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ABBREVIATIONS

PBCV-1, Paramecium Bursaria Chlorella Virus 1; PEDV, Porcine Epidemic Diarrhea Virus; HCV, Hepatitis C Virus; YNB, Yeast Nitrogen Base; PGK, Phosphoglycerate Kinase; K2P, Two Pore domain Potassium

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) Vpu is an integral membrane protein that belongs to the viroporin family. Viroporins interact with cell membranes triggering membrane permeabilization and promoting release of viral particles. *In vitro* electrophysiological methods have revealed changes in membrane ion currents when Vpu is present, however, *in vivo* the molecular mechanism of Vpu at the plasma membrane is still uncertain. We used the yeast *Saccharomyces cerevisiae* as a genetic model system to analyze how Vpu ion channel impacts on cellular homeostasis. Inducible expression of Vpu impaired cell growth, suggesting that this viral protein is toxic to yeast cultures. This toxicity decreased with extracellular acidic pH. Also, Vpu toxicity diminished as extracellular K^+ concentration was increased. However, expression of the Vpu protein suppresses the growth defect of K^+ uptake-deficient yeast (Δ*trk*1,2). The phenotype rescue of these highly hyperpolarized cells was almost total when grown in medium supplemented with high concentrations of KCl (100 mM) and pH 7.0 but significantly reduced when extracellular K^+ concentration or pH was decreased. These results indicate that Vpu has the ability to modify K^+ transport in both yeast strains. Here, we show also that Vpu confers tolerance to the aminoglycoside antibiotic hygromycin B in Δ*trk*1,2 yeasts. Our results suggest that Vpu interferes with cell growth of *wild-type* yeast, but improves proliferation of hyperpolarized *trk1,2* mutant by inducing plasma membrane depolarization.

Furthermore, evaluation of ion channel activity of Vpu protein in Δ*trk*1,2 yeast could serve to develop a high-throughput screening assay for molecules that target the

retroviral protein.

The yeast *Saccharomyces cerevisiae* has been widely recognized as a model system for ion homeostasis studies of eukaryotic cells. Control of expression levels with inducible promoters and use of host strains with orthologous gene deletions facilitate functional studies with heterologous protein in yeast. In addition, alkali metal cation transport and homeostasis in yeast has been characterized in great detail. Depending on specific characteristics of the heterologous channel, phenotypic changes in host cells may vary widely. Fine analysis performed with M2 of Influenza virus, Kcv of PBCV-1 and PEDV

ORF3 have proven the usefulness of the *S. cerevisiae* model for characterizing ion channel activity of viroporins [1-3].

The Vpu protein of human immunodeficiency virus type 1 (HIV-1) is an oligomeric type I integral membrane protein [4;5]. Vpu enhances release of virus particles by a still unclear mechanism [6]. At present, when trying to explain the role that Vpu plays at this stage during virus replication cycle, two reported activities for the viral protein stand out. First, many studies report enhanced release of virus particles after Vpu antagonism with the IFN inducible factor tetherin (BST2) [7]. A second approach focusses on similarities between Vpu and other small hydrophobic viral proteins (viroporins) that have been suggested to promote viral particle release by plasma membrane permeabilization (as reviewed in [8;9]). Like other viroporins, Vpu associates with the plasma membrane permeabilizing it to non-permeating molecules [10;11]. Evidence has been provided that Vpu oligomers form ion channels or pores in the plasma membrane [12;13]. Besides Vpu, several other viroporins, including Influenza M2, HCV p7, PBCV-1 KcV and Coronavirus E protein among others, have membrane permeabilization activity and also ion channel capacity [14-17]. However, these viroporin-formed ion channels differ in the number of assembled-monomer units, subcellular localization and membrane topology [9]. Early *ex-vivo* and *in vivo* studies demonstrated that Vpu forms cation-selective channels in lipid bilayers and also permeabilizes *Escherichia coli* plasma membrane to proline [18]. Further studies performed in *Xenopus* oocytes suggested that Vpu ion channels are selective to monovalent cations over anions [19]. NMR spectroscopy and computer simulations pointed to an homopentamer structure with some resemblance to potassium channels and weak cation selectivity of Vpu channels [20-22]. 5-(N,N-hexamethylene) amiloride (HMA) blocks Vpu-induced cation currents in planar lipid bilayers and inhibits HIV-1 replication in macrophages [23;24]. A novel inhibitor, BIT225, also inhibits the Vpu ion channel activity [25]. Notably, Vpu-mediated tetherin antagonism is not affected by this viroporin inhibitor [26]. In addition, ion channel activity and tetherin antagonism activities localize at different positions in the Vpu transmembrane domain [27].

Vpu interacts with the mammalian TASK-1, a weak inward K^+ rectifier and their

interaction leads to mutual functional destruction [28]. In lymphoid cells, Vpu inhibits TASK-1 conductance enhancing virus particle release. Besides, in the absence of Vpu, overexpression of the first transmembrane segment from TASK-1 causes a similar enhancing effect on the virus release. Further studies have demonstrated that the efficiency of HIV-1 release is inversely correlated with membrane potential stability [29].

