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The YheI/YheH heterodimer from *Bacillus subtilis* is a multidrug ABC transporter

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ABSTRACT

ABC (ATP-binding cassette) transporters form the largest family of membrane proteins in micro-organisms where they are able to transport a wide variety of substrates against a concentration gradient, in an ATP-dependent process. Two genes from the same putative *Bacillus subtilis* operon, *yheI* and *yheH*, encoding possibly two different ABC transporters, were overexpressed in *Escherichia coli* in high yield, either separately or jointly. Using membrane vesicles, it is shown here that both subunits were required to detect, (i) the transport of four structurally unrelated drugs, and (ii) a vanadate-sensitive ATPase activity. Mutation of the invariant Walker-A lysine to an alanine residue in both subunits led to an inactive transporter. Moreover, after membrane solubilization by detergent, both wild-type subunits co-purified on a Ni-Agarose affinity column while only the YheH subunit contained a hexa-histidine tag. This shows that YheI and YheH are indeed able to interact together to form a heterodimer. Importantly, expression of both *yheI* and *yheH* genes in *B. subtilis* could be strongly stimulated by addition of sub-inhibitory concentrations of various unrelated antibiotics. Therefore, *B. subtilis* YheI/YheH forms a new heterodimeric multidrug ABC transporter possibly involved in multiple antibiotic resistance *in vivo*.

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1. Introduction

ABC (ATP-binding cassette) transporters belong to a large superfamily of membrane proteins widely spread among all living organisms [1–3]. They are able to translocate, either as an import or an export mechanism, a wide range of substrates across membranes using ATP hydrolysis as a power source [4]. All ABC transporters share the same basic architecture with two cytosolic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP, and two transmembrane domains (TMD) that translocate the substrates. These four domains can be part of a single polypeptide (full-length transporters such as human P-glycoprotein) or be borne on separate subunits, up to four [5]. Some transporters have one NBD fused to a TMD and these so-called half-size transporters work either as homodimers (two identical half-size subunits assemble together to form a functional transporter) or as heterodimers (two different half-size subunits associate together as a functional transporter) [6,7]. While the TMDs are quite variable in sequence and topology among ABC transporters, their NBDs show a remarkable similar 3-D structure [8], resulting from the conservation at the sequence level of several motifs including notably the Walker A and B and the ABC signatures [9].

Drug resistance has been recognized as a major impediment in therapy for a long time but the involvement of drug transporters as major players in this mechanism, especially in multidrug resistance (MDR) phenotypes, has only been gaining credit in recent years [10,11]. In contrast to eukaryotes where identified MDR transporters belong to the ABC family [12], the vast majority of bacterial MDR transporters reported so far are secondary transporters [13]. However, the discovery of the first bacterial ABC transporter about ten years ago by Konings' group, LmrA from *Lactococcus lactis* [14], and the advent of the genomic era have paved the way to the study of many more putative MDR bacterial ABC transporters. Hence, we and others have participated in the characterisation of these new MDR transporters including BmrA from *B. subtilis* [15], HorA from *Lactobacillus brevis* [16], OmrA from *Oenococcus oeni* [17], and VcaM from *Vibrio cholerae* [18]. These are all half-size transporters that presumably worked as homodimers and this has been demonstrated biochemically for both LmrA and BmrA [19,20] and confirmed by 3-D structures of related bacterial transporters, Sav1866 or MsbA [21,22]. Alternatively, it was shown that two different half-size bacterial ABC transporters are sometimes required to observe a MDR phenotype suggesting that they work as heterodimers. These pairs include EfrA/B from *Enterococcus faecalis* [23], LmrC/D from *L. lactis* [24] and AbcA/B from *Bifidobacterium breve* [25] (see [26] for a recent review).

The genome of *B. subtilis*, the archetype of Gram positive bacteria, encodes for at least 78 ABC transporters that have been split in 38 importers and 40 exporters, and classified into 11 sub-families according to their putative function [27]. Of these, 8 ABC transporters of *B. subtilis* have been classified in the MDR-like sub-family with 4 members predicted to function as homodimers, YvcC, YwjA,

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YgaD and SunT, while the 4 others, CydD/C, YfiB/C, YheI/H, YknU/V were putative heterodimers. We have shown that YvcC is indeed a MDR ABC transporter and renamed it BmrA (for *B*acillus *m*ultidrug *r*esistance *A*TP, [15], see above), while SunT belongs to the sublancin operon and is likely involved in the export of the sublancin 168 lantibiotic [28]. For the putative heterodimeric ABC transporters of this MDR-like sub-family of *B. subtilis*, little information is yet available concerning their possible function, except for the CydD/C proteins. They are encoded in the *cydABCD* operon which is maximally expressed under low oxygen tension [29]. In *E. coli*, the CydD/C transporter is required for the proper cytochrome bd quinol assembly and it exports reductants into the periplasm [30]. Concerning the YheI/YheH couple, a recent report suggested that it was somehow involved in the sporulation process of *B. subtilis* because its overexpression reduced the sporulation efficiency possibly by modulating the function of KinA [31]. On the other hand, overexpression of *B. subtilis yheI/H* was observed in the presence of few antibiotics [32,33], suggesting a possible role of YheI/H in multiple drug resistance.

The aim of this work was to functionally characterize YheI/H, and thanks to a high level of overexpression in *E. coli*, we show here that these proteins associate to form a new multidrug ABC transporter, the first one to function in *B. subtilis* as a heterodimer. Moreover, the expression of both *yheI/yheH* in *B. subtilis* can be turned on by the presence of many structurally unrelated drugs, which strongly hints at a physiological role of YheI/YheH as an ABC transporter involved in cellular detoxification.

