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Full Length Research Paper

Presence of Walker B-like signature sequences on ABC-transporter proteins in the genome of *Pseudomonas aeruginosa* lytic phage and *Enterococcus faecalis* V583

Zhabiz Golkar[1]* and Nusrat Jamil[2]

[1]South Carolina Center for Biotechnology, Claflin University, Orangeburg, SC, 29115, USA.
[2]Department of Microbiology, Karachi University , Karachi, 75270, Pakistan.

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In this study, the lytic phage for multidrug-resistant (MDR) *Pseudomonas aeruginosa* was isolated; this phage belongs to the *Myoviridae* and *Siphoviridae*. Analysis of its genome sequences highlighted by different antibiotic resistance gene primers offered the most direct and sensitive method of determining the therapeutic status of this lytic phage. Interestingly, this approach reveals the existence of *vanA* cassette in the genome of this lytic phage. Whereas *van B* gene primers highlighted the polymerase chain reaction (PCR) product of 1248 bp, which has shown a relation with ABC transport proteins of *Enterococcus faecalis V583* instead of the ligase meant for the *van B* resistance trait. BLASTn analysis of the sequenced product has shown the existence of small stretches on *Pseudomonas* phage PCR product. These predicted signature sequences of phage PCR product are 100% identical with signature sequences of the same size but located at different sites on the genome of *E. faecalis V583* corresponding to ABC transporter proteins. Six signature sequences were identified. These signatures are different from Walker A and human ABC signatures. Presumably, these signatures reflected the relation with Walker B sequence. Our data suggested that *Pseudomonas* lytic phage has some proteins that have partial homologous structure to ABC-transporter with Walker B motifs. The existence of these unique signature sequences once in different ABC transporter in *E. faecalis V583* and phage genome has reflected the presence of some functional domains on these proteins that have not yet been identified, and their function need to be elucidated.

Key words: Bacteriophage, lytic phage, *van B*, ABC-transporters, Walker B signature.

INTRODUCTION

ATP-binding cassette (ABC) transporters are widespread among living organisms and comprise one of the largest protein families. For example, components of ABC transporters are encoded by approximately 5% of the *Escherichia coli* and *Bacillus subtilis* genomes (Linton and Higgins, 1998; Young and Holland, 1999). These transporters are found in all species and they are evolutionarily related (Hellen and Richard, 2004). However, they are functionally diverse and have roles in a wide range of important cellular functions.

The different ABC transporters can be assigned to classes, families and subfamilies on the basis of phylogenetic analysis (Dassa and Bouige, 2001). Interestingly, the proteins having ABC transporters that are located in the inner membrane of Gram-negative bacteria are capable of stimulating specific immune response. An examination of the antibody responses in convalescent-phase sera from individuals that had been infected by vancomycin-resistant *Enterococcus faecium* led to the selection of plasmid DNA clones coding for two amino acid sequences containing ABCs (Burnie et al., 2002).

The roles of some ABC transporters in bacterial

*Corresponding author. E-mail: zhabizg@yahoo.com or zgolkar@claflin.edu.

virulence indicate that the components of the transporters may be suitable targets for mutation for the development of live attenuated antibacterial vaccines. In one such study, mice immunized with a *Brucella abortus* mutant having a deletion in a virulence gene encoding the ABC-containing protein *ExsA* exhibited superior protective immunity against virulent *B. abortus* challenge compared to mice immunized with commercial vaccine strains (Rosinha et al., 2002). Furthermore, since ABC transporters may be immunogenic, they might also be exploited as candidate for developing subunits vaccine against pathogenic bacteria. Vaccines of this nature have been evaluated for *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* (Lefèvre et al., 1997).

MATERIALS AND METHODS

Purity determination of bacterial culture

Bacterial cultures of *Pseudomonas aeruginosa (P5* and *P6*) were streaked on Luria-Bertani (LB) agar (Sigma Co.-USA) to get isolated colonies. The purity of these isolated colonies was determined by Gram-staining, biochemical reactions, colonial and morphological characteristics.

Isolation and purification of bacteriophage

Bacteriophages were isolated from clinical specimen (urine sample of urinary tract infected patient) and raw sewage water from a local sewage treatment plant by plaque assay and spot assay techniques.