Here we used *S. cerevisiae* as a genetic model to analyze the ion channel capacity of Vpu protein. We found functional complementation of K^+-uptake mutant phenotypes by endogenously synthesized Vpu protein. Vpu complementation was dependent on extracellular pH and mediated by depolarization of the plasma membrane.

EXPERIMENTAL PROCEDURES

Plasmid construction and microbial strains. The *vpu* gene sequence from BH10 clone of HIV-1 [30] was cloned into the yeast expression vector pEMBLyex [31]. Following standard procedures, viral sequence was inserted at *Bam*HI and *Hind* III sites in the polylinker. The exogenous gene is controlled by the *CYCGAL1* promoter which is tightly repressed by glucose and strongly induced by galactose. The *E. coli* DH5α strain [32] was used for the construction of the *E.coli*-yeast shuttle vector. Two *S. cerevisiae* strains were used, W303-

1B [33] and isogenic mutant WΔ3 (*trk1::LEU2*, *trk2::HIS3*) [34] that were generously provided by Dr. L. Carrasco (CBM. Universidad Autónoma de Madrid. Spain) and Dr. A. Rodríguez Navarro (ETSI Agrónomos. Universidad Politécnica de Madrid. Spain), respectively.

Yeast media, *transformation and induction.* Yeast cells were grown at 30ºC, with orbital shaking at 300 rpm, in standard YNB glucose medium supplemented with 20 mg/l Ltryptophan, 40 mg/l adenine (W303-1B strain) or 20 mg/l (WΔ3 strain), and 20 mg/l Lhistidine (only W303-1B strain). For production of competent yeast and transformation, we used the standard lithium acetate protocol [35]. Transformants of W303-1B strain were maintained on selective medium (containing 20 mg/l L-leucine). To achieve *vpu* expression, yeast cells were cultured in YNB medium containing 2% galactose and required supplements (inducing medium). To avoid extraneous cations in the growth media, we adjusted pH using arginine [36]. When required, solid medium was prepared by the addition of 1.5 % purified agar (Difco) to the broth.

Assay conditions. All experiments were independently repeated at least three times, using at least three cultures derived from independent colonies, with consistent results. Growth curves represent average data from three experiments. Before any experiments, cells were grown in non-inducing medium, adjusted to pH 6.5 with arginine, to the exponential phase. After washing with MilliQ water [36], cells were diluted in fresh inducing medium and cell density was properly adjusted. The cell growth was estimated by measuring optical density at 600 nm (OD_{600}) (Genesys 10 VIS Thermo Scientific Spectrophotometer). Incubations were at 30 ºC, with shaking at 300 rpm. Mutant cells were grown in medium supplemented with 100 mM KCl to fulfill their K^+ requirements. Liquid cultures were started with exponentially growing cells that were diluted in inducing medium to OD_{600} 0.005

(corresponding to 1.5 x 10^5 cells/ml). In growth kinetic assays, after 24 h of incubation (T= 0 h), samples were collected and OD_{600} was determined. Agar plate assays were performed with exponentially growing cells. After cell washing, cultures were diluted with fresh medium to OD_{600} 0.2 (glucose) or 0.4 (galactose).

Drop tests were performed by spotting 3 or 4 µl of serial dilutions of the cell suspension onto agar plates. These tests used the same (non-inducing or inducing) medium, adjusted to pH 7.0, to make dilutions and also prepare agar plates. Plates were incubated for indicated time at 30 ºC and then scanned.

Statistical analysis. To compare growth differences between control and transfected cultures, scatter plots were created with data from replicated experiments. The growth phase was divided into two or three segments that were independently fitted to linear regression lines and slopes with correlation coefficient were calculated with the aid of

Microsoft Excel.

Protein extraction and Western blot. Exponentially growing cells were diluted in YNB (2% Galactose) to OD_{600} 0.005 before incubation. When cultures reached OD_{600} 1.0, 1 ml samples were collected for protein extraction using the following procedure. Cells were pelleted and then resuspended in 1 ml lysis buffer [0.2 M NaOH (Merck), 0.1 M βmercaptoethanol (Merck) and 0.1 mM PMSF (Roche)]. After 5 min of incubation on ice, 2 µl of 100 % TCA were added and cell lysate was incubated at 65 ºC for 5 min and then incubated at 4 ºC for 5 min. The non-soluble fraction was collected by centrifugation and washed with acetone at -20 ºC (Merck). The pellet was speed-vac dried and then protein extract was resuspended in 100 µl sample buffer [0.16 M Tris-HCl pH 6.8 (Merck), 13.3 % glycerol (Merck), 2 % SDS (Serva), 1.5 % DTT (Sigma), 0.033 % bromophenol blue (Sigma)]. Finally, samples were sonicated and heated to 100 ºC for 5 min. Total protein content of each extract was assessed using Protein assay kit (Bio-Rad). All cellular extracts were adjusted to equal protein concentrations (1mg/ml), before loading onto a 20 % polyacrylamide gel for separating proteins by SDS- PAGE. After gel running, Vpu and PGK proteins were detected by Western blotting. Proteins were transferred to nitrocellulose membranes (Trans-blot Transfer Medium Bio-Rad) by wet blotting. The efficiency of protein transference was checked by staining proteins on the membrane using Ponceau S.

