Biochimica et Biophysica Acta 1840 (2014) 2123–2127

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Inorganic phosphate uptake in unicellular eukaryotes ☆

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form 27 February 2014
Accepted 17 March 2014
Available online 25 March 2014

Keywords:
P_i transporter
Saccharomyces cerevisiae
Neurospora crassa
Trypanosoma cruzi
Trypanosoma rangeli
Plasmodium falciparum

ABSTRACT

Background: Inorganic phosphate (P_i) is an essential nutrient for all organisms. The route of P_i utilization begins with P_i transport across the plasma membrane.
Scope of review: Here, we analyzed the gene sequences and compared the biochemical profiles, including kinetic and modulator parameters, of P_i transporters in unicellular eukaryotes. The objective of this review is to evaluate the recent findings regarding P_i uptake mechanisms in microorganisms, such as the fungi *Neurospora crassa* and *Saccharomyces cerevisiae* and the parasite protozoans *Trypanosoma cruzi*, *Trypanosoma rangeli*, *Leishmania infantum* and *Plasmodium falciparum*.
Major conclusion: P_i uptake is the key step of P_i homeostasis and in the subsequent signaling event in eukaryotic microorganisms.
General significance: Biochemical and structural studies are important for clarifying mechanisms of P_i homeostasis, as well as P_i sensor and downstream pathways, and raise possibilities for future studies in this field.

1. Introduction

One important role for cellular transport systems is to allow the entry of all essential nutrients into the cytoplasmic compartment and, subsequently, into organelles, allowing for the metabolism of exogenous sources of carbon, nitrogen, sulfur, and phosphorus [1]. Monitoring the external environment is necessary for all living cells, particularly microorganisms [2]. The extra- or intracellular environment of unicellular parasites must adapt to changes. The transport of ions and organic solutes is an important modification that allows the parasite to react to modifications in the external conditions, such as the acquisition of nutrients, the elimination of metabolic waste products, and the regulation of cell volume [3].

Inorganic phosphate (P_i) is an essential nutrient required for a large number of cellular functions. In addition, P_i is involved in many biochemical reactions related to the transfer of phosphoryl groups [4–6]. Low availability of P_i in the environment is a limiting compound for the growth of several organisms [7].

Active P_i uptake by the plasma membrane is central for the maintenance of P_i homeostasis and is the initial point for the utilization of this anion [8,9]. Because of the negative electrochemical potential across the cell membrane, anionic P_i cannot accumulate in the cytosol by simple diffusion. Therefore, P_i uptake must be coupled to an inwardly directed Na^+ or H^+ gradient to facilitate the transport of P_i against the gradient [10].

Two important P_i-responsive P_i transporter families in unicellular eukaryotes are (i) the inorganic phosphate transporter (PiT) family (TCDB# 2.A.20) and (ii) the H^+ symporter (PHS) family (TCDB# 2.A.1.9). The PiT family consists of functionally characterized P_i transporters that catalyze P_i either by H^+ or by Na^+ symport. Members of the PiT family have 354 to 681 amino acid residues and 10 to 12 transmembrane domains. The PHS family is part of the major facilitator superfamily (MFS, TCDB# 2.A.1) and comprises P_i:H^+ symporters of 400–600 amino acid residues and 12 to 14 transmembrane domains [1].

This review provides a critical overview of the recent findings regarding P_i-responsive P_i uptake in eukaryotic microorganisms.

2. *Neurospora crassa*

The filamentous fungus *N. crassa*, which plays a crucial role in modern genetics, was the first microorganism in which the P_i uptake mechanism was identified. It possesses two non-homologous, high-affinity phosphate permeases, PHO-4 and PHO-5 [11]. Similar to other transport systems that have been studied in *Neurospora*, the phosphate transport system appears to depend on metabolic energy [12].

In cells previously grown in P_i-free medium, P_i transport increased significantly, with an 8-fold increase over the original rate [13]. When

☆ This work is dedicated to Adalberto Vieyra on his 70th birthday.

