Many heterodimeric ATP-binding cassette (ABC) exporters evolved asymmetric ATP-binding sites containing a degenerate site incapable of ATP hydrolysis due to noncanonical substitutions in conserved sequence motifs. Recent studies revealed that nucleotide binding to the degenerate site stabilizes contacts between the nucleotide-binding domains (NBDs) of the inward-facing transporter and regulates ATP hydrolysis at the consensus site via allosteric coupling mediated by the D-loops. However, it is unclear whether nucleotide binding to the degenerate site is strictly required for substrate transport. In this study, we examined the functional consequences of a systematic set of mutations introduced at the degenerate and consensus site of the multidrug efflux pump EfrCD of Enterococcus faecalis. Mutating motifs which differ among the two ATP-binding sites (Walker B, switch loop, and ABC signature) or which are involved in interdomain communication (D-loop and Q-loop) led to asymmetric results in the functional assays and were better tolerated at the degenerate site. This highlights the importance of the degenerate site to allosterically regulate the events at the consensus site. Mutating invariant motifs involved in ATP binding and NBD closure (A-loop and Walker A) resulted in equally reduced transport activities, regardless at which ATP-binding site they were introduced. In contrast to previously investigated heterodimeric ABC exporters, mutation of the degenerate site Walker A lysine completely inactivated ATPase activity and substrate transport, indicating that ATP binding to the degenerate site is essential for EfrCD. This study provides novel insights into the split tasks of asymmetric ATP-binding sites of heterodimeric ABC exporters.

Introduction

ATP-binding cassette (ABC) exporters are ubiquitous transmembrane proteins found in all living cells [1]. They minimally consist of two nucleotide-binding domains (NBDs), responsible for ATP binding and hydrolysis and two transmembrane domains (TMDs), which form the substrate permeation pathway across the membrane. In a typical bacterial ABC exporter, a TMD is fused to a NBD constituting a half-transporter which homo- or heterodimerizes to form the active transporter. Many eukaryotic ABC exporters encode the four domains in a single polypeptide chain and can therefore be seen as fused heterodimers. In order to transport substrates, the TMDs alternate between an inward- and an outward-facing conformation. These movements are fueled by ATP hydrolysis at the NBDs, which are connected to the

Abbreviations

ABC, ATP-binding cassette; AMP-PNP, adenosine 5’-β,γ-imido triphosphate; BCECF-AM, 2’,7’-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester; ISOV, inside-out membrane vesicle; NBD, nucleotide-binding domain; NBS, nucleotide-binding site; TMD, transmembrane domain.
TMDs via coupling helices. The NBDs dimerize by sandwiching two nucleotides at their interface via Walker A, Walker B, A-loop, and switch loop from one NBD, and D-loop and ABC signature from the opposite NBD and vice versa, thereby creating two composite nucleotide-binding sites (NBSs; Fig. 1). Closed NBDs are associated with TMDs adopting the outward-facing or outward-occluded state. ATP hydrolysis at the closed NBDs destabilizes the NBD dimer, leading to their dissociation and a concomitant transition to inward-facing TMDs.

The conserved motifs fulfill various functions in the transport process (Fig. 1) [2]. The A-loop carries a conserved aromatic residue (typically a tyrosine), which interacts with the adenine ring of bound ATP via π-stacking. The Walker A motif wraps around the phosphates of bound ATP and contains a highly conserved lysine residue, which interacts with the β- and γ-phosphates of ATP. The interactions mediated by the Walker A lysine contribute to binding affinity and orient ATP such that they are optimally placed for NBD closure. In a closed NBD dimer, the ABC signature motif of the opposite NBD is juxtaposed against the Walker A motif thereby occluding the phosphates of ATP from both sides. ATP sandwiching at the NBD dimer interface is a prerequisite for ATP hydrolysis, which is mediated by a catalytic dyad consisting of the Walker B glutamate and the switch loop histidine. The D-loops play an important role in the allosteric coupling of the ATP-binding sites. The Q-loop

**Fig. 1.** Heterodimeric NBDs of EfrCD. Upper half: In the closed NBD dimer, two ATP molecules (dark green) are sandwiched at the interface, thereby forming two composite NBSs named degenerate and consensus site. The three noncanonical sequence motifs of the degenerate site are framed. Mutations characterized in this study are specified after the sequence motif labels. Lower half: Close-up view of a typical consensus site with highlighted conserved sequence motifs. AMP-PNP is shown as sticks and Mg²⁺ as green sphere [based on the crystal structure of McjD (PDB: 4PL0)].

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**Split tasks of asymmetric nucleotide-binding sites**

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senses the presence of the nucleotide and is noncovalently connected to the coupling helices of the opposite polypeptide chain thereby mediating cross-communication between TMDs and NBDs.