Blots were quickly destained in distilled water and then incubated with a 1:5,000 dilution of Vpu polyclonal antibody [10] and PGK monoclonal antibody (Molecular Probes). After incubation with a 1:10,000 dilution of a secondary peroxidase-conjugated goat (antirabbit/anti mouse) IgG antibody (Pierce), labelled proteins were detected using ECL™ Western Blotting Detection Reagents (Amersham). Blots were exposed to X-ray films to visualize chemiluminescence signal.

Hygromycin B sensitivity. Changes in membrane potential in yeast plasma membrane were tested by measuring the entry of the antibiotic hygromycin B (Roche). Cells were grown to exponential phase and then washed with MilliQ water and resuspended in inducing medium, adjusted to pH 7.0 and supplemented with KCl 30 or 100 mM. Final cell density was adjusted to OD_{600} 0.003. Increasing concentrations of hygromycin B were added to cells. After 45 or 48 h of incubation, the cellular density of each culture was determined by measuring the OD_{600}. Relative growth was calculated as the ratio between growth in the presence and absence of added antibiotic and expressed as a percentage.

RESULTS

Inducible expression of Vpu protein in S. cerevisiae. Our initial rationale was that structural

and functional similarities between yeast and human cell membranes [37;38] might help characterize *in vivo* how Vpu-plasma membrane interactions permeabilize cells. We used high copy plasmid, pEMBLyex4, to express Vpu in *S. cerevisiae* W303. Phosphoglycerate kinase (PGK), one of the most abundant proteins in the cell, enabled us to monitor the relative content of the viral protein. As shown in Fig. 1A, yeast growth in induction liquid medium yielded cells producing high levels of Vpu protein when bearing *vpu*-plasmid. Clones bearing *vpu*-plasmid or empty plasmid (control cells) were grown in solid minimal medium containing glucose and also in induction medium containing galactose (Fig. 1B). In glucose medium, both clones grew at the same levels. However, in induction medium, cells expressing *vpu* gene showed impaired growth relative to that of control cells. The growth defect of Vpu-expressing cells disappeared after longer incubation (data not shown); even so, the drop test documented growth differences of about a two-fold dilution.

This finding suggests that HIV-1 Vpu exhibits deleterious effects on yeast cells but cultures can recover from such stress probably using their adaptive plasticity in response to changes in the ionic fluxes [39].

Sensitivity to extracellular conditions. Some channels expressed in yeast are only functional over a narrow pH range [40]. We assessed the growth characteristics on liquid cultures of both clones (bearing empty or *vpu*-plasmids) at various pHs (Fig. 2). Time course comparison of both transformants at pH 5.4 revealed a nearly indistinguishable growth pattern, although growth of *vpu*-expressing cells was slightly delayed. Thus, growth differences were shown from the beginning of the growing (slopes S1). At more neutral pHs, growth differences were more evident. Thus, major differences in growth rates between both cultures were reached at pH 7.0. The highest rate of growth was reached by *vpu*-expressing cells just after control cells shifted from fast to slow growth (pH 6.5 and 7). Western blot analysis, with PGK serving as a loading control, demonstrated that Vpu levels did not vary with pH of medium (data not shown). However, after longer adaptation periods as the pH became more neutral, *vpu*-expressing cultures reached the same growth as the control. These results suggested that *vpu*-expression were unable to adapt to neutral pH_{out} as well as control cells, indicating that deleterious effect of Vpu was pH-dependent.

Having established that Vpu-induced growth impairment is modulated by extracellular pH, we were interested in investigating how Vpu protein may affect transport systems at the plasma membrane. Potassium is the major inorganic cation in the cell cytoplasm. Although yeast cells can grow in media with a wide range of extracellular potassium concentrations (low mM- µM), intracellular stores are always maintained around 200 to 300 mM by means of a mechanism of transport that may vary depending on the growth conditions [41]. We analyzed Vpu effect when yeasts were cultured with varying potassium concentrations, by supplementing growth media with 10 or 100 mM KCl (Fig. 3). Both *vpu*-expressing and control cultures reached faster growth rates with increasing potassium concentrations. However, under conditions of standard medium (7 mM K^+) growth delay of *vpu*-expressing cells was very significant; they hardly reached the highest growth rate when control culture shifted to slower growth. When growth medium was supplemented with 10 mM KCl, growth of *vpu*-expressing yeast was also delayed, though the slope differences between both cultures were reduced. When growth medium was

supplemented with 100 mM KCl, *vpu* expression slightly delay the early growth but cells recover rapidly from it and growth differences were almost indistinguishable between both transformants. These results suggest that the Vpu protein interferes with potassium uptake or its accumulation inside

cells.