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http://dx.doi.org/10.1016/j.bbagen.2014.03.014
0304-4165/

N. crassa is grown with an adequate supply of phosphorus, phosphate ions are brought into the cells by the phosphate transport system I (PTSI — cyclohexamide-insensitive). This constitutive system has a low affinity for phosphate. The affinity decreases further with increasing pH, and the system barely functions above pH 7 [12]. In contrast, when the organism is grown under conditions of limiting phosphorus, a number of derepressible proteins are synthesized that are necessary for scavenging phosphorus from the environment. These proteins include a high-affinity phosphate permease, which is part of a functional entity called the phosphate transport system II (PTSII — cyclohexamide-sensitive) [13].

For the PTSII, Mann et al. [14] identified an ORF of *PHO-4* that encodes a membrane carrier protein. PHO-4p is a large hydrophobic polypeptide of 590 aa, with 12 transmembrane domains, and a K_m for P_i of 2.56 ± 0.19 μM [11]. In addition, *PHO-5* encodes a high-affinity phosphate permease, with a K_m value of 37.4 ± 2.75 μM [11]. This permease is 569 aa in length with 12 transmembrane domains [15]; however, there is no similarity between *PHO-4* and *PHO-5* [11]. Strains of *N. crassa* containing null alleles of both Δ*pho-4* and Δ*pho-5* are unable to grow under restrictive conditions, indicating that both comprise the high-affinity phosphate transport system (PTSII) of *N. crassa* [15]. PHO-5 is active at neutral pH, whereas PHO-4 is active in alkaline pH, providing P_i during alkaline stress. Moreover, PHO-4 P_i uptake is coupled to the Na^+-gradient, whereas PHO-5 is a H^+-phosphate symporter, suggesting that PHO-4 and PHO-5 may use different mechanisms for phosphate transport [16,11].

Activation of PTSII is regulated by the phosphorus acquisition system, which includes four regulatory genes, *NUC-2*, *PREG*, *PGOV* and *NUC-1* [17]. NUC-1 is a transcription factor that requires nuclear localization to activate the transcription of structural genes related to P_i acquisition [18]. Under high phosphate conditions, a cytoplasmic interaction occurs between PREG and PGOV (a cyclin-like protein and a mitogen-activated protein kinase, respectively), and the PREG–PGOV complex scavenges NUC-1 in the cytosol [19]. However, under low phosphate conditions, an ankyrin repeat protein, NUC-2, inhibits the PREG–PGOV complex and NUC-1 is translocated to the nucleus, thereby activating the transcription of P_i responsive genes [20].

Recently, it was shown that a MAPK activation cascade, the MAK-2 signaling pathway, is also related to the activation of P_i-repressible genes. In *N. crassa*, the MAK-2 signaling pathway includes at least two other MAPKs, NRC1 and MEK-2, which are involved in the hierarchical activation of MAK-2 [21]. During P_i shortage, the two MAPKs are functional and inactive under abundant P_i conditions. Moreover, the nuc-2 mutant strain of *N. crassa* presents similar levels of MAPK transcription, which suggests that the MAPK transcription rate is dependent on NUC-2 activity and indicates the complexity of the metabolic phosphorus-sensing network [22].

3. *Saccharomyces cerevisiae*

Because unicellular eukaryotes, such as yeast, interact directly with the environment, the regulation of P_i transport is maintained solely by the transduction of nutrient signals across the plasma membrane [9]. The phosphate signal transduction pathway (PHO pathway) is responsible for regulating the expression of several genes responsive to P_i that are involved in the scavenging and specific uptake of P_i from extracellular sources in yeast [5]. P_i response, mediated by the PHO pathway, is determined by the activity and localization of the transcription factor PHO4, a homolog of NUC-1 in *N. crassa*. Its localization is controlled by the phosphorylation activity of the cyclin and cyclin dependent kinase (CDK) PHO80–PHO85 complex, which is homologous to PGOV–PREG in *N. crassa*. During P_i starvation, the CDK inhibitor PHO81 acts on the PHO80–PHO85 complex to inactivate it. This allows dephosphorylated PHO4 to localize to the nucleus and associate with other transcription factor, such as PHO2, thus inducing the expression of P_i responsive genes [23,24]. P_i responsive genes codify high affinity transporters (PHO84 and PHO89), secreted acid phosphatases (PHO5, PHO11, and PHO12) and other proteins related to P_i metabolism [25].

