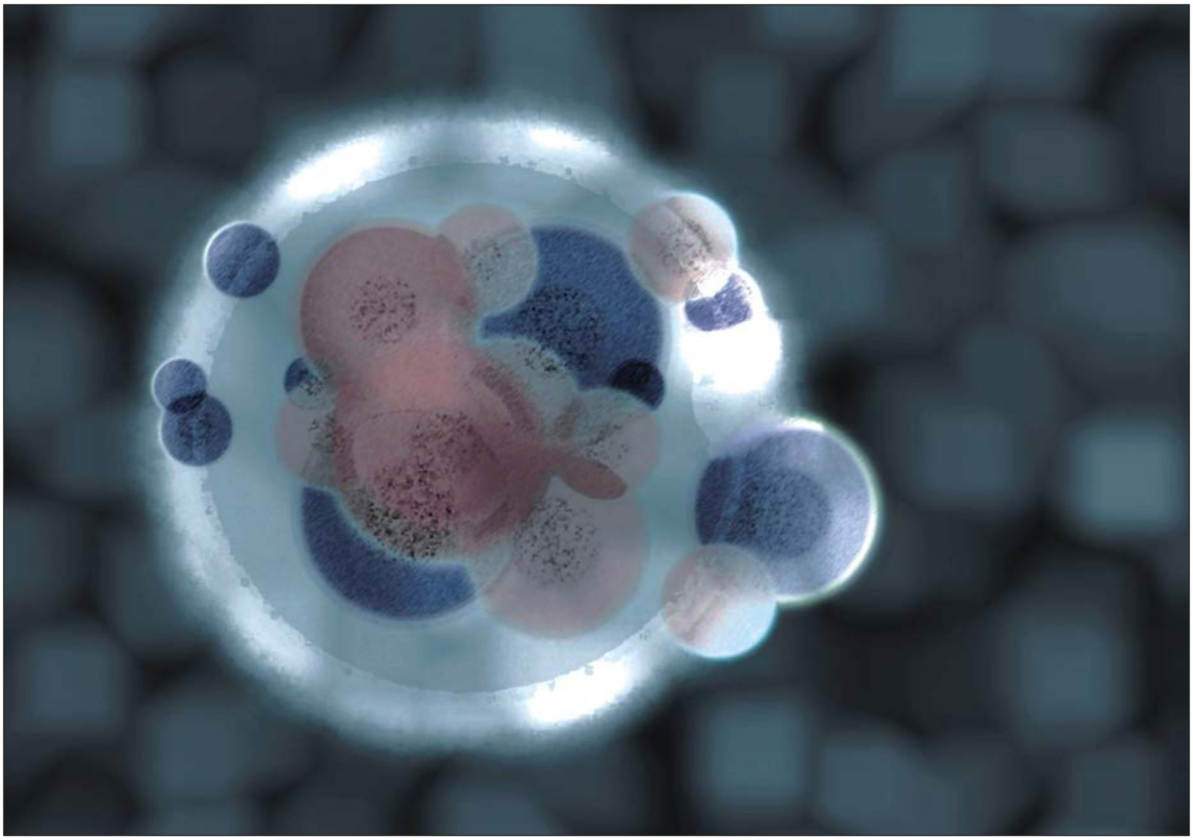

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Effects of Increasing Concentration of Salts on the Expression of *omp40* Gene Encoding a Major Porin of *Acidithiobacillus ferrooxidans*

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Abstract: Effects of increasing concentration of NaCl, KCl and Na_2SO_4 on the growth of *Acidithiobacillus ferrooxidans* cells and on the expression of *omp40* gene, encoding one of the major outer membrane proteins of *A. ferrooxidans*, were examined to obtain information on the response of *A. ferrooxidans* NASF-1 cells to environmental changes and to improve the bioleaching system operated in salt-containing environments. Although NaCl and KCl inhibited growth of *A. ferrooxidans* NASF-1 cells at the concentration of 0.3 M, *A. ferrooxidans* NASF-1 could grow in medium supplemented with 0.5 M Na_2SO_4. An analysis of outer membrane proteins by a polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate revealed that the relative amount of Omp40 was increased when cells were grown in Fe^{2+}-medium supplemented with NaCl. Northern blot hybridization analyses also revealed that the transcription of *omp40* gene was stimulated in cells incubated in medium supplemented with NaCl and KCl, but not with Na_2SO_4.