Channel activity in K^+-uptake defective cells. The conditional negative phenotype of *Δtrk1,2* cells has been used to detect function of heterologous potassium channels by restoration of growth [40]. Therefore, the auxotrophic double mutant strain, WΔ3, was used for phenotypic complementation studies, allowing us to specifically test for the K^+ channel activity of Vpu. In this strain, the deletion of TRK1 and TRK2 transporters abolished highaffinity uptake of extracellular potassium, resulting in impaired cellular growth on potassium–limiting medium. We transfected the K^+-uptake defective strain with control or *vpu*-containing plasmids. As shown in Fig. 4, when grown in the presence of galactose, high levels of Vpu protein were synthetized by cells transfected with plasmid containing the retroviral gene. Notably, these *vpu*-expressing cells grew to a greater extent than control cells. Drop test showed that both cultures differed by more than a two-fold dilution. These results revealed that Vpu can rescue the growth phenotype of *Δtrk1,2*. Vpu complementation with yeast Trk-system corroborated its ion permeation ability. Unfortunately, it was not possible to prove the capacity of HMA to inhibit this Vpuinduced growth enhancement; the intrinsic stimulation of yeast growth by the DMSO solvent was too high to permit reliable detection of growth differences induced by the dissolved drug (data not shown).

Double mutant yeasts exhibit hypersensitivity to low extracellular pH that can be

suppressed by the addition of high concentrations of potassium to the medium [42]. We tested Vpu impact on growth behavior of mutant cells at different extracellular pHs ranging from 5.4 to 7.0 and in the presence of 100 mM KCl (Fig. 5). When grown at pH 5.4, mutant cells could hardly grow but a slight advantage of *vpu*-expressing culture was detected for early growth compared with control mutant. As pH increased, the Vpu-induced growth advantage became more pronounced. At pH 7.0, OD_{600} difference between both cultures reached 8.7 ± 0.35 fold (50h); in the absence of Vpu protein, the log-phase was delayed but Vpu endowed mutant cells with growth characteristics similar to those of isogenic *wild-type* cells (shown in Fig. 3). Thus, these observations demonstrated that Vpu restored the growth of K^+ uptake–deficient cells.

To gain further insights into the mechanism by which Vpu is involved in rescuing the mutant phenotype, potassium requirements for growth stimulation of Trk-deficient cells were tested (Fig. 6). Growth of mutant cells depended on the presence of high concentrations of extracellular K^+ but Vpu endowed mutant cells with a growth advantage, even at low extracellular potassium (10 mM KCl). We observed that K^+-uptake mutants exhibited growth defects. The final OD_{600} and growth slope of mutants are much smaller than isogenic *wild type* values (Fig. 3). However, under a non-limiting potassium concentration (100 mM KCl) Vpu protein shortened the lag-phase and increased growth yield of mutant strain, which now showed similar growth kinetics to those of the *wild-type* strain. These results indicated that Vpu protein was able to catalyze low-affinity K^+ uptake.

Impact of Vpu on low-affinity K^+ transport. Similar ionic properties of potassium and ammonium permit, under limiting potassium concentrations, the entry of ammonium via potassium channels. Thus, ammonium inhibits K^+ low-affinity uptake in *Δtrk1,2* cells [34]. In order to avoid any competitive ammonium inhibition, we used arginine as the unique nitrogen source during culture of transformed mutant cells [36]. K^+-uptake deficient cells grew to a lesser extent in ammonium-free medium than in ammonium containing medium (Fig. 7). Even though Vpu ameliorated potassium requirements in both media, differences in K^+ requirements were much more appreciable in the absence of ammonium. This dramatic increase in tolerance to low K^+ concentration indicates a possible competition between potassium and ammonium during Vpu-facilitated uptake.

The knockout of both Trk potassium transporters has been reported to result in high membrane hyperpolarization leading to increased sensitivity to hygromycin B [34]. We used the sensitivity towards this cationic antibiotic as an indirect indicator of plasma membrane potential of *Δtrk1,2* transformants (Fig. 8). Under low K^+ conditions (30 mM KCl) vpuexpressing cells exhibited high tolerance to 10 mg/ml hygromycin B in comparison with control cells. However, under near-physiological K^+ conditions (100 mM KCl), differences in hygromycin B tolerance between both transformants were significantly reduced. Thus, given that resistance to the positively charged aminoglycoside is closely correlated with a depolarization of the cellular membrane potential [43], the presence of Vpu protein should decrease the membrane potential of K^+ uptake-deficient cells.