Two major types of transporters are responsible for P_i incorporation in *S. cerevisiae*, a high-affinity and a low-affinity transporter system. The low-affinity transporter system comprises PHO87, PHO88 and PHO90, and has an apparent K_m for external phosphate of approximately 1 mM. It has also been proposed that this system is constitutively expressed system due to its insensitivity to P_i starvation conditions [26,27].

The high-affinity transporter system consists of two P_i transporters, PHO-84 and PHO-89. PHO-84 has the highest affinity (K_m = 8.2 μM), whereas PHO-89 has a low K_m value (K_m = 770 μM) [28]. PHO-84 is a H^+:P_i symporter with high activity under acidic conditions [29]. PHO-89 is a Na^+:P_i symporter that is active under alkaline conditions, having a strong preference for Na^+ [30]. *PHO-84* expression and *PHO-89* expression are regulated by PHO system activation during P_i starvation [31].

PHO-84 belongs to the phosphate:H^+ symporter (PHS) family (TC No. 2.A.1.9.1). The protein encoded by *PHO-84* ORF contains 596 amino acid residues and has a molecular size of 65 kDa, homologous to *PHO-5* in *N. crassa* [29]. This symporter is responsible for the largest amount of P_i uptake and is very sensitive to phosphate starvation conditions [32]. Wykoff et al. [33] showed that deletion of *PHO-84* causes the loss of almost all phosphate transport, indicating that low-affinity transport is down regulated in response to phosphate limitation. PKA activation is essential for down-regulation and PHO-84 degradation. Moreover, inhibition of PKA decreases PHO-84 clearance from the plasma membrane in response to exogenous P_i increases [34]. PHO-84 is part of the P_i sensor machinery and involved in the cellular responses to the exogenous P_i concentration [25]. In addition, PHO-84 acts as a transceptor, suggesting that it also has a nutrient sensor function. However, PHO-84 uses the same phosphate-binding site for transport and signaling. Using a nontransported P_i agonist, it was possible to determine that signaling requires a specific conformational change that may be part of the conformational changes that occur during transport but does not require the complete transport cycle [35].

Moreover, Δpho84 cells under high-P_i conditions overexpress PHO87, PHO90, or PHO91, thereby increasing the P_i-uptake ability and suppressing constitutive PHO5 phosphatase expression [33]. This is a major distinction from the *N. crassa* homolog PHO-5, which presents no compensatory effects in Δpho-5 cells [15].

Table 1
Kinetic parameters of P_i transport in trypanosomatid parasites.

	T. rangeli		*T. cruzi*		*L. infantum*	
	H^+:P_i	Na^+:P_i	H^+:P_i	Na^+:P_i	H^+:P_i	Na^+:P_i
$K_{0.5,\ Na}$ (mM)	–	1.2	–	4.5	–	nd
$K_{0.5,\ Pi}$ (μM)	45	58	73	9	16	nd
V_{max} (*)	7	17	13	13	9.4	nd
P_i	$H_2PO_4^-$	$H_2PO_4^-$	$H_2PO_4^-$	$H_2PO_4^-$/HPO_4^{-2}	$H_2PO_4^-$/HPO_4^{-2}	nd
ATPase	H^+-ATPase	Na^+-ATPase	(H^+ + K^+) ATPase	Na^+-ATPase	(H^+ + K^+) ATPase	nd

*pmol $P_i \times min^{-1} \times (10^7\ cells)^{-1}$.
nd — not detected.