Key words: Outer membrane protein, acidophile, iron-oxidizing bacterium, western blotting analysis, osmotic pressure

INTRODUCTION

An outer membrane of gram-negative bacteria is a structure exposed to environmental changes by external stimuli. The bacteria make use to specialized nonspecific pore-forming proteins (porins) to facilitate the passage of small hydrophilic molecules through their outer membranes (Nikaido, 2003). General diffusion porins in *Escherichia coli*, OmpC and OmpF, have been well studied in response to osmotic pressure. The regulation is mediated via the OmpR/EnvZ two-component system, resulting in the qualitative and quantitative changes of outer membrane proteins (Nikaido, 2003).

Acidithiobacillus ferrooxidans (formerly *Thiobacillus ferrooxidans*) is a gram-negative, acidophilic and chemolithotrophic bacterium able to oxidize ferrous ion or reduced inorganic sulfur compounds (Rawlings, 2002; Rohwerder *et al*., 2003). This peculiar metabolism makes it one of the most important bacteria in bioleaching, the biological solubilization of metal ions from sulfide ores. The outer membrane proteins are exposed to strongly acidic environments and are involved in response to environmental changes. It is very useful for the improvement of the bioleaching system using *A. ferrooxidans* to understand the mechanism involved in the response or the adaptation of *A. ferrooxidans* to environmental changes. A major outer membrane protein having an apparent molecular mass of 40 kDa (Omp40) in *A. ferrooxidans* has been known to be organized in a trimeric structure and to form a slightly anionic channel (Jerez *et al*., 1992; Silva *et al*., 1992; Guiliani and Jerez, 2000). The structure of Omp40 protein is similar to the *E. coli* OmpC involved in response to osmotic pressure (Guiliani and Jerez, 2000). Environmental factors, such as pH and phosphate starvation have been reported to affect the level of Omp40 synthesis (Amaro *et al*., 1991; Jerez *et al*., 1992; Seeger and Jerez, 1993). The relative content of Omp40 in the outer membrane has been known to be increased when cells grown at pH 3.5 were shifted to pH 1.5 or cells were grown under phosphate-starved condition. Since Omp40 is a major outer membrane protein and has a similar structure to the *E. coli* OmpC, Omp40 is expected to be involved in response to osmotic pressure. However, the detailed information on effects of increasing concentration of salts on the synthesis of Omp40 is not available. Since the bioleaching system is operated in salt-containing environments, information on the response of *A. ferrooxidans* cells to increasing

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concentrations of salts is valuable to improve the bioleaching system operated in salt-containing environments. In this report, we examined effects of NaCl, KCl and Na_2SO_4 on the growth of *A. ferrooxidans* NASF-1 cells and on the expression of the *omp40* gene.

MATERIALS AND METHODS

Bacterial strain and growth conditions: This study was carried out from April 2003 to March 2006 at Department of Botany and Microbiology, Division of Science, Graduate school of Natural Science and Technology, Okayama University. The iron-oxidizing bacterium used in this study was *A. ferrooxidans* strain NASF-1 (NBRC101131). Cells were grown at 30°C under aerobic condition in iron (Fe^{2+})-medium (Wakai *et al*., 2004) supplemented with different concentrations of NaCl, KCl or Na_2SO_4. The number of cells in the medium was counted with a microscope and a hemacytometer (Kayagaki Irika Kogyo Co., Tokyo).

Preparation of outer membrane fraction and analysis of the proteins: Outer membranes were prepared from cells grown in Fe^{2+}-medium with or without 0.2 M NaCl according to the method of Silva *et al*. (1992). The outer membrane proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE). The relative amount of protein detected by Coomassie Blue-staining was estimated by densitometry image analysis using Malti-analyst software (Bio-Rad).