Isolation of phage from sewage

Coliphage was isolated from a sewage sample using a double agar layer technique (Qureshi and Qureshi, 1991; Rajala-Mustonen and Heinonen-Tanski, 1994). Forty-five milliliters of sample was centrifuged at 4000 rpm for 20 min to remove solid matter and the supernatant was filter-sterilized through 0.45 µM membrane (EMD Millipore Co.). To this filter-sterilized sample, 5 ml of BHI 10× supplemented with $CaCl_2$ and $MgSO_4$ (0.5 M final concentration, respectively) and 100 µL of 4 h young growth culture of *Escherichia coli* was added, and the mixture was incubated in a shaker incubator at 37°C for overnight.

Isolation of phage from clinical specimen

Urine sample from a patient (female athlete, 24 years old) was centrifuged at 6000 rpm to remove solid matters and the supernatant was then passed through a 0.45-µM pore size nitrocellulose filter (EMD Millipore Co.). Afterward, 50 µL of filtrate and 100 µL of 4 h growth of *P. aeruginosa* were mixed in 3 ml of melted L.B soft agar and plated on to L.B agar plate. It was incubated at 37°C for overnight.

Plaque purification and bacteriophage titers

Phages were purified by successive single plaque isolation and propagation. In general, a single plaque was picked from a plate using a sterile capillary tube and added to a mid-log-phase *Pseudomonas* culture (10^8 CFU/ml) supplemented with 0.1 M $CaCl_2$. In brief, 10 µL of culture mixture and phage mixture were incubated at 37°C overnight. The lysate was filtered through a 0.45-µM pore-size sterile filter (EMD Millipore Co.). Serial dilutions were made, and plaques were allowed to form on a lawn of the same host culture. Single plaques were purified through 3 successive rounds of plaquing and repeated three additional times, after which purified phages were obtained.

Determination of phage interaction with bacteria

Spot assay and plaque assay were used to determine the host range of phages as well as to check the cross infectivity.

Plaque assay by double layer method

Briefly, 100 µL of 4 h growth of *P. aeruginosa*, 50 µL of respective lysate, $CaCl_2$ and $MgSO_4$ (0.1 M final con. respectively) were added to 3 ml of melted LB soft agar tube. This was then poured on LB agar plate and incubated at 37°C for overnight. For the control sample, only lysate was omitted.

Spot assay by double agar layer method

Briefly, 100 µL of the 4 h growth culture of *P. Aeruginosa* was mixed into 3 ml of melted LB soft agar and plated on a LB agar plate. After solidification, 10 µl of phage lysate were applied on the bacterial lawn and incubated at 37°C overnight.

Transmission electron microscopy

Particle morphology was studied by precipitating the lysate with PEG 6000 (Promega Co. USA) and NaCl to a final concentration of 8 and 4%, respectively, and then incubated at 4°C overnight. The pellet was re-suspended in 100 µL of double deionized distilled water. Four hundred mesh carbon coated grids were negatively stained with 2% uranyl acetate for 30 s and examined in a GOEL-JEM-1200 EXII transmission electron microscope.

Phage DNA extraction

DNA from the phage was extracted from PS5 and PS6 phage lysate using DNA extraction kit (Promega Co., USA).

Polymerase chain reaction (PCR) amplification

The phage DNA as aforementioned was used as PCR templates. PCR products were generated by using *vanB* F:5'-AAG CTA TGC AAG AAG CCA TG-3' and R: 5'-CCG ACA ATC AAA TCA TCC TC-3' primer set (Table 1). The protocol of PCR used was denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing temperature 60°C for 30 s and extension at 72°C for 2 min. The amplified products were sized by 2% agarose gel electrophoresis and photographed.

DNA sequencing and computer analysis

Sequencing reaction was performed using MACROGEN-advancing through genomics. For alignment and comparison of similar new sequences, ClustalW.2 was used. The similarity between our data sequence and the sequence database was assessed by the use of BLAST-NCBI.

Table 1. Vancomycin gene targeted primer used for PCR.