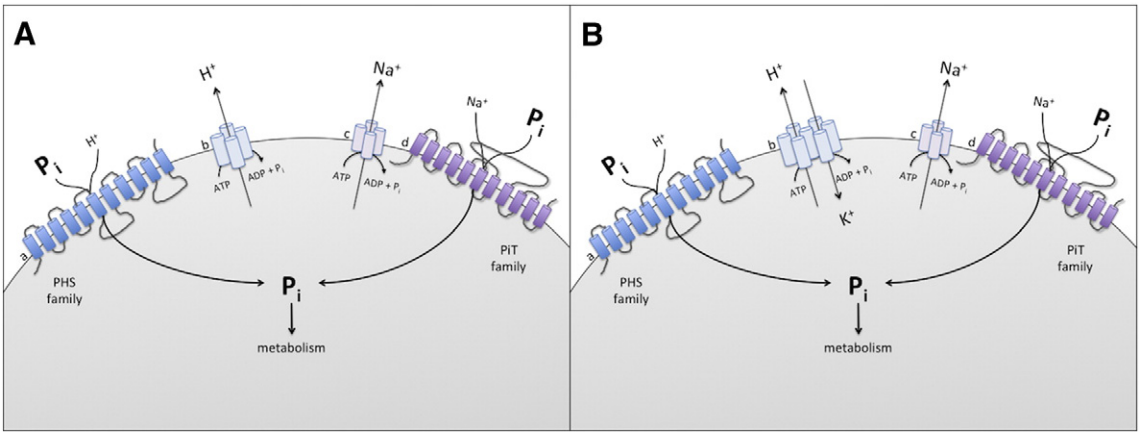

Fig. 1. Schematic model for P_i uptake mechanisms in trypanosomatids. (a) Predicted topographical model of a PHS transporter derived from the hydropathy plots of *Trypanosoma cruzi* TcPho84, *T. rangeli* TrPho84 and *Leishmania infantum* LiPho84 P_i transporter sequences. These transporters require a proton motive force provided by H^+-ATPase in *T. rangeli* (b, panel A) or (H^+ + K^+)-ATPase in *T. cruzi* and *L. infantum* (b, panel B). (c) Predicted topographical model of a PiT transporter derived from the hydropathy plots of *T. cruzi* TcPho89 and *T. rangeli* TrPho89. In this case, a Na^+-ATPase pump (d) is responsible for providing the sodium motive force required for P_i uptake through PiT transporters.

PHO-89 belongs to the inorganic phosphate transporter (PiT) family (TCDB # 2.A.20.2.2). This transporter has 574 amino acids and 12 transmembrane domains and is homologous to *PHO-4* in *N. crassa* [22,36]. Similar to PHO-84, PHO-89 is also regulated by P_i starvation via the PHO pathway [30]. Using a Δ*pho-84* mutant strain, it was shown that PHO-89 is able to catalyze Na^+-coupled P_i uptake, with a K_m for P_i of 0.5 μM [31]. Moreover, the PHO-84 transporter is maximally active at pH 5.0, a pH at which the PHO-89 transporter is largely inactive. In contrast, the PHO-89 transporter is active in the alkaline pH range, with a maximal activity at pH 9.5 [9]. Recently, using a reconstituted system, the kinetics of PHO89 were determined. It was shown that PHO89 utilizes ΔpNa^+ as a driving force for P_i uptake (maximum amount at 25 mM NaCl) and presents with an apparent K_m for P_i of 64.1 ± 23.3 μM [37]. It was suggested that the electrogenic nature of P_i incorporation via PHO-89 is because of P_i uptake stimulation by low Na^+ concentrations. In this scenario, the transport of a positively charged complex resulting from the stoichiometry of two Na^+ ions with one monovalent phosphate ion is driven by the prevalent electrical gradient (Δp) across the membrane [38].

4. *Candida* sp.

Candida albicans is an opportunistic eukaryotic pathogen that resides in the gastrointestinal tract and the oral and vaginal mucosa of many, if not all, healthy individuals [39]. There is limited information about the P_i starvation response in *C. albicans*. Cassone et al. [40] observed indirect evidence for the reduction of phosphate containing compounds in the hyphae as well as the appearance of hyphal growth in the absence of external P_i. Recently, it was shown that various isolates of *C. albicans* could respond to phosphate limitation with enhanced virulence, resulting in host death [41].

Table 2
The PiT or PHS members present in unicellular eukaryotes.

Species	PiT member	PHS member
N. crassa	PHO-4	PHO-5
S. cerevisiae	PHO89	PHO84
C. glabrata	nd	Predicted CgPHO84
P.falciparum PfPIT	nd	
T. rangeli	Putative TrPHO89	nd
T. cruzi	Putative TcPHO89	Putative TcPHO84
L. infantum	Putative LiPHO89	Putative LiPHO84

nd — not detected.