2. Materials and methods

2.1. Bacteria and growth conditions

B. subtilis 168 strain was used for *yheI* and *yheH* amplification. *E. coli* TOP10 (Invitrogen) and *E. coli* BL21(DE3) (Novagen) strains were used for gene cloning and protein overexpression, respectively. Unless otherwise stated, *B. subtilis* and *E. coli* cells were grown in Luria Bertani (LB) medium at 37 °C.

2.2. Cloning and sequencing

Genomic DNA was prepared from *B. subtilis* cells by an adapted cetyltrimethylammonium DNA purification method [34]. *YheI* and *yheH* genes (accession no. BG13041 and BG13040, respectively) were amplified by PCR using Pfu Turbo DNA Polymerase (Stratagene) (primers: **YheI**-5′AAG GAT CCG TTT TCA GTT TTG AAA AAG CTT GGC TGG, **YheI**-3′ AA CTC GAG TGC CCC TGC TCC CCC TTC TTC CGC, **YheH**-5′ AAG GAT CCG AAA ATA GGA AAA ACG TTA TGG AG, **YheH**-3′ AA CTC GAG TGC AAT GGA ATG TTT CTG TCC C; restriction sites are underlined). The YheI/YheH (YheI/H) construction was obtained by DNA amplification using **YheI**-5′ and **YheH**-3′ primers. Sequence verifications were made by DNA sequencing (Genome Express Corporation, France). The genes were cloned into pET21b(+) vector (Novagen), allowing the addition of six consecutive histidine residues on the carboxyl terminus of the proteins.

2.3. Site-directed mutagenesis of YheI/YheH

The pET21b(+)/YheI/H plasmid constructed as described above, was used as a template for site-directed mutagenesis using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Primers used were as follows: **K377A-YheI** GT AAA ACC GGT AGC GGA GCA ACG ACA ATT ATT AAG CAG C; **K469A-YheH** GGC CAT ACC GGA TCA GGA GCT AGC TCG ATT TTG AAT CTT C. (the mutated bases are underlined and the reverse-complementary primers are not listed).

The pET21b(+)/YheI K377A/YheH K469A double mutant was obtained by introducing the MscI/XhoI fragment from the K469A construction into the plasmid coding for the K377A mutant.

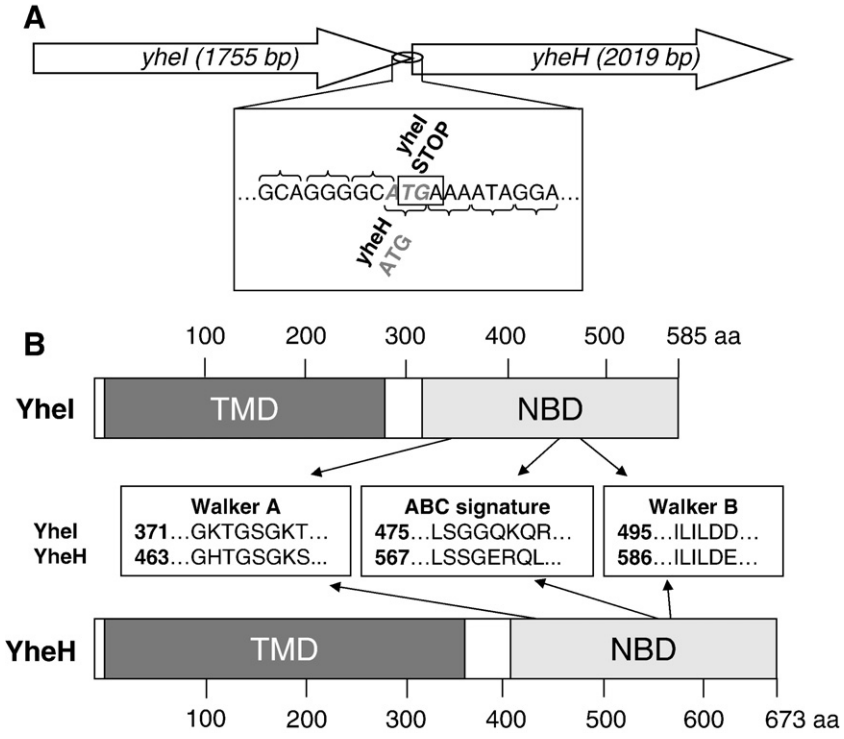

Fig. 1. Organization of the *yheI/yheH* operon in *B. subtilis*, and predicted topology with conserved motifs in YheI and YheH. A, the overlap between *yheI* stop codon and *yheH* start codon is highlighted. B, the conserved motifs of the ABC transporter superfamily are indicated for both proteins; TMD and NBD correspond to the transmembrane domain and nucleotide-binding domain, respectively. The TMD contains 6 and 4 predicted α-helices for YheI and YheH, respectively [27].

2.4. Protein overexpression and inside-out membrane vesicles (IMV) preparation

Freshly transformed colonies were inoculated into Turbo Broth™ medium (AthenaES) and grown at 37 °C with shaking at 220 rpm. 0.7 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added when the OD_{600} nm reached 1.8. The culture was further grown overnight at 25 °C and bacteria were collected by low-speed centrifugation. Subsequent bacterial lysis and IMV preparation were carried out using a French press as previously described [35]. Protein overexpression was checked by Coomassie brilliant blue-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Purification of YheI/YheH

IMV containing overexpressed YheI and hexahistidine-tagged YheH were prepared as described above and solubilized for 2 h on ice in buffer A (50 mM Tris–HCl pH 8, 15% glycerol, 100 mM NaCl, 5 mM β-mercaptoethanol) supplemented with 10 mM imidazole and 1% *N*-dodecyl-β-D-maltoside (DDM) at a protein concentration of 1 mg/ml. After centrifugation for 1 h at 200,000*g*, the supernatant was incubated with 1/10 (v/v) of Ni^{2+}-NTA resin (Qiagen) for 1 h. Resin was washed with 20 column volumes of buffer A supplemented with 0.05% DDM and 20 mM imidazole. Elution was carried out in the same buffer but in the presence of 250 mM imidazole.