Primer	Function	Sequence	Annealing temperature (ºC)	PCR product size (bp)
Van B	Ligase	Forward 5'-AAG CTA TGC AAG AAG CCA TG-3'	60	1248
		Reverse 5'-CCG ACA ATC AAA TCA TCC TC-3'	60	868

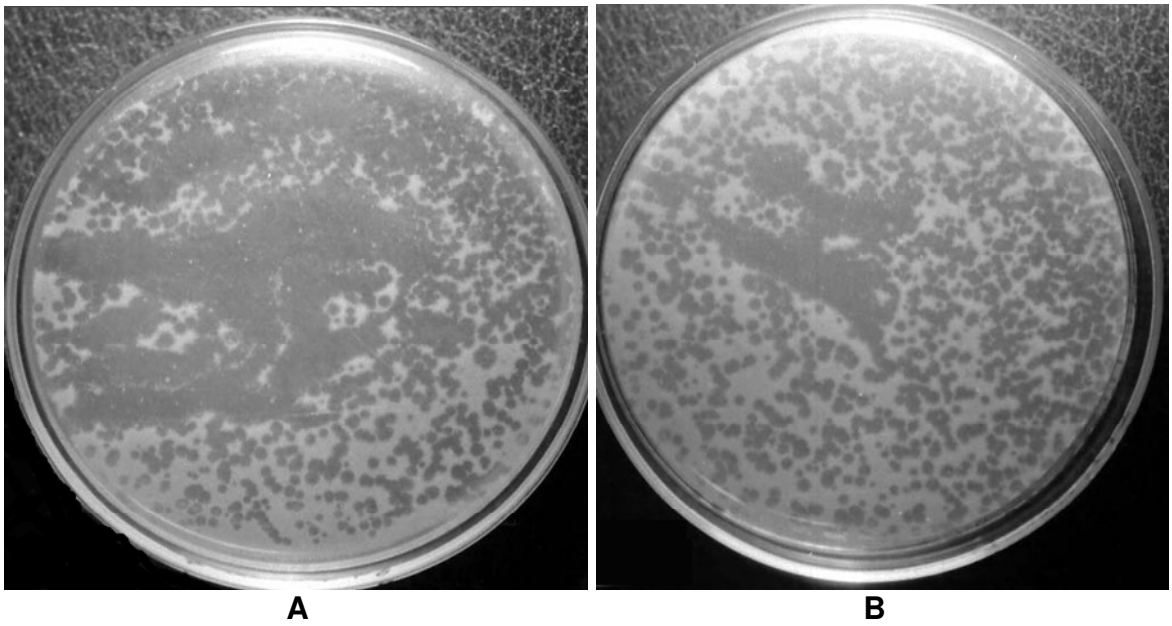

Figure 1. Plaque assay of lytic phage on the lawn of different strains of multiple drug resistance *Pseudomonas* spp. A: Plaque assay of lysate U1 on the lawn of *P5*; B: Plaque assay of lysate U2 on the lawn of *P6*.

RESULTS AND DISCUSSION

In this study, phages were isolated from a clinical specimen (urine sample) from a 24 years old female athlete suffering from urinary tract infection (UTI). From the urine sample, *E. coli* was isolated but no *Pseudomonas* was detected. Further investigation was done by using the *Pseudomonas* spp. as a bacterial lawn for detecting the host-phage interaction. Plaque assay of filter sterilized urine sample on the lawn of *P5* and *P6* exhibited enormous clear plaques due to lytic activity. (Figure 1) The electron microscopic examination indicated that these phages belong to *Siphoviridae* and *Myoviridae* phage based on morphology (Figure 2). The PCR product amplified by *van B* primer demonstrated 100% identity of four, 113 bp stretches of the phage genome with 13 bp motif of *Staphylococcus aureus MRSA252* genome that represented 1-phosphatidylinositol phosphodiesterase, a phospholipase C specific for phosphoinositide occurring in all tissues. Similarly, 114 bp stretches of phage genome has 100% identity with 12 bp related to *LysR* family regulatory protein and super antigen-like protein 7 of *S. aureus MRSA252* genome.

BLASTn analysis of this PCR product also exhibited sequence similarity with various proteins of *E. faecalis V583*. It has been documented that ABC-transporters are found in all species and are evolutionary related (Christophe and Loomis, 2002). ABC transporters are responsible for target export and import of a variety of allocrites across the cytoplasmic membrane or capsular polysaccharide in Gram-negative bacteria (Helen and Richard, 2004). Our result has shown that phage P5 has some shared similarity with membrane protein (transport protein) like ABC-transporter protein in *E. faecalis V583* (Figure 2). ATP-binding cassette (ABC) proteins of both eukaryotic and prokaryotic origins are implicated in the transport of lipids (Helen and Richard, 2004; Young, 1999; Antje et al., 2005; Schneider, 2001; Hosie and Poole, 2001).