Regarding non-*Candida albicans Candida* (NCAC) species, *Candida tropicalis* is an NCAC species frequently isolated from candidiasis [42]. Therefore, *C. tropicalis* grown in P_i limited culture medium shows increased phosphate (P_i) uptake, with maximal uptake rates after 2 to 3 h of P_i-deprivation. It was shown that *C. tropicalis* has two proteins involved in P_i transport: F1 plays a role in P_i uptake at neutral pH, whereas F2 may be involved in P_i transport at acidic pH (pH 5.2–5.4, the physiological pH for growth) in the yeast *C. tropicalis* [43]. It was also demonstrated that the high-affinity P_i transporter system consists of a P_i-binding protein located near the cell surface within the cell wall and a plasmalemma-specific P_i carrier of 30 kDa. This hypothesis is supported by the observation that P_i uptake in *C. tropicalis* cells is inhibited equally by whole anti-PiBP2 antibody molecules and by their corresponding Fab fragments [44].

Another NCAC species, *C. glabrata*, has a PHO pathway similar to *S. cerevisiae*, but for P_i transporters, it does not have a *PHO-84* homolog, a predicted *CgPHO84* (Cg systematic name CAGL0B02475 g), or an ortholog to *PHO-89* [45].

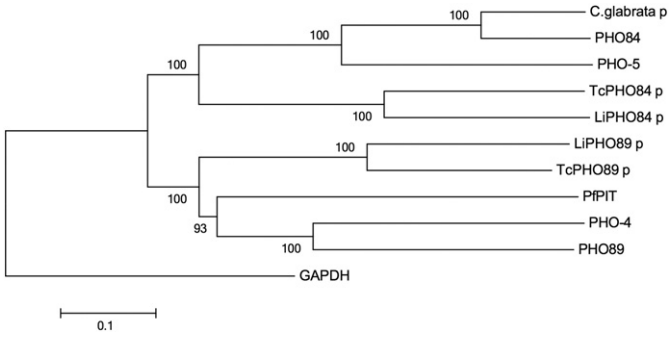

Fig. 2. Phylogenetic analysis of the PHS and PiT family proteins. Amino acid sequences from different unicellular eukaryotic species were aligned and phylogenetic analysis was performed using MEGA 5.2.2 software. The PHS family members (with their respective GenBank information) are as follows: TcPHO84 p from *Trypanosoma cruzi* (GenBank: XM_809326.1, [Dick et al., 2013]), LiPHO84 p from *Leishmania infantum* (GenBank: AFJ96967.1, [Russo-Abrahão et al., 2013]), *C. glabrata* p from *Candida glabrata* (GenBank: XM_445078.1, [Kerwin & Wykoff, 2009]), PHO-5 from *Neurospora crassa* (GenBank: AAA74899.1, [Versaw 1995]), and PHO84 from *Saccharomyces cerevisiae* (GenBank: CAA89157.1, [Bun-Ya et al., 1991]). The PiT family members (with their respective GenBank information) are as follows: PHO-4 from *N. crassa* (AAA33607.1, [Mann et al., 1989]), LiPHO89 p from *L. infantum* (GenBank: XP_001466587.1, [Russo-Abrahão et al., 2013]), TcPHO89 p from *T. cruzi* (GenBank: XP_813912.1, [Dick et al., 2013]), PfPIT from *Plasmodium falciparum* (CAE30463.1, [Saliba et al., 2006]), and PHO89 from *S. cerevisiae* (GenBank: NP_009855.1, [Martinez & Persson, 1998]). p = putative sequence with functional measurements. Outgroup: Human GAPDH (GenBank: NP_002037.2).

5. Protozoan parasites

The characterization of P_i transporters in protozoa species is a recent development. The intraerythrocytic parasite *Plasmodium falciparum*, a malaria parasite, depends on an external supply of P_i to maintain normal growth [46]. For the first time in 2006, Saliba et al. [47] characterized a plasma membrane P_i transporter (PfPIT) in *P. falciparum*, which is a member of the PiT family (TCDB # 2.A.20.2.5). PfPIT has 669 aa, is 78 kDa and contains 12 transmembrane domains. It allows the intracellular parasite to take up P_i through a Na^+ electrochemical gradient, with a stoichiometry of $2Na^+$:$1P_i$, an apparent preference for $H_2PO_4^-$ over HPO_4^{-2} and an affinity for P_i of 20 ± 2 mM. Moreover, phylogenetic analysis reveals that *PfPIT* is closer to Na^+-dependent transporters, such as *PHO-89* of *S. cerevisiae*, than H^+-dependent P_i transporters. The role of Na^+ ions in the P_i-uptake of *P. falciparum* has been studied as well. Recent data show that the growth of this parasite in low-Na^+ culture medium did not modify P_i uptake. This observation suggested that Na^+ ions were not essential to P_i-uptake. In addition, parasites could grow in culture medium containing sucrose and K^+ (~140 mM), which indicates that this parasite can be supported by a wide variety of ions [48].