2.6. ATPase activity

The ATPase activity was measured by Pi release using a colorimetric method described by Heinonen and Lahti [36], slightly modified by Tomaszek and Schuster [37]. Briefly, membrane control or membranes containing overexpressed YheI, YheH or YheI/YheH (200 μg of proteins) were incubated at 30 °C for 30 min in 50 mM Hepes/KOH pH 8.0, 7 mM ATP, 7 mM $MgCl_2$, 2 mM NaN_3, and in the presence when indicated of 200 μM ortho-vanadate (Sigma) (final volume of 200 μl). Membranes obtained from an *E. coli* strain transformed with an empty pET21 plasmid were used as a control. After an incubation period, 4 volumes of freshly prepared acetone-acid-molybdate (AAM) reagent (3 parts acetone, 1 part 10.0 N H_2SO_4 and 1 part 15.0 mM ammonium molybdate) were added. 45 s after AAM addition, 0.4 volumes of 1 M citric acid were added. The absorbance of the phosphomolybdate complex was measured at 355 nm.

2.7. Fluorescent drugs transport

For drug transport assays, variations in fluorescence intensity were monitored with a Photon Technology International Quanta Master I fluorimeter. Excitation and emission wavelengths were of 355 and 457 nm for Hoechst 33342 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2, 5′-bis-1*H*-benzimidazole; Sigma), 480 and 590 nm for doxorubicin (Sigma) and 608 and 690 nm for mitoxantrone (Sigma), respectively. Membrane vesicles (200 μg of protein) were added into a 2 ml cuvette containing 1 ml of 50 mM Hepes/KOH pH 8.0, 2 mM $MgCl_2$, 8.5 mM NaCl, 40 μg pyruvate kinase (Roche) and 4 mM phosphoenolpyruvate. After 1 min incubation at 30 °C, the drug was added, and its fluorescence was recorded for 1–2 min. ATP (2 mM) was then added and the fluorescence intensity was monitored for several minutes.

2.8. BCECF uptake into inside-out membrane vesicles

Accumulation of BCECF (2′,7′-bis-(3-carboxyethyl)-5-(and-6)-carboxyfluorescein; Biochemika) in IMV was performed in buffer containing 50 mM Hepes/KOH pH 8, 250 mM sucrose, 8.5 mM NaCl, 7 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 20 μg/ml pyruvate kinase (Roche), with addition of 7 mM ATP or 7 mM AMPPNP in control samples. BCECF was added to a final concentration of 12.5 μM. The samples (with membrane vesicles at 1 mg protein/ml) were incubated at 30 °C for 30 min in darkness. The reaction was stopped by addition of an iced-cold stop solution (50 mM Hepes/KOH pH 8, 250 mM sucrose, 20 mM EDTA) to give a final EDTA concentration of 12 mM, followed by a subsequent centrifugation (100,000 ×*g*, 30 min). The supernatant was removed and the vesicle pellet was washed three times with the stop solution before being resuspended in 1 ml of 1% (v/v) Triton X-100 in 50 mM Hepes/KOH pH 8. BCECF fluorescence was then measured with the wavelengths set at 494 nm and 521 nm for the excitation and emission, respectively. Fluorescence intensity was found to be linear with BCECF concentration in the range considered. Experiments were performed in triplicate.

Table 1
Sequence comparison between YheI/YheH and selected ABC transporters

Organism	Subunit	Identical+strongly similar residues (in %) [a]	
		YheI	YheH
Bacillus subtilis	YheI	–	48.2
	YheH	48.2	–
Enterococcus faecalis	EF1733	70.8	49.6
	EF1732	55.4	59.2
Listeria monocytogenes	Lmo1652	69.5	49.0
	Lmo1651	53.6	60.3
Streptococcus pneumoniae R6	Spr1657	69.2	51.0
	Spr1651	55.7	60.4
Serratia marcescens	SmdA	65.4	45.8
	SmdB	49.3	54.9
Escherichia coli	MdlA	63.7	44.9
	MdlB	49.3	54.8
Lactococcus lactis	LmrC	53.5	45.9
	LmrD	46.0	51.7
Enterococcus faecalis	EfrA	53.2	47.5
	EfrB	55.1	52.2
Streptococcus pneumoniae	SP2075	54.7	46.6
	SP2073	51.6	48.7