ABC transporters are remarkably conserved in terms of the primary sequence and the organization of the domains or subunits. Characteristically, ABC transporters have a highly conserved ATPase domain (the ABC, also

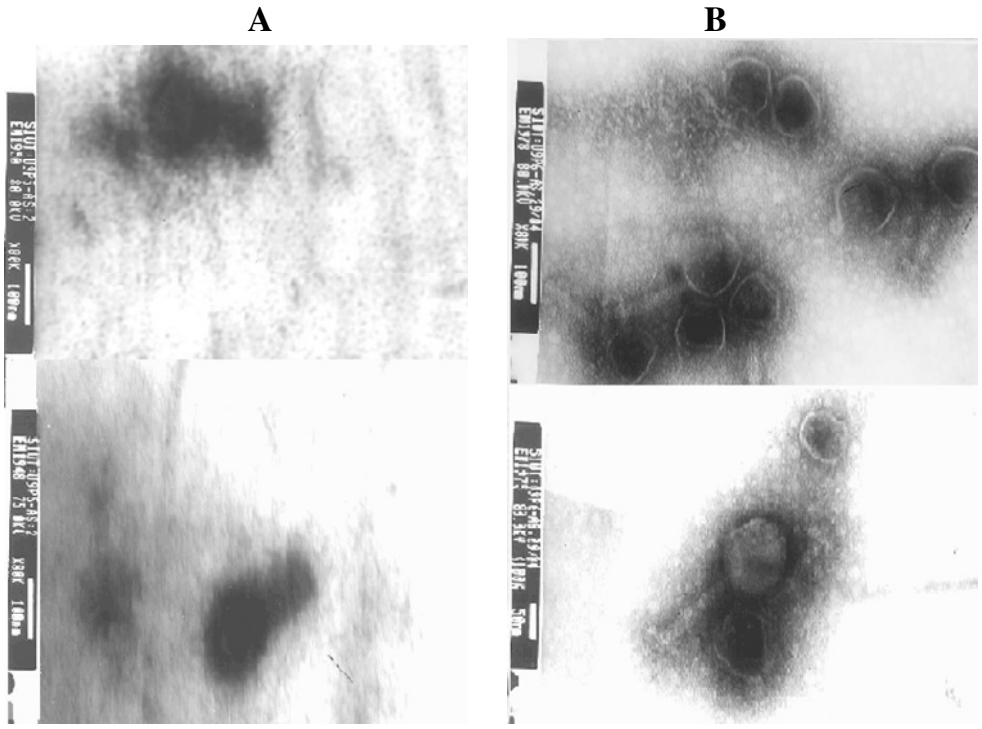

Figure 2. Electron micrograph of amplified lysates PS5 and PS6. A: lysate PS5; B: lysate PS6.

Table 2. Signature identified on phage genome and ABC-transporter of *Enterococci V583* genome.

DNA	Primer	Motifs sequence	Amino acid sequence	Description
PS5	Van B1	TGTTCAATATAATTT	TSYIK	ABC-transporter protein ATP-binding protein
		TCTTCATATCATACCATT	RSIVW-	ABC-transporter protein ATP-binding protein
	Van B	CACGCATGATGTTCAA	VRTTS	ABC-transporter protein ATP-binding protein
		TGTTCAATATAATTT	TSYIK	ABC-transporter protein ATP-binding protein
	Van B1	ATTAATACAACCCGATCA	-LCWAS	ABC-transporter protein ATP-binding protein
		GCAAGCAATAAATTTTCT	RSLFKR	ABC-transporter protein ATP-binding protein