Preparation of probe and Southern hybridization: Genomic DNA (gDNA) was extracted from NASF-1 cells, as described previously (Wakai *et al*., 2004). Since a gene encoding Omp40 in *A. ferrooxidans* ATCC 18959 cell has already been sequenced (Guiliani and Jerez, 2000), primers,Omp40-F(5'-GTGTTCGGTTACGCCCAGAT-3') and Omp40-R (5'-CCCATCACGTCGTCGGCATG-3'), were constructed and used to amplify part of the *omp40* gene from NASF-1 by PCR. The PCR reaction was as follows: 3 min at 95°C, followed by 25 cycles at 95°C for 25 sec, 55°C for 30 sec and 72°C for 45 sec and then 3 min at 72°C. After the electrophoresis of PCR-amplified DNA fragments, the fragments were purified with a GeneClean Kit (Q·BIOgene) and directly sequenced, as described previously (Wakai *et al*., 2004). The PCR-amplified fragments were labeled with digoxigenin (DIG) by using a DIG DNA Labeling and Detection Kit (Roche) and used as a probe for Southern and Northern blot hybridization experiments.

Southern blotting analysis was performed with *A. ferrooxidans* NASF-1 total DNA digested with different restriction enzymes, SmaI, SacI or SalI. Hybridization signals were detected with the colorimetric reactions using a DIG DNA labeling and Detection Kit (Roche), as described previously (Wakai *et al*., 2004).

RNA isolation and Northern blot hybridization: Total RNA of strain NASF-1 cells was extracted using an RNeasy Kit (Qiagen) with an RNase-free DNase treatment, as described previously (Wakai *et al*., 2004). After the electrophoresis of RNA on formaldehyde gel, RNA was transferred to a positively charged nylon membrane (Hybond-N^+, Amersham Biosciences) by using Trans-Blot Cell system (Bio-Rad). A DIG-labeled probe for *omp40* gene was used to detect Omp40-mRNA. Prehybridizition and hybridization with the DIG-labeled probe were performed under stringent conditions. RNA hybridized with the probe was detected with the colorimetric reactions using a DIG DNA labeling and Detection Kit (Roche). The relative amount of mRNA detected by the hybridization was estimated by densitometry image analysis using Malti-analyst software (Bio-Rad).

Intensity of rRNA stained with ethidium bromide after electrophoresis was used to confirm the quantity and the quality of RNA used for Northern blot hybridization analysis.

Database analysis: Nucleotide sequences for downstream and upstream of the *omp40* gene from *A. ferrooxidans* ATCC 23270 were obtained from The Institute for Genomic Research (TIGR, http: //www.tigr.org/tdb/mdb/mdbinprogress.html).

RESULTS AND DISCUSSION

Effect of salts on growth of *A. ferrooxidans* NASF-1 cells: *A. ferrooxidans* is an obligate chemolithoautotroph and does not incorporate organic substances. The growth is strongly inhibited in medium containing organic substances (Tuovinen and Kelly, 1972). The inhibitory effect of chloride ion on growth and ferrous iron oxidation of *A. ferrooxidans* cells has also been recognized (Lazaroff, 1963; Huber and Stetter, 1989; Harahuc *et al*., 2000). A concentration of 0.14 M was reported as being toxic to this bacterium (Razzel and Trussell, 1963). On the other hand, sulfate does not show such an inhibitory effect on growth at 0.14 M and the concentration may reach as high as 1.25 M during bioleaching of sulfide minerals (Espejo and Romero, 1997). Since the growth inhibition of *A. ferrooxidans* cells by organic substances is very strong, NaCl, KCl and Na_2SO_4 were used to examine the effect of osmotic pressure on the expression of *omp40*. Strain NASF-1 was grown in Fe^{2+}-medium supplemented with different concentrations of NaCl, KCl and Na_2SO_4. Compared with growth in Fe^{2+}-medium without NaCl, growth yields after the cultivation in Fe^{2+}-medium with 0.1 M, 0.2 M, 0.3 M. NaCl for 7 days were inhibited by 30, 70 and 80%, respectively and no growth was observed in Fe^{2+}-medium with 0.5 M NaCl (Fig. 1A). Strain NASF-1 could grow in Fe^{2+}-medium supplemented with Na_2SO_4 at the concentration up to 0.5 M, although the growth yield after the cultivation for 7 days were 25% of that in Fe^{2+}-medium without Na_2SO_4 (Fig. 1B). KCl showed a relatively strong inhibitory effect on growth of NASF-1 cells. The growth yield after the cultivation in Fe^{2+}-medium with 0.1 M KCl for 7 days was 25% of that in the medium without KCl.