[a] Percentage of identical plus strongly similar residues were obtained from pairwise sequence alignments using the Clustal W program on the http://npsa-pbil.ibcp.fr/ web site with the predefined parameters. In this program, the residues defined as strongly similar belong to either of the following groups (see also http://bips.u-strasbg.fr/fr/Documentation/ClustalX/): Ser, Thr or Ala; Asn, Glu, Gln or Lys; Asn, His, Gln or Lys; Asn, Asp, Glu or Gln; Gln, His, Arg or Lys; Met, Ile, Leu and Val; Met, Ile, Leu and Phe; His or Tyr; Phe, Trp or Tyr. Sequences used were obtained from the following web servers: *Bacillus subtilis* YheI and YheH, http://genolist.pasteur.fr/SubtiList/index.html; *Listeria monocytogenes* (Lmo1651 and 1652), http://genolist.pasteur.fr/ListiList/index.html; *Streptococcus pneumoniae* strains R6 (Spr 1656 and 1657) and Tigr4 (SP 2073 and 2075), http://genolist.pasteur.fr/StreptoPneumoList/index.html; *Serratia marcescens* SmdA and SmdB, http://www.ncbi.nlm.nih.gov/Genbank/ (accession codes, BAF79679.1 and BAF79680.1, respectively); For the other sequences, the Swiss-Prot entries (given in parenthesis) were used: MdlA and MdlB from *E. coli* (P77265 and P0AAG5, respectively); LmrC and LmrD from *Lactococcus lactis* (Q9CIP6_LACLA and Q9CIP5_LACLA, respectively); EfrA, EfrB, EF1732 and EF1733 from *Enterococcus faecalis* (Q82ZX7_ENTFA, Q82ZX8_ENTFA, Q834D4_ENTFA and Q834D3_ENTFA, respectively). The top six transporters are the most closely related ones and, among them, the Lmo1652/Lmo1651 pair is somehow involved in the virulence of *Listeria* [46], and insertional inactivation of *lmo1652* leads to a strongly attenuated *in vivo* intracellular replication of the knockout mutant [47]. For the EF1733/EF1732 pair, its expression has been shown to be strongly up-regulated by addition of either erythromycin or chloramphenicol [48]. The three remaining transporters are more distantly related to YheI/YheH but they all have been shown to be involved in MDR phenotypes [23,24,43,44].

2.9. Measurement of expression values of yheI and yheH

The relative mRNA levels of the genes *yheI* and *yheH* in *B. subtilis* were determined using whole-genome microarrays based on a two-color fluorescence technology (Eurogentec, Seraing, Belgium). *B. subtilis* 168 growing in Belitzki minimal medium [38], to OD_{600} = 0.4 at 37 °C were treated with antibiotics for 10 and 40 min, respectively. The preparation of untreated and antibiotics-treated samples, the image acquisition via the Axon GenePix® 4000A confocal laser scanner (Axon Instruments, Foster City, CA) and the relative signal

Table 2
Antibiotics-induced expression of *yheI* and *yheH* in *B. subtilis*

Compound	Concentration (μg/ml)	Time (min)	Expression ratio	
			yheI	*yheH*
Azithromycin*	2	10	2.61	2.51
		40	6.88	6.52
Cerulenin	32	10	1.57	2.23
		40	2.86	4.63
Chloramphenicol*	4	10	2.98	5.20
		40	2.25	3.54
Doxycycline*	0.3	10	6.42	5.92
		40	n.d.	n.d.
Erythromycin*	0.1	10	9.27	12.82
		40	6.17	7.91
Fluorouracil	0.2	10	1.24	1.09
		40	3.09	3.07
Fusidic acid*	0.08	10	6.08	4.38
		40	9.30	5.99
Gentamicin*	0.26	40	1.18	1.06
		40	1.73	2.12
Linezolid*	0.5	10	10.93	6.20
		40	20.70	13.65
Nalidixic acid	25	10	1.51	1.12
		40	2.38	1.63
TAN 1057*	0.048	10	4.84	5.55
		40	1.22	1.16

Average expression values (ratios) of *yheI* and *yheH* higher than the value 2.00 derived from *B. subtilis* transcriptome measurements with 46 different antibiotics. In addition to the eleven compounds listed above, the following antibiotics were tested: corallopyronin, streptovaricin, rifampicin, actinonin, gentamicin, kanamycin, pactamycin, puromycin, moiramide B, triclosan, azaserine, novobiocin, ciprofloxacin, sulfamethoxazole, trimetoprim, trovafloxacin, moxifloxacin, cefoxitin, methicillin, mersacidin, oxacillin, ristocetin, ramoplanin, vancomycin, phenyl-thiazolylurea derivative, mupirocin, actinomycin D, ethidium bromide, N-ethylmaleimide, nitrofurantoin, nisin, netropsin, polymyxin B, showdomycin, tunicamycin.
* Compounds known as protein biosynthesis inhibitors. n. d., not determined.

intensity calculation (treated versus untreated sample) by the software Expressionist® Pro 2.0 (Genedata, Basel) have been published previously [39,40]. Each average expression value (ratio) was derived from triplicates of microarray experiments.

3. Results and discussion

3.1. The yheI/yheH operon in B. subtilis

Analysis of the genome of *B. subtilis* 168 strain using the Subtilist web server (http://genolist.pasteur.fr/SubtiList/) revealed the presence of two overlapping open reading frames for the *yheI* and *yheH* genes. Thus, the STOP codon (*TGA*) of *yheI* became part of the A*TG* start codon of the downstream *yheH* gene, by a frameshift of one nucleotide (Fig. 1A). Overlapping has been widely observed in organisms with a limited genome size, and although it was initially considered as a mechanism to increase the amount of information per unit length [41], it is also now viewed as a way to tightly coordinate the expression of overlapping genes [42]. Both *yheI* and *yheH* genes encode half-size ABC transporters containing a putative transmembrane region followed by a nucleotide-binding domain. The latter shows the characteristic motifs of the ABC superfamily, namely the Walker A and B motifs and the ABC signature (Fig. 1B). Therefore, the genomic organization of their coding regions and the predicted topology of the two putative half-size transporters, YheI and YheH, suggest the possibility that they might interact together to form a functional heterodimeric unit.