DISCUSSION

Vpu induces changes in composition and functioning of plasma membrane that may lead to the enhancement of virus particle release from the plasma membrane [29;44]. This viroporin enhances even the release of unrelated viruses, such as Sindbis virus, Ebola Virus, etc [45;46]. A debate is opened about the dual pore/ion channel functioning of Vpu viroporin during virus replication cycle [12;13]. We previously demonstrated that HIV-1 Vpu enhances permeability of the plasma membrane in prokaryotic and mammalian cells [10]. Here, transfected yeasts produce high levels of Vpu that result in growth impairment similar to that reported for the Influenza M2 protein [2]. Notably, the Influenza viroporin affected the transmembrane proton flux in *wild type* strain. Instead, we found that the HIV-1 viroporin modifies the potassium transport in both *wild type* and K^+-uptake mutant strains. Defective growth phenotype of *trk1*, *trk2* null mutant cells is fully rescued by expressing the Vpu protein, in agreement with observations reported with other viroporin channels such as Kcv of PBCV-1 and PEDV ORF3 [1;3].

Vpu protein complements the potassium uptake deficient phenotype in a pH_{out} dependent manner. This behavior is similar to that reported for mouse inward K^+ rectifying channel, Kir2.1 [47]. In yeast, Trk1 and Trk2 are involved in the response of the membrane potential

to changes in the external pH, increasing the permeability to H^+ [34]. This may be the reason why pH-dependence of Vpu activity was stronger in the defective mutant than in the *wildtype* strain. The K^+ uptake complementation by Vpu is more evident in the absence of ammonium than when present. This suggests that Vpu may function as a non-specific cation channel with inward transport of K^+ and also ammonium ions.

The knockout of both Trk potassium transporters has been reported to result in high plasma membrane hyperpolarization [34]. At extracellular pH 7 and high potassium concentration, Vpu-expressing mutants grow even better than non-expressing *wild-type* cells in conditions of K^+ restriction, but not control mutants. The Vpu-induced restoration of growth phenotype in mutant cells suggested a restoration of membrane potential as a result of increased inward transport of ectopic potassium. *Wild type* yeasts are sensitive to hygromycin B and become resistant to the drug by defects in the plasma membrane H^+ATPase, that result in membrane depolarization [43]. Thus, the hyperpolarized *tkr1*Δ*tkr2*Δ

strain is hypersensitive to hygromycin B [34]. Notably, our results show that Vpu confers hygromycin B resistance to the hypersensitive *tkr1*Δ*tkr2*Δ strain. This evidence suggests a depolarizing role for the Vpu protein in eukaryotic cells. Our results suggest that Vpu interferes with the signaling regulation of potassium transport [48]. It is possible that depolarization by Vpu protein destabilizes Trk system at the plasma membrane. Thus, under low K^+ conditions Vpu would interfere with potassium homeostasis by accelerating the turnover of essential transporters.

Taken together, these data support a model in which Vpu depolarizes plasma membrane by forming channels with weak selectivity for K^+. The ion channel activity of Vpu should affect functioning of cellular channels involved with ion homeostasis and eventual membrane disturbance. In fact, recent studies in human cells showed that vesicle release

was promoted as a result of silencing K2P channels [49]. Similarly, Vpu channel may depolarize the budding membrane, making particle release an energetically favorable process, as previously proposed. Thus, our results are compatible with suggested changes in composition and functioning of plasma membrane that may lead to the Vpu-induced

enhancement of virus particle release from the plasma membrane [29;44].

Contrary to most studies, which have used the transmembrane domain of Vpu or a fusion protein including full sequence, here we used genuine Vpu protein already synthetized in a cellular system. Although other models could just show a slight cation preference of Vpu channels, here we specifically demonstrate that Vpu modifies K^+ transport through plasma membrane. Owing to its good phenotype complementation, *trk1 trk2* null mutants of *S. cerevisiae* represent a promising model for screening of inhibitors of channel-forming Vpu protein and also other viroporins.

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FIGURE LEGENDS

Figure 1. Growth impairment of cells expressing *vpu* gene. Cells, transformed with control plasmid (-) or *vpu*-plasmid (+), were inoculated into glucose or galactose containing medium. A) Yeasts were grown in liquid media with vigorous aeration. Expression levels of Vpu protein were analyzed in each culture by Western blot, using PGK or Vpu antibodies. B) Drop test analysis with series of two-fold dilutions. Before digitally scanned, plates were incubated for 2 (glucose) or 3 (galactose) days.

Figure 2. pH_{out} sensitivity of the Vpu activity in *S. cerevisiae*. Cells were grown in inducing media adjusted with arginine to the corresponding pH. OD_{600} was measured at indicated times. Control (■) and Vpu (□). Growth curves represent the average of three independent experiments with error bars indicating standard deviation (mean ± SD). Linear regression lines were fitted to the replicate data included into two or three segments indicated with arrows in each curve. S1, S2 and S3 indicate the corresponding values obtained for slope (R^2) of the early (1), middle (2) and late (3) segments respectively.