Effect of salts on the transcription of *omp40* in *A. ferrooxidans* NASF-1 cells: To analyze effects of increasing concentration of salts on the transcription of *omp40* by Northern blotting, a specific probe for *omp40* was prepared. DNA fragments amplified by PCR had an expected length (447 bp) of the corresponding part of the *omp40* gene. Since the nucleotide sequence was the same as that of *omp40* previously determined by Guiliani and Jerez (2000). The fragments were labeled by DIG to use as a probe for *omp40* gene. A specificity of the DIG-labeled probe was examined by Southern hybridization analysis. The only one hybridization signal was detected in each gDNA digested with endonucleases, indicating the specificity of the probe (Fig. 2). Therefore, the probe was used to analyze the transcription of *omp40* gene.

An analysis of nucleotide sequence for the *omp40* gene obtained from the TIGR pre-released genomic data of *A. ferrooxidans* ATCC 23270 revealed that the open reading frame was preceded by a plausible ribosome-binding site with an AGGA sequence and -10 and -35 promoter sequences. A stem-loop structure followed by a T-rich sequence was found downstream from the stopping UAA codon, representing a rho-independent transcriptional terminator. Therefore, the inferred length of transcribed mRNA is expected to be about 1.3 kb. One hybridization signal having the expected length was detected by Northern blot hybridization. Compared with the transcriptional level of *omp40* in NASF-1 cells grown in Fe^{2+}-medium without NaCl, the transcriptional level was increased about 1.6 times in cells grown in Fe^{2+}-medium with 0.2 M NaCl (Fig. 3A). To examine whether the increased level of transcription of *omp40* gene resulted in the increased amount of Omp40 protein, outer membranes were prepared from cells grown in Fe^{2+}-medium with or without 0.2 M NaCl and analyzed by

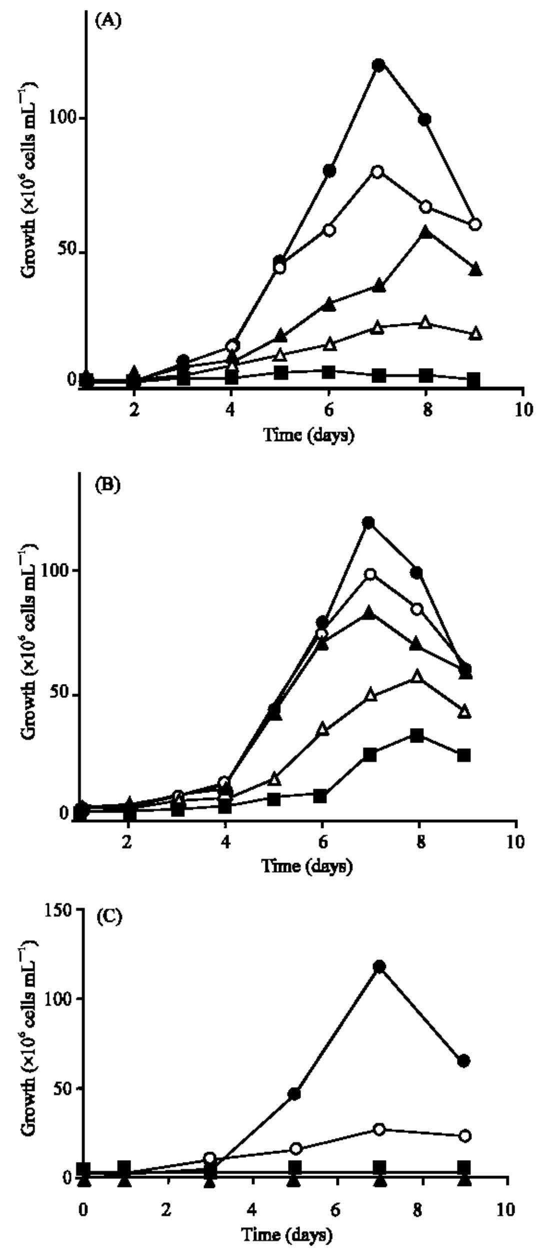

Fig. 1: Effect of NaCl (A), Na_2SO_4 (B), or KCl (C) on growth of *A. ferrooxidans* NASF-1 cells. Cells were grown in Fe^{2+}-medium with 0 M (●), 0.1 M (○), 0.2 M (▲), 0.3 M (Δ) and 0.5 M (■) of each salt