A search for orthologues of YheI/YheH in other bacterial species was undertaken. Notwithstanding the related *Bacilli* species which all possess closely related pairs of ABC transporters, orthologues of YheI/YheH were also found in some pathogenic species such as Gram positives *Listeria monocytogenes* (Lmo1652/Lmo1651), *Streptococcus pneumoniae* (Spr1657/Spr1656) and *Enterococcus faecalis* (EF1733/EF1732), or Gram negatives *Serratia marcescens* (SmdA/SmdB) and *E. coli* (MdlA/MdlB; see Table 1). Within this closely related cluster, only the SmdA/SmdB heterodimer has been shown very recently to likely confer a MDR phenotype using an *E. coli* reporter strain [45].

3.2. YheI/yheH induction upon antibiotics exposure

Previous results have shown that addition of sub-inhibitory concentrations of few drugs might induce the expression of both *yheI* and *yheH* [32,33]. Reporter strains were then built using the upstream sequence of *yheI* gene [49] or *yheH* gene [50], but induction by antibiotics was only seen for the former strain, which is in agreement with both genes being co-transcribed from the same promoter (upstream sequence of *yheI*). We then decided to confirm and extend these studies by addressing the effect of 46 different antibiotics on the transcriptome of *B. subtilis*. As shown in Table 2, eleven of these were able to increase significantly the expression level of both *yheI/yheH*. Importantly, we found no other ABC transporters or secondary transporters putatively, or known to be, involved in MDR phenotypes with such a high level of induction upon drug exposure (data not shown), despite the occurrence of many hypothetical MDR transporters in *B. subtilis* genome [51]. Notably, and in accord with previous results obtained with the *yheI* promoter [49], most of the compounds that increase the expression of both genes are antibiotics that target the ribosomes. This might suggest that *yheI/yheH* induction is somehow a consequence of a ribosome-related signaling pathway, and it has been proposed that the antibiotic-inhibited ribosome may affect the transcription apparatus by triggering the stringent response [52]. Consistent with this idea, *yheI/yheH* overexpression has also been reported during the stringent response of *B. subtilis* [53]. Accordingly, treatment of *B. subtilis* with antibiotics affecting the 50S and/or 30S ribosomal subunits has been reported to induce accumulation of (p)ppGpp, a hallmark of the stringent response [54]. However, the transcriptome data obtained here with antibiotics affecting protein synthesis (data not shown) show no correlation with the transcriptional program triggered by the stringent response in *B. subtilis* [53]. This implies that the signaling pathways elicited by

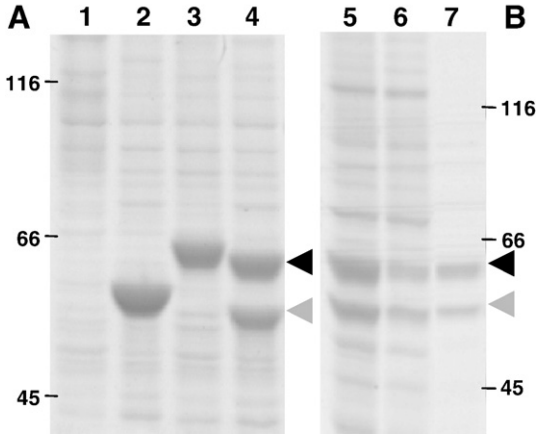

Fig. 2. Overexpression of YheI and/or YheH in inside-out *E. coli* vesicles and co-purification of YheI/YheH. *A*, 10% SDS-PAGE stained with Coomassie Blue of inside-out membrane vesicles prepared from an *E. coli* strain containing the pET21b empty plasmid (lane 1), or overexpressing YheI (lane 2), YheH (lane 3) or both YheI/YheH (lane 4); 8 μg of membrane proteins were loaded into each lane. Black arrowhead, YheH; grey arrowhead, YheI. The small difference in electrophoretic mobility for YheI (lane 2 as compared to lane 4) is due to the presence of an additional $(His)_6$-tag in lane 2. In the case of YheH, an additional T7-tag is present in lane 3 as compared to lane 4. B, 10% SDS-PAGE stained with Coomassie Blue of inside-out membrane vesicles prepared from an *E. coli* strain overexpressing YheI and YheH-6xHis (lane 5); soluble fraction obtained after incubation with 1% *N*-dodecyl-β-D-maltoside (lane 6); fraction eluted from the Ni^{2+}-NTA column in the presence of 250 mM imidazole (lane 7). The positions of the molecular weight markers are indicated on each side of the figure (in kDa).

ribosome-targeting antibiotics and by the stringent response are different.

3.3. Overexpression of YheI and/or YheH in E. coli and ATPase activities of membrane vesicles

To investigate the function of YheI and YheH, these proteins were overexpressed in a BL21(DE3) *E. coli* strain, either separately or jointly, and inside-out membrane vesicles were prepared. A very high level of expression was obtained for the two proteins expressed alone, YheI or YheH, when their respective gene was cloned separately into an *E. coli* expression vector under the control of a T7 promoter, and each protein appeared correctly addressed to the membrane (Fig. 2A). Similarly, when the whole *B. subtilis yheI/yheH* operon was cloned into the same *E. coli* expression vector, both proteins YheI and YheH were readily overexpressed (Fig. 2A, lane 4). This result is clearly different from the scenario observed in the case of the DrrA/DrrB ABC transporter from *M. tuberculosis* where the original overlapping of *drrA* and *drrB* genes was detrimental to the co-expression of both subunits in *E. coli*; the co-expression of both subunits was only observed after the creation of an artificial operon, with a new ribosome-binding site being introduced between both genes [55]. Besides, the level of expression reported here for YheI/YheH is much higher than that reported for two heterodimeric half-size ABC transporters that have been previously overexpressed in *Lactococcus lactis,* LmrC/D from *L. lactis* and AbcA/B from *Bifidobacterium breve* [24,25], but in either case the two genes were not overlapping at the genomic level. To our knowledge, this is probably the highest expression level reported so far for an ABC transporter, that seems even better than that of BmrA in *E. coli* which amounted to ~50% of total membrane proteins [15].