Figure 3. Extracellular K^+ concentration dependency of growth impairment by Vpu protein. Cells, transformed with control or *vpu*-plasmid, were grown in inducing media adjusted to pH 7.0 and containing different K^+ concentrations. The OD_{600} is plotted versus time (in hours). Control (■) and Vpu (□). Growth curves represent the average of three independent experiments with error bars indicating standard deviation (mean ± SD). Linear regression lines were fitted to data included into two or three segments indicated with arrows in each curve. S1, S2 and S3 indicate the corresponding values obtained for slope (R^2) of the early

(1), middle (2) and late (3) segments respectively.

Figure 4. Complementation of growth defect by *vpu* expression in a Δ*trk1* Δ*trk2* yeast mutant. Cells, transformed with control (-) or *vpu*-plasmid (+) were inoculated into glucose or galactose medium, supplemented with 100 mM KCl. A) Cells were grown in liquid media with vigorous aeration. Western blot used PGK or Vpu antibodies. B) Drop test analysis of serial two-fold dilutions. Before digitally scanned, plates were incubated for 2 (glucose) or 3 (galactose) days.

Figure 5. Acid sensitivity of Vpu channel activity. Δ*trk1* Δ*trk2* cells were grown in inducing media, supplemented with KCl 100 mM and adjusted with arginine to appropriated pHs. OD_{600} was measured at indicated times. Control (■) and Vpu (□).

Growth curves represent the average of three independent experiments with error bars indicating standard deviation (mean ± SD). Linear regression lines were fitted to data included into two segments indicated with arrows in each curve. S1, S2 and S3 indicate the corresponding values obtained for slope (R^2) of the early (1), middle (2) and late (3) segments respectively.

Figure 6. Modulation of Vpu phenotype complementation in *trk*1,*trk*2 null mutants by extracellular K^+ concentration. Δ*trk1* Δ*trk2* cells, transformed with control or *vpu*-plasmids, were grown in inducing media adjusted to pH 7.0 and containing different K^+ concentrations. The OD_{600} is plotted versus time (in hours). Control (■) and Vpu (□). Growth curves represent the average of three independent experiments with error bars indicating standard deviation (mean ± SD). Linear regression lines were fitted

to data included into two segments indicated with arrows in each curve. S1 and S2 indicate the corresponding values obtained for slope (R^2) of the early (1), middle (2) and late (3) segments respectively.

Figure 7. Influence of type of nitrogen source on the release from potassium requirements of mutants by Vpu channel. Δ*trk1* Δ*trk2 c*ells, transformed with control or *vpu*-plasmids, were suspended in standard medium ($+NH_4^+$) and also in modified medium ($-NH_4^+$); both media adjusted to pH 7.0. Drop test analysis with series of 5-fold dilutions. Before digitally scanned, plates were incubated for 5 ($+NH_4^+$) or 8 ($-NH_4^+$) days.

Figure 8. Influence of Vpu protein on the Hygromycin B sensitivity of *trk*1,*trk*2 null mutants. Control cells (black) and Vpu cells (white) were grown in the presence of increasing hygromycin B concentrations (µg/ml). Drug sensitivity was tested using induction medium supplemented 30 or 100 mM KCl. OD_{600} was determined after 45 and 48 h of incubation. Data are represented as mean ± SD of each pair of measurements.