SDS-PAGE. The relative content of Omp40 increased 1.4 times when cells were grown in Fe^{2+}-medium with 0.2 M NaCl (Fig. 4). To confirm that the increased level of the transcription was not due to changes in culture phases, SDS-PAGE analysis was performed with outer membrane proteins prepared

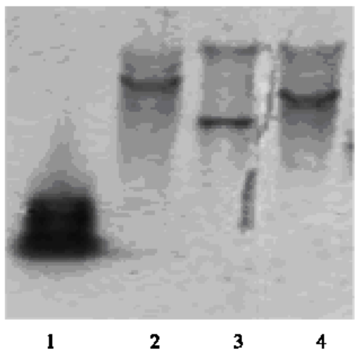

Fig. 2: Southern blot hybridization analysis of *omp40* gene. Partial *omp40* fragments amplified by PCR (lane 1) and DNA fragments digested with endonucleases, SmaI (lane 2), SacI (lane 3) or SalI (lane 4) were analyzed by 1% agarose gel electrophoresis. Southern blot hybridization was carried out by using a DIG-labeled *omp40* probe

from cells grown in middle-log phase (4 days-culture), stationary phase (7 days-culture) and late-stationary phase (14 days-culture) in Fe^{2+}-medium without NaCl. The relative amount of Omp40 was not changed in cells under various culture phases (data not shown), indicating that the presence of NaCl in Fe^{2+}-medium stimulated the *omp40* expression. The stimulation was observed not only in cells grown in Fe^{2+}-medium with 0.2 M NaCl for 7 days, but also in cells incubated in Fe^{2+}-medium with 0.2 M NaCl for 5 h (Fig. 3B, lane 4).

Since the level of *omp40* expression was increased in cells incubated in Fe^{2+}-medium supplemented with NaCl, effects of KCl and Na_2SO_4 on *omp40* transcription were analyzed by Northern blot hybridization using RNA prepared from cells incubated in Fe^{2+}-medium with KCl or Na_2SO_4 at 30°C for 5 h. Although the level of omp40 transcription was increased 1.4 times in cells incubated with 0.2 M KCl (Fig. 3C, lane 1), the level in cells incubated with 0.1 M Na_2SO_4 (Fig. 3C, lane 2) was similar