The functionality of the overexpressed proteins was then studied by measuring the ATPase activity of the different vesicles. As shown in Fig. 3, vesicles containing both YheI/YheH had a ~2.5–3 fold higher ATPase activity compared to vesicles containing each protein alone. This ATPase activity attributable to YheI/YheH was reduced to ~20% in the presence of 200 μM ortho-vanadate, a well-known inhibitor of ABC transporters [56,57]. Membrane vesicles prepared from *E. coli* cells containing an empty pET21b plasmid (control vesicles) showed a higher level of background ATPase activity than the vesicles containing each protein alone, but it should be noted that the same amount of total membrane proteins were used for each assay leading probably to an overestimation of the ATPase activity due to 'contaminating' ATPases in these control vesicles. In contrast to the vesicles containing both YheI and YheH, none of the other vesicles showed an ATPase

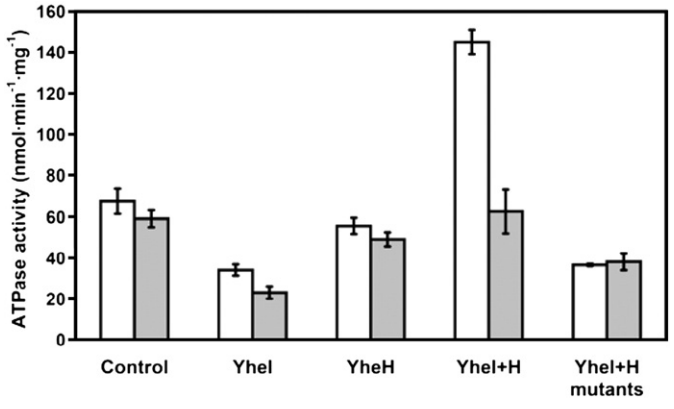

Fig. 3. ATPase activity of *E. coli* inside-out membrane vesicles containing overexpressed YheI, YheH or both YheI+YheH wild-type or double mutant (YheI K377A/YheH K469A). Control vesicles were obtained from an *E. coli* strain transformed with an empty pET21 plasmid. The ATPase activities were measured as described under "Materials and methods", in the absence (empty boxes) or presence (filled boxes) of 200 μM ortho-vanadate. The values are expressed as the mean±SD for three independent experiments.

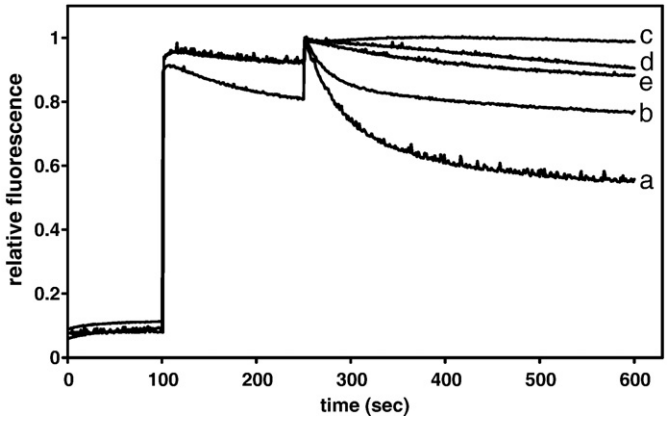

Fig. 4. Transport of Hoechst 33342. 2 μM Hoechst 33342 was added at $t \sim 100$ s to inside-out membrane vesicles containing overexpressed wild-type YheI plus YheH (traces a and b), YheI (trace c), YheH (trace d), or YheI K377A/YheH K469A double mutant (trace e). Trace b was obtained in the presence of 200 μM vanadate. After ~ 150 s of further incubation, 2 mM ATP was added to initiate Hoechst 33342 transport.

activity readily inhibited by vanadate, thus indicating that if YheI or YheH alone were able to hydrolyse ATP to some extent, this hydrolysis would be unrelated to that normally displayed by native ABC transporters. An additional control was made where the invariant Walker A lysine, known to be essential in ABC transporters [58], was mutated to alanine in both YheI and YheH, thus creating a K377A (YheI)/K469A (YheH) double mutant. Although the membrane vesicles prepared from an *E. coli* strain overexpressing the K377A/K469A double mutant showed a slightly reduced level of mutated subunits as compared to an *E. coli* strain overexpressing the wild-type YheI/YheH transporter (data not shown), the level of ATPase activity of the vesicles containing the double mutant was strongly reduced to ~25% of that of the wild-type membranes. More importantly, the level of ATPase activity of the vesicles containing the double mutant was unaffected by vanadate addition. Overall, these data are consistent with the hypothesis that YheI and YheH are required together to create a functional transporter.