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Figure 1

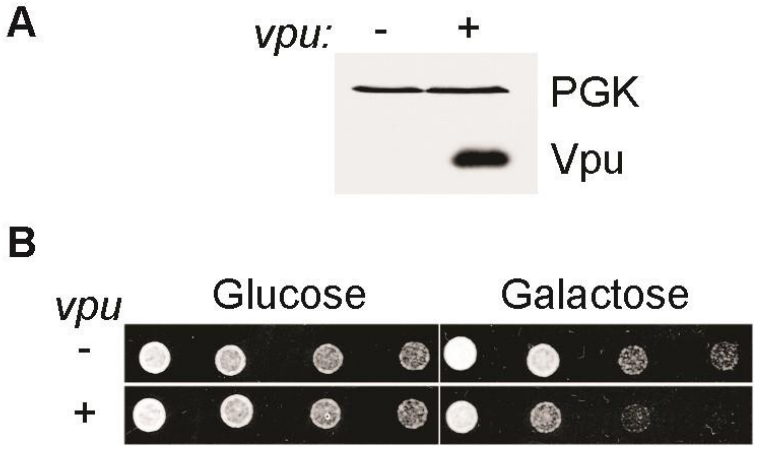
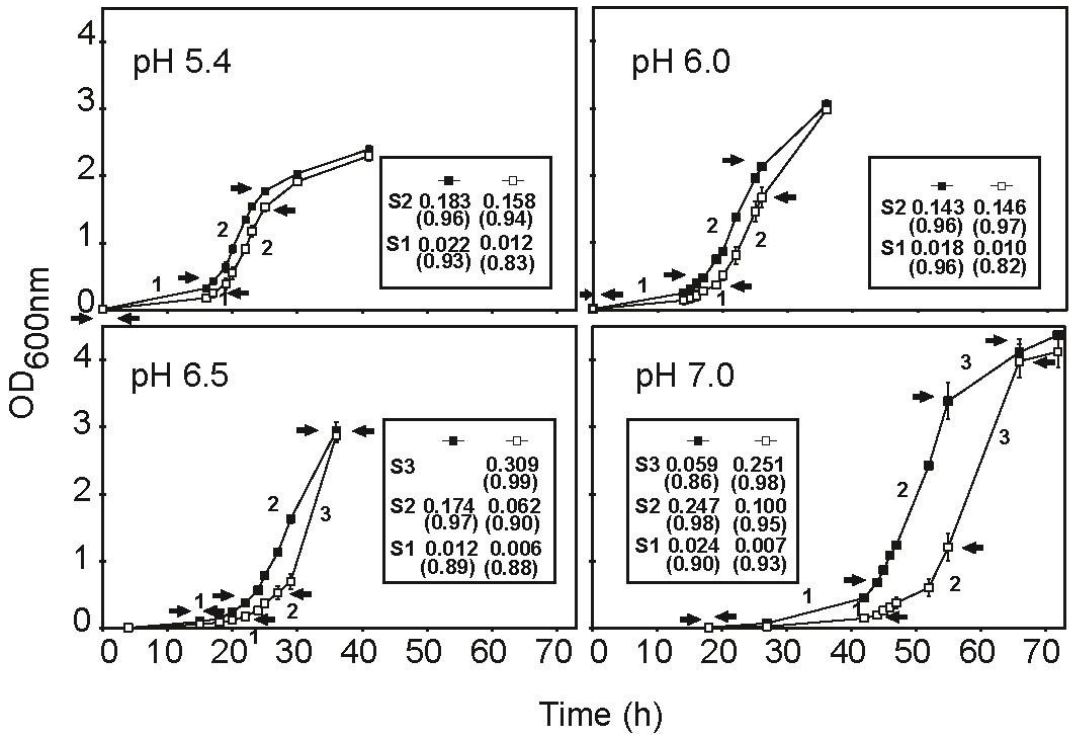


Figure 2



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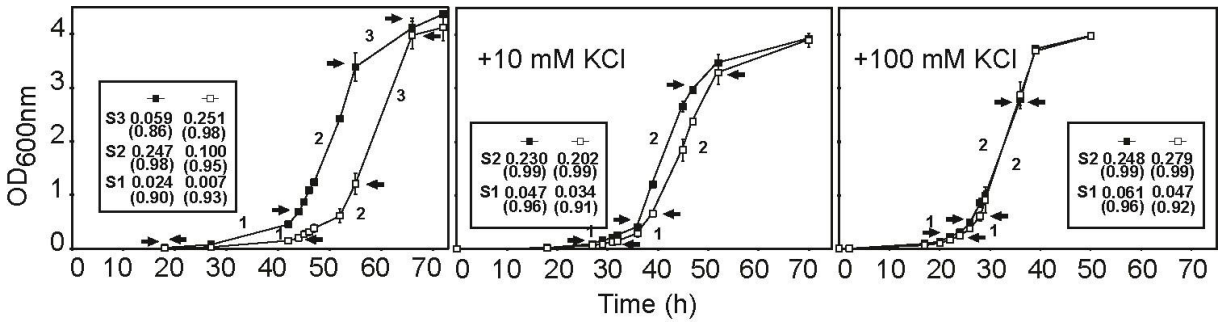

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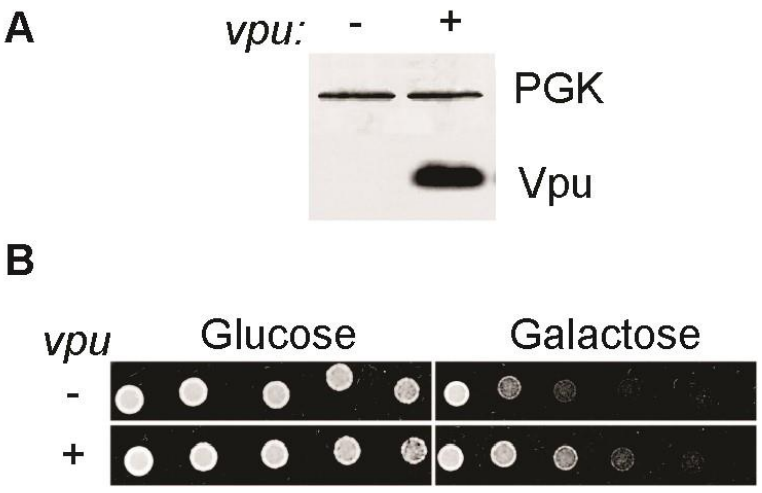