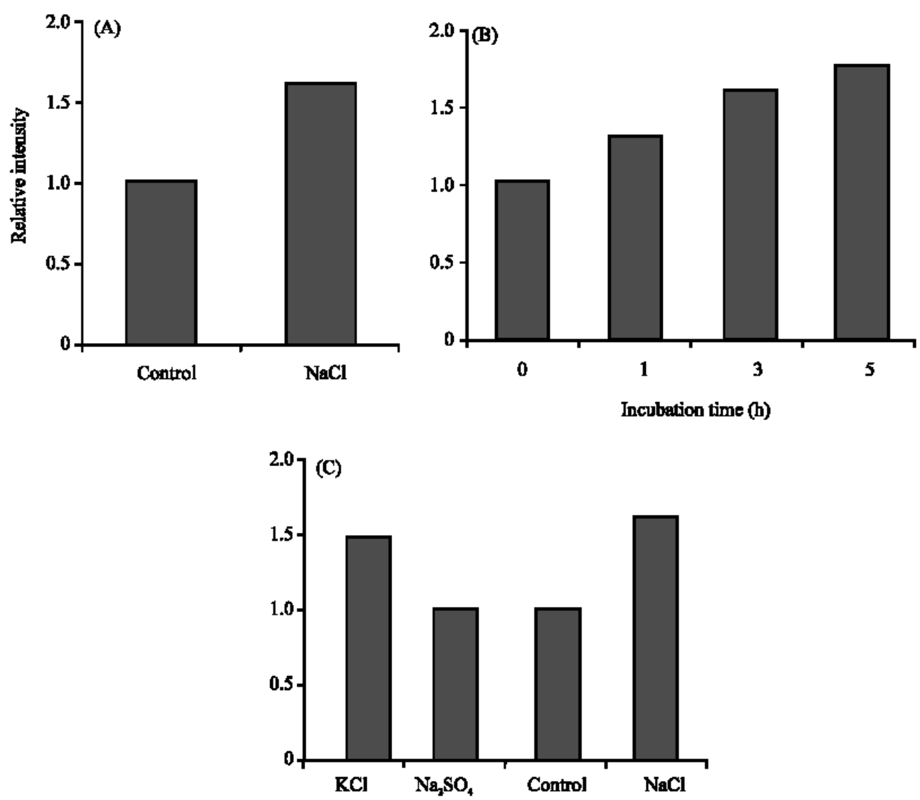

Fig. 3: Effects of salts and incubation periods on the transcription of *omp40* gene in *A. ferrooxidans* NASF-1 cells. (A) RNA was prepared from cells grown in Fe^{2+}-medium with 0 M (control) and 0.2 M (NaCl) of NaCl for 7 days. (B) RNA was prepared from cells incubated in Fe^{2+}-medium with 0.2 M NaCl for 0, 1, 3 or 5 h and (C) RNA was prepared from cells incubated for 5 h in Fe^{2+}-medium with 0.2 M KCl (KCl), 0.1 M Na_2SO_4 (Na_2SO_4), 0 M NaCl (control), or 0.2 M NaCl (NaCl). Hybridizations were carried out with a DIG-labeled *omp40* probe. The intensities of each hybridization signal were densitometrically determined. The level of *omp40* transcription was shown as the relative intensity. Intensity of rRNA stained with ethidium bromide after electrophoresis was used to confirm the quantity and the quality of RNA used for Northern blot hybridization analysis

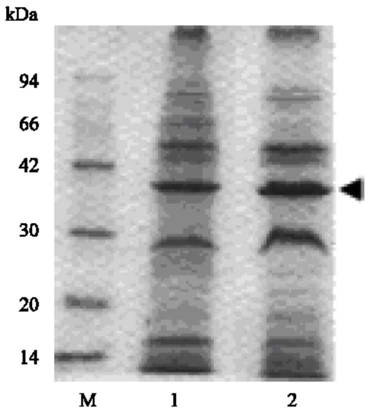

Fig. 4: SDS-PAGE analysis of outer membranes prepared from cells grown in Fe^{2+}-medium with NaCl. Outer membranes from cells grown in medium with (lane 2) or without 0.2 M NaCl (lane 1) for 7 days were analyzed. Molecular mass in kDa is indicated on the left (lane M). Gel was stained with Coomassie Blue. Arrowheads indicate the positions of Omp40 protein

to that in cells incubated without salts (Fig. 3C, lane 3). Since the same concentration of Na^{2+} was used to examine the effect of salts on *omp40* transcription, the result suggested that the stimulation of *omp40* transcription by NaCl or KCl was due to the increased concentration of chloride ion in medium, not due to the change in osmotic pressure.

It has been reported that the Omp40 channel does not discriminate well K^+ and Cl^- in KCl solution, similar to general diffusion porins (Silva *et al.*, 1992). When *A. ferrooxidans* ATCC 19859 cells grown at pH 3.5 were shifted to pH 1.5, the Omp40 synthesis has been upregulated by the pH shift (Amaro *et al.*, 1991). The characterization of Omp40 identified a large external L3 loop that could control the size of the entrance to the pore and ion selectivity at the entrance (Guiliani and Jerez, 2000). The calculated charge of this loop is postulated to control influx of proton across the outer membrane. Compared to that in the cells grown on ferrous iron, a relative content of Omp40 in *A. ferrooxidans* CCM 4253 cells has been recently reported to be increased in sulfur-grown cells, (Bouchal *et al.*, 2006). Since pH in the medium become acidic due to the production of sulfate during sulfur oxidation, the increased amount of Omp40 in sulfur-grown cells has implied that Omp40 is involved in the adaptation of the cell to acidic conditions. Since an increased concentration of chloride ion has an inhibitory effect on growth of NASF-1 cells, the cell must develop the mechanism allowing it to control the free passage of chloride ion from the outside. The results obtained in this study implied that Omp40 is also involved in the adaptation of cell to the increased concentration of chloride ion, as well as in the adaptation to acidic conditions.

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