3.4. YheI and YheH physically interact to form a heterodimer

The possible interaction between YheI and YheH was then investigated by trying to co-purify YheI and YheH while only the latter subunit contained a poly-histidine tag. First, membrane fractions containing both YheI and YheH were solubilized in the presence of *N*-dodecyl-β-D-maltoside and the soluble fraction was loaded onto a nickel affinity chromatography column. After a washing step, both YheI and YheH co-eluted from the column in the presence of high concentration of imidazole (Fig. 2B). This result confirms that a physical interaction was maintained between YheI and YheH throughout the procedure and that both proteins form a stable heterodimer. Similar conclusions were drawn from the studies of LmrC/LmrD and AbcA/AbcB [24,25]. However, it is possible that some loss of the untagged subunit (i.e. the YheI subunit) had occurred here because when we performed an extensive washing step, the amount of YheI subunit recovered decreased as compared to the YheH subunit (data not shown), showing that the stability of the heterodimer can be challenged by the purification procedure.

3.5. YheI/YheH is a MDR transporter

Next, transport assays of typical fluorescent substrates of MDR ABC transporters were performed using inside-out membrane vesicles [59], according to the continuous fluorescence assay described for the study of these transporters. Among the tested compounds, the strongest transport activity was seen with Hoechst 33342. This

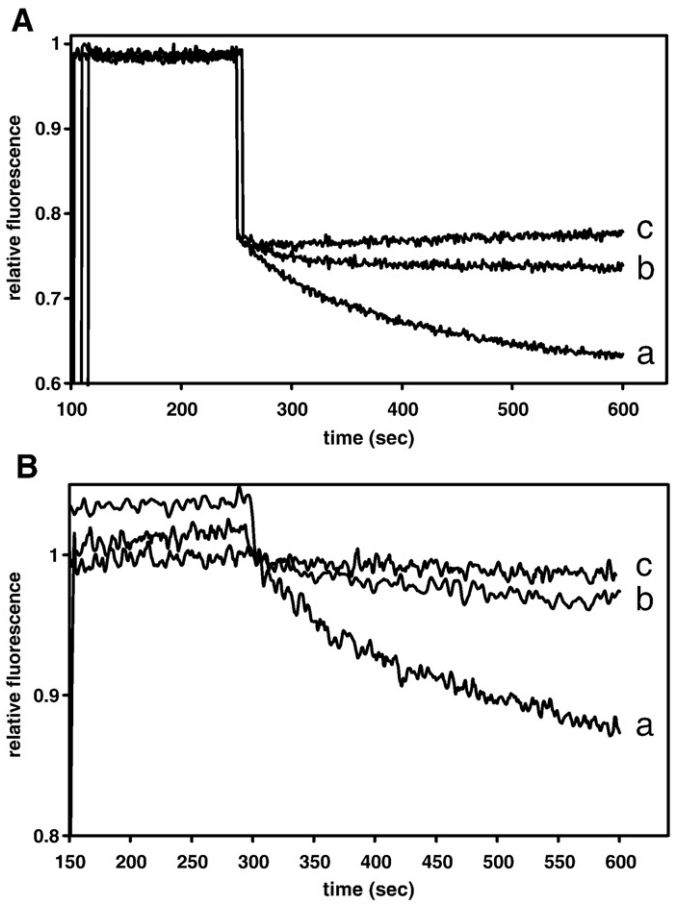

Fig. 5. A. Transport of doxorubicin (A) and mitoxantrone (B). A, After ~ 100 s incubation at 30 °C, 10 μM doxorubicin was added to inside-out membrane vesicles containing overexpressed wild-type YheI/YheH, in the absence (a) or presence of 200 μM vanadate (b), or YheI K377A/YheH K469A double mutant (c). Transport of doxorubicin was initiated by addition of 2 mM ATP at t ~ 250 s. B, 5 μM mitoxantrone was added to inside-out membrane vesicles containing overexpressed wild-type YheI/YheH, in the absence (a) or presence of 500 μM vanadate (b), or YheI K377A/YheH K469A double mutant (c). Transport of mitoxantrone was initiated by addition of 2 mM ATP at *t* ~ 300 s.

membrane-permeable dye is fluorescent when bound to the membrane but not in an aqueous medium, allowing movement of the dye to be monitored by real time fluorescence intensity [24,35,60–62]. As shown in Fig. 4, transport of the fluorophore by YheI/YheH from the membrane into the lumen of the vesicles was revealed by a rapid fluorescence decay observed when ATP was added, and this activity was highly reduced in the presence of vanadate (compare traces *a* and *b*, respectively). In contrast, membrane vesicles containing the Walker A lysine double mutant, YheI K377A/YheH K469A, showed no transport activity at all (trace *e*). Likewise, vesicles containing YheI or YheH alone did not show any significant transport activity of Hoechst 33342 (traces c and d, respectively). Using a similar approach to monitor the transport of other fluorescent substrates [15], the wild-type YheI/YheH was also shown to transport doxorubicin and mitoxantrone (Fig. 5A and B, respectively, traces a), while the Walker A lysine double mutant failed to transport any of these drugs (Fig. 5A and B, traces c). Again, addition of vanadate strongly attenuated the transport of doxorubicin or mitoxantrone by wild-type YheI/YheH (traces b).

Finally, we used an accumulation assay described for vesicles enriched in multidrug-related protein MRP1 [63], to monitor the transport of fluorescent BCECF by wild-type YheI/YheH (Fig. 6). Replacing ATP by a non-hydrolysable analogue, AMPPNP, significantly reduced the transport of this drug by the wild-type YheI/YheH (by ~35%) and a similar result was obtained in the presence of vanadate (not shown). Moreover, this transport ability was even further reduced (to ~ 55%) when the YheI K377A/YheH K469A double mutant was used.