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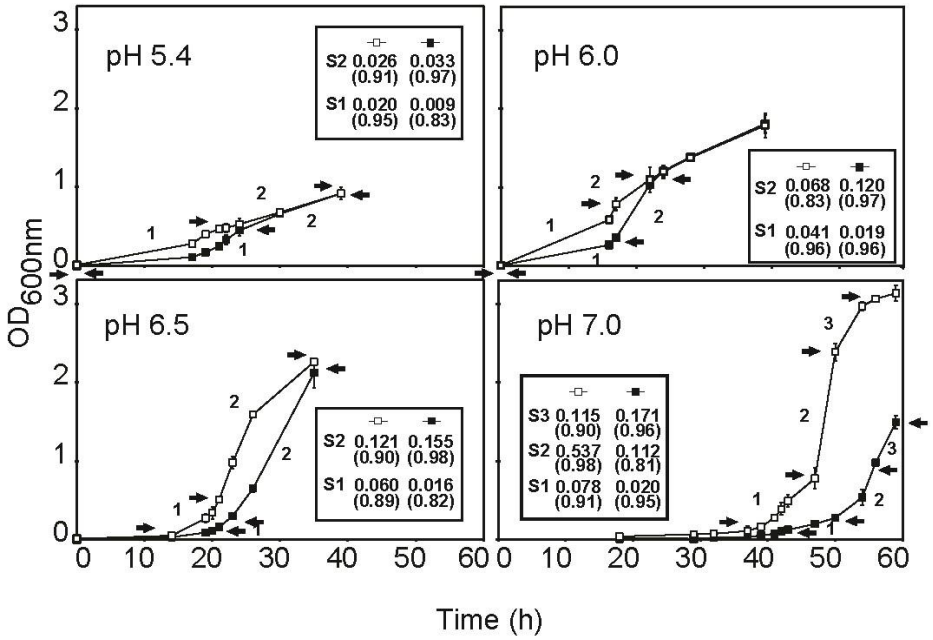

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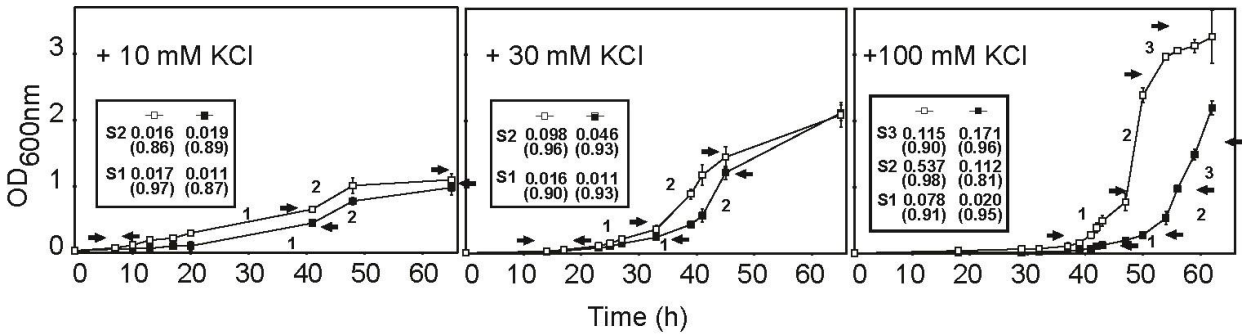

Figure 7

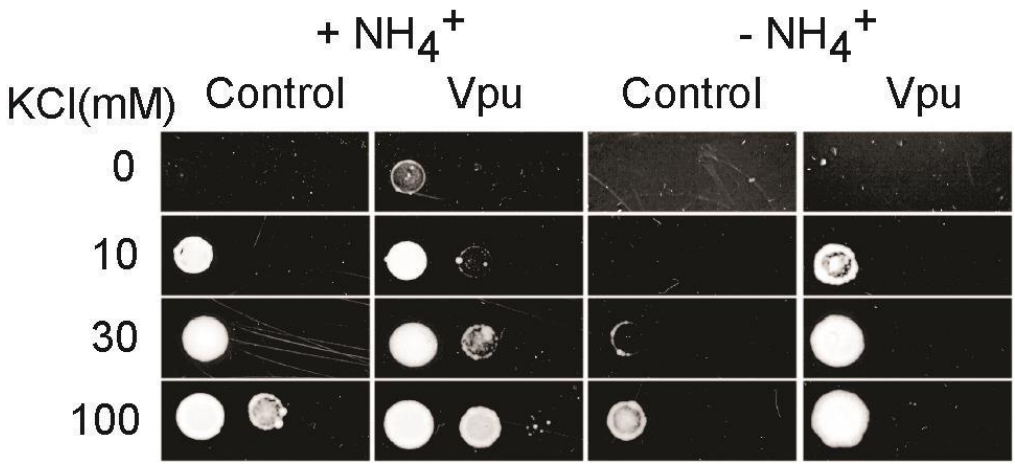

Figure 8