Another class of protozoan parasite, the trypanosomatids, is also dependent on the presence of P_i in the culture medium for development. These parasites include *Trypanosoma rangeli* [49], *Trypanosoma cruzi* [50] and *Leishmania infantum* [51]. The presence of a system of transport and metabolism of P_i in these organisms has been hypothesized. The kinetic parameters of P_i internalization are described in Table 1.

T. rangeli possesses two independent P_i incorporation mechanisms: Na^+-independent P_i uptake that is most likely H^+-dependent and Na^+-dependent P_i uptake (facilitated by TrPHO89). *TrPHO89* shows great similarity to other putative P_i transporters encoded by trypanosomatids and the well-characterized *P. falciparum* transporter. Moreover, *TrPHO89* is similar to the well-characterized *S. cerevisiae* transporter *PHO89*. Regarding the P_i uptake mechanism, Na^+:P_i and H^+:P_i transporters facilitate P_i entry into the cytosol, which allows P_i to be utilized by metabolic pathways. Coupled H^+ extrusion by a bafilomycin A_1-sensitive ATP-driven pump and Na^+ extrusion mediated by the ouabain-resistant and furosemide-sensitive Na^+-ATPase allow for a continuous steady influx of P_i in both a Na^+-independent and a Na^+-dependent manner, according to the metabolic requirements [52].

Similar to *T. rangeli*, *T. cruzi* expresses *TcPHO84*, a H^+:P_i-symporter, and *TcPHO89*, a Na^+:P_i-symporter. As in *T. rangeli*, the furosemide-sensitive ouabain-resistant Na^+-ATPase of *T. cruzi* is responsible for providing the Na^+ electrochemical gradient. In contrast, the SCH28080-sensitive (H^+ + K^+)ATPase provides steady electrochemical gradients for H^+. Both gradients are utilized to power the secondary active mechanisms of P_i influx. In addition, the presence of a high affinity Na^+-dependent component for P_i suggests that it is important for the parasite to incorporate P_i under P_i-starvation conditions [53].

Unlike *T. cruzi* and *T. rangeli*, *L. infantum* possesses only one Na^+-independent P_i uptake mechanism. mRNA expression of *LiPHO89* is disproportionately lower than that of *LiPHO84*. In *L. infantum*, the system of P_i transport is not dependent on Na^+-ions. Therefore, LiPHO84 contributes to the acquisition of P_i by promastigote forms of *L. infantum* and to growth and survival. Furthermore, this transport is modulated by P_i deprivation [54,51]. The various mechanisms for P_i uptake in trypanosomatids are summarized in Fig. 1.

This intriguing difference in P_i transport among parasites of the same evolutionary branch may be because of differences in the expression of the PiT and PHS transporter genes.

6. Concluding remarks

P_i is a central and limiting compound in the metabolism of all organisms, including unicellular eukaryotes. Although many elements of the PHO pathway have been well elucidated for some microorganisms, biochemical and structural studies are important for clarifying the regulation of P_i homeostasis. There remain many gaps in our understanding of the role of P_i and P_i acquisition in the life cycle and pathogenesis of these organisms, as well in signaling pathways. Here, we thoroughly summarized the presence of P_i responsive P_i transporters in unicellular eukaryotes and the presence of transporter homologues in each species (Table 2). The presence of some of these transporters may be related to the evolutionary branch to which the species belongs (Fig. 2), whether the organism is intracellular or free-living, or environmental conditions. However, additional studies are needed for improved correlations. This review should stimulate further research into P_i uptake and utilization in metabolic pathways for different microorganisms.

Acknowledgements

This work was supported by grants from the Brazilian Agency Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Science Without Borders process numbers 245456/2012-0 and 245823/2012-3, the Carlos Chagas Filho Rio de Janeiro State Research Foundation process number E-26/103.040/2011(FAPERJ), the Brazilian Federal Agency for Support and Evaluation of Graduate Education process number 1973/2010(CAPES) and the National Institutes of Science and Technology, process number 161026/2012-5 (INCT), Brazil.

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