In conclusion, we have shown here that YheI/YheH forms a heterodimeric ABC transporter able to work as a new multidrug transporter *in vitro*, with four structurally unrelated drugs identified here as its substrates. The drug transport ability was impaired in the double Walker A mutant, or strongly reduced by vanadate addition in the wild-type transporter, showing that ATP hydrolysis is required to power YheI/YheH, in agreement with results obtained on two other MDR bacterial ABC transporters, BmrA or LmrC/D [15,64]. Due to the abundance of putative, or proven, MDR transporters in many bacteria (for instance 20 transporters out of 37 putative MDR were indeed capable of transport drugs in *E. coli*, [65]), it has been proposed that most of them have another physiological function and that their drug transport abilities are merely an opportunistic feature [66,67]. This was exemplified with the Bmr and Blt secondary transporters of *Bacillus subtilis*: they share 51% of sequence identity and both transporters can act as a MDR transporter with similar multidrug specificity [68]. However, in contrast to Bmr whose expression is upregulated by many drugs and is likely to be a 'true' MDR transporter, Blt expression is not induced by drugs [68], and this transporter is more likely devoted to spermidine efflux in *Bacillus* than to multidrug efflux [69].

On the other hand, classification of new a transporter as a multidrug transporter based solely on a high degree of sequence similarity with a related transporter known to be a MDR should not be taken for granted. Indeed, it has been shown that although human MDR1 and MDR2 share ~75% identity (~70% in the case of orthologous transporters, mdr1 and mdr2, from mouse), the former only is able to efflux drugs while the latter fails to confer a MDR phenotype and its physiological role has been assigned to a phosphatidylcholine translocase [70,71]. Therefore, bioinformatic classification can give us some clues about the possible function of unknown transporters but only biochemical evidence should be used to ascertain the role of putative MDR transporters.

In the case of *yheI/yheH*, it is noteworthy that their expression level can be strongly induced upon exposure to several classes of structurally unrelated antibiotics. Such a high level of induction brought about by some antibiotics was unmatched for any other putative or proven MDR transporters in *B. subtilis* that belonged either to the ABC superfamily or to secondary transporter superfamilies [32],

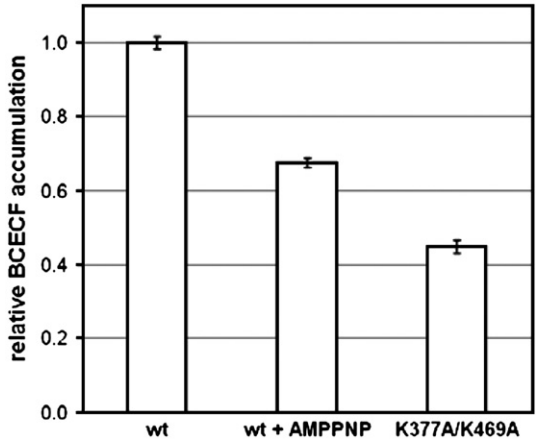

Fig. 6. BCECF uptake into inside-out membrane vesicles. Inside-out membrane vesicles (1 mg/ml) containing wild-type YheI/YheH (***wt***) or YheI K377A/YheH K469A double mutant (***K377A/K469A***) were incubated at 30 °C with 12.5 μM BCECF for 30 min in the presence of either 7 mM ATP or 7 mM AMPPNP as a control (***wt+AMPPNP***). After centrifugation and washing steps, the pellet containing the vesicles was resuspended in 1% Triton X-100, and BCECF accumulation was estimated by fluorescence quantification (see Materials and methods).

including the well known multidrug secondary transporter Bmr [72]. To the best of our knowledge, this is the first time that a putative multidrug bacterial ABC transporter is shown to be so much up-regulated by different drugs. Based on the assumption that the only true bacterial multidrug transporters are those that are up-regulated by many drugs (see [66] for a review), our results suggest that the YheI/YheH pair functions *in vivo* as a multidrug transporter. In order to investigate this further, we created a Δ*yheI*/*yheH* mutant of *Bacillus subtilis* and compared its growth properties to that of wild-type *B. subtilis* in the presence of different concentrations of many drugs, including antibiotics listed in Table 2. In contrast to what might be expected, no clear difference was observed between both strains regarding their sensitivity towards the tested compounds (results not shown). Although this could be interpreted as a lack of MDR function for the YheI/YheH pair *in vivo*, it is noteworthy that *B. subtilis* is one of the micro-organisms that contain the highest amount of putative MDR transporters in its genome (>30; [73]). Thus, it is possible that the presence of other MDR genes mask the effect of deleting the *yheI/yheH* in this knockout mutant. Moreover, a recent report using a collection of ~4,000 single-gene knockouts in *E. coli* revealed that among the 20 exporters which have been shown previously to extrude drugs when overexpressed in a drug-sensitive *E. coli* host mutant [65], only the deletion of the well characterized AcrAB pump led to a significant increase of sensitivity towards three of seven antimicrobial compounds tested [74]. This latter report emphasizes how difficult it can be to ascertain the physiological role of putative MDR transporters based on knockout experiments. Congruent with this, a Δ*bmrA* (formely known as Δ*yvcC*) *Bacillus* mutant also showed no increase in sensitivity as compared to the wild-type strain whatever antimicrobial compound was screened (Cédric Orelle and J.-M. J, unpublished results), and despite the ability of BmrA to function efficiently *in vitro* as a multidrug transporter [15,35]. Thus, regarding the YheI/YheH pair, although its features are consistent with a function in multidrug efflux, we cannot rule out that its physiological role might be linked to a more specific cellular pathway triggered by the exposure to antibiotics, and further work will be needed to investigate this point. With the ever-growing number of bacterial genomes available, it is likely that the discovery of heterodimeric (either 'true' or opportunistic) multidrug ABC transporters in many more bacteria, including pathogenic species, will become an emerging theme in this research field.

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