known as nucleotide-binding domain) which binds and hydrolyze ATP to provide energy for the import and export of a wide variety of substrates (or allocrites) (Young, 1999). The ABC contains two highly conserved motifs, the walker A and walker B motifs, which together form a structure for binding ATP (Helen and Richard, 2004). The ABC protein super family functions range from the acquisition of nutrients and the excretion of waste products to the regulation of various cellular processes. Generally, ABC proteins are low capacity but high affinity transporters, capable of transporting substrates against a concentration gradient of up to 10,000 fold. Hydrolysis of ATP is required for substrate transport (Antje et al., 2005). In bacteria, ABC transporters have a diverse range of functions that may be required in response to the environments in which different bacteria find themselves. They import a variety of allocrites, including sugars and other carbohydrates (Schneider, 2001), amino acids (Hosie and Poole, 2001), peptides (Detmers et al., 2001), polyamines (Igarashi et al., 2001), metal ions (Claverys, 2001), sulfate (Kertesz, 2001), iron (Self et al., 2001) and molybdat (Köster, 2001). ABC transporters are also responsible for the targeted export of other allocrites across the cytoplasmic membrane (for example, capsular polysaccharide in Gram- negative bacteria) (Silver et al., 2001). Other exporters are responsible for the secretion of antibiotics in some extracellular toxins. Members of another class of ABC systems have roles in cellular processes, such as translational regulation (Méndez and Salas, 2001) and DNA repair (Veen et al., 2001).

Six signature sequences of FVENVF, -PLFGQFI, TSYIK, PSIVW- , VRTTS, -LCWAS, and RSLFKP were identified on ABC-transporter protein of *E. faecalis V583* (Table 2). All these amino acids are found to be hydrophobic in nature except E and N. These signatures

are different from Walker A and human ABC signatures and presumably these signatures reflected the relation with Walker B sequence, as their pattern seems to be closed to “hhhhD” motifs where “h” stands for hydrophobic. Our data suggested that *Pseudomonas* lytic phage has some proteins that have partially similar structure to ABC- transporter with Walker B motifs. The existence of these unique signature sequences once in different ABC-transporter in *E. faecalis V583* and phage genome has reflected the presence of some functional domain on these proteins that is yet to be identified, and their function need to be elucidated. It can be presumed that phage can modulate the uptake system of host by coding membrane transport protein.In order to enhance the nutritional requirement and biochemical potential of the host and uptaking the variety of allocrites during the lysogen stage of phage, this is important for phage survival and morphogenesis. Similarly, UniProtKB/TrEMBL and JCVI Gene annotation analysis has highlighted the motifs related to alcohol dehydrogenase by *vanB* primer. Oxidation-reduction is an important regulator of various metabolic function of cell. Comparative data analysis of our phage indicated that the presence of motifs related to alcohol dehydrogenase domain on phage genome can encode for energy production by phage genome and can be a tool of the phage for additional energy providing oxidation-reduction systems. The presence of this domain may play a functional role in the phage that can benefit phages by temporarily optimizing the functionality before lysis.

In this study, comparative analysis of PCR products *Pseudomonas* phage genome and database gives us a new window into phage-host interactions and their evolutionary implication. The presence of alcohol dehydrogenase domains in phage genome has indicated the independency of phage in the evolutionary tree and show that phage is fully equipped with genetic tools that not only regulates its host’s biochemical machinery which it utilizes for its own morphogenesis, but also make it capable for molecular interaction with eukaryotic cells of its host microbe. Our findings suggest that evolving site for new strains of phages is in the human body especially gut which provides an ecosystem for genetic mixing of phage and different bacteria(Gram-positive and Gram-negative) that are the numerically predominant in human colon and may act as very important reservoirs for evolution of MDR genes. The eternal host-parasite struggle has blossomed into innumerable fauna and flora, while defensive means against parasites did likewise. The prime directive of speciation is maintenance of genome integrity but this could not be achieved without symbiosis of primitive phages with the evolving host genomes (Bagasra and Pace, 2010). Our present studies provide a tiny glimpse into this Darwinian evolutionary picture.

Our analysis of the phage genome has implications not only for phage fitness, but also evolution of hosts that are influenced by genes carried by phages. There is evidence that phages may have mediated horizontal gene transfer between host and phages could have implications for evolution of both hosts and its parasitic phages, and it may represent a more general phenomenon of metabolic facilitation of host functions. Our analyses points towards a co-evolutionary pattern between phages and its host microbes where phages appear to not only regulate its host microbe’s biochemical machinery for its own morphogenesis but also make it capable of molecular interaction with eukaryotic cells. PCR product analysis of the genome of our lytic phage provide (data unpublished) the evidence that, this phage relies on its bacterial host for a number of energy dependent functions and other uptake activities during their vegetative growth or morphogenesis as well phage genes may allow for the host bacterial evolution, making them more fit for survival against its eukaryotic cells that surrounds them

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REFERENCES

Antje P, Devaux, Philippe F, Herrmann Andreas (2005). Function of prokaryotic and eukaryotic ABC proteins in lipid transport. BBA-Molecular and Cell Biology of Lipids vol. 1733 issue 1, p. 29-52.

Bagasra O, Pace DG (2010). Back to the Soil: Retroviruses and Transposons. In Biocommunication of soil-bacteria and viruses. Guenther Witzany, Ed. Chapter 6. Springer Press (p. 161-188).

Burnie J, Carter T, Rigg G, Hodgetts G, Donohoe M, Matthews R (2002). Identification of ABC transporters in vancomycin-resistant *Enterococcus faecium* as potential targets for antibody therapy. FEMS Immunol. Med. Microbiol. 33:179-189.

Christophe A, Loomis F (2002). Evolutionary Analyses of ABC Transporters of *Dictyostelium discoideum.* Eukaryot Cell. 4:643–652.

Claverys J (2001). A new family of high-affinity ABC manganese and zinc permeases. Res. Microbiol. 152:231-243.

Dassa E, Bouige P (2001). The ABC of ABCs: A phylogenetic and functional classification of ABC systems in living organisms. Res. Microbiol. 152:211-229.

Detmers F, Lanfermeijer C, Poolman B (2001). Peptides and ATP binding cassette peptide transporters. Res. Microbiol. 152:245-258.

Helen S, Richard W. Titball (2004). ATP-Binding Cassette Transporters Are Targets for the Development of Antibacterial Vaccines and Therapies. Infect. Immun. 12:6757-6763.

Hosie A, Poole S (2001). Bacterial ABC transporters of amino acids. Res. Microbiol. 152:259-270.

Igarashi K, Ito K, Kashiwagi K (2001). Polyamine uptake systems in *Escherichia coli.* Res. Microbiol. 152:271-278

Kertesz M (2001). Bacterial transporters for sulfate and organosulfur compounds. Res. Microbiol. 152:279-290.

Köster W (2001). ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B_{12}. Res. Microbiol. 152:291-301.

Lefèvre P, Braibant M, De Wit L, Kalai M, Röeper M, Grötzinger J, Delville J, Peirs M, Ooms P, Huygen K, Content J (1997). Three different putative phosphate transport receptors are encoded by the

Mycobacterium tuberculosis genome and are present at the surface of *Mycobacterium bovis* BCG. J. Bacteriol. 179:2900-2906.
Linton K. J, Higgins T (1998). The *Escherichia coli* ATP-binding cassette (ABC) proteins. Mol. Microbiol. 28:5-13.
Méndez C, Salas A (2001). The role of ABC transporters in antibiotic-producing organisms: drug secretion and resistance mechanisms. Res. Microbiol. 152:341-350.
Qureshi M, Qureshi A (1991). Polyvalent coliphages in sewage. Wat. Sci. Technol. 24:255-259.
Rajala-Mustonen R, Heinonen-Tanski H (1994). Sensitivity of host strains and host range of coliphages isolated from Finnish and Nicaraguan wastewater. Wat. Res. 2:1811-1815.
Rosinha G, Freitas D, Miyoshi A, Azevedo A, Campos E, Cravero S, Rossetti G. Splitter G, Oliveira S (2002). Identification and characterization of a *Brucella abortus* ATP-binding cassette transporter homolog to *Rhizobium meliloti* ExsA and its role in virulence and protection in mice. Infect. Immun. 70:5036-5044.
Schneider E (2001). ABC transporters catalyzing carbohydrate uptake. Res. Microbiol. 152:303-310
Self W, Grunden A, Hasona B, Shanmugam K (2001). Molybdate transport. Res. Microbiol. 152:311-321.
Silver R, Prior K, Nsalai C, Wright L (2001). ABC transporters and the export of capsular polysaccharides from Gram-negative bacteria. Res. Microbiol. 152:357-364
Veen HW, Higgins CF, Konings W (2001). Multidrug transport by ATP binding cassette transporters: a proposed two-cylinder engine mechanism. Res. Microbiol. 152:265-274.
Young J, Holland B (1999). ABC transporters: bacterial exporters—revisited five years on. Biochim. Biophys. Acta1461:177-200.