Many ABC exporters, such as PatAB [3], LmrCD [4], TM287/288 [5], TmrAB [6], CFTR [7], and TAP1/2 [8], feature one NBS called the degenerate site, in which the Walker B glutamate, the switch loop histidine, and the ABC signature motif deviate from the consensus sequence. The second NBS agrees with the consensus sequence of ABC-type NBDs and is therefore denoted as the consensus site. The degenerate site binds nucleotides tightly, but is unable to hydrolyze ATP [9,10]. This notion was supported by the structure of inward-facing TM287/288, which featured adenosine 5′-β,γ-imido)triphosphate (AMP-PNP) bound exclusively to the degenerate ATP-binding site [5]. Further structural and biochemical analyses of TM287/288 revealed that nucleotide binding stabilizes the cross-NBD contacts of the inward-facing transporter in an allosteric fashion involving the D-loops [11,12], thereby preventing full separation of the NBDs as seen in inward-facing ABC exporters featuring two consensus sites [13–15]. Mutations introduced at the degenerate site are generally better tolerated than analogous mutations at the consensus site [16,17]. This was also observed for Walker A lysine mutants of TAP1/2, CFTR, and MRP1 [8,10,18,19], suggesting that nucleotide binding to the degenerate site is not a strict requirement for NBD closure and substrate transport.

Recently, the heterodimeric ABC exporter EfrCD of Enterococcus faecalis was identified as an important drug efflux pump in this opportunistic human pathogen [20]. EfrCD contains a complete set of noncanonical substitutions in the degenerate site, namely an aspartate instead of a glutamate in the Walker B motif, a glutamine instead of a histidine in the switch loop and a threonine instead of a glycine in the middle position of the ABC signature motif (Fig. 2). In this study, we introduced 15 point mutations into the NBDs of EfrCD and studied their functional consequences with regard to transport and ATPase activity. Our results provide evidence for a regulatory role of the degenerate site in modulating the activity of the consensus site as well as for the functional necessity of ATP binding to the degenerate site.

**Results**

Point mutations were introduced into the coding sequence of the multidrug ABC exporter EfrCD (Fig. 1) [20]. Taking advantage of the robust drug transport activity and high basal ATPase activity of the purified transporter, the mutants were studied in three different assays: (a) Transport of the fluorescent dyes ethidium, Hoechst 33342, and 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) in Lactococcus lactis cells, (b) Hoechst 33342 transport in inside-out membrane vesicles (ISOVs), and (c) activity measurements with detergent-purified protein to determine apparent K_m and v_max of ATP hydrolysis and co-operativity between the two ATP-binding sites.

**Walker B motif**

The Walker B glutamate of the consensus site was exchanged for glutamine (E512QEfrD). The resulting mutant was incapable of transporting fluorescent dyes in cells and inactive in terms of ATPase activity of the purified protein (Fig. 3, Table 1). This finding is in full agreement with the literature on heterodimeric ABC exporters [5,8,16,17,20–22]. In all experiments conducted in this study, wild-type and E512QEfrD mutant were included as controls and represented the boundaries (fully active vs. inactive) in the functional assays.

When the equivalent noncanonical aspartate of the degenerate site was mutated to asparagine (D492N_EfrC), the functional consequences were less severe. The capability of transporting Hoechst 33342 and BCECF-AM in cells was only marginally reduced, while the mutant had a reduced capability of ethidium transport. Hoechst 33342 transport in ISOVs was clearly impaired, indicating that this assay is more sensitive to mutations than Hoechst 33342 transport in cells and inactive in terms of ATPase activity of the purified protein (Fig. 3). This finding is in full agreement with the literature on heterodimeric ABC exporters [5,8,16,17,20–22]. In all experiments conducted in this study, wild-type and E512QEfrD mutant were included as controls and represented the boundaries (fully active vs. inactive) in the functional assays.

When the equivalent noncanonical aspartate of the degenerate site was mutated to asparagine (D492N_EfrC), the functional consequences were less severe. The capability of transporting Hoechst 33342 and BCECF-AM in cells was only marginally reduced, while the mutant had a reduced capability of ethidium transport. Hoechst 33342 transport in ISOVs was clearly impaired, indicating that this assay is more sensitive to mutations than Hoechst 33342 transport in cells. The purified D492N_EfrC mutant exhibited a 5-fold decreased v_max and a 2.6-fold increased K_m of basal ATPase activity as compared to the wild-type transporter (Table 1). The same aspartate residue was also substituted by glutamate (D492E_EfrC). The ATPase activity and apparent ATP affinity of the D492E_EfrC mutant was almost identical to the D492N_EfrC mutant, indicating that introduction of a
glutamate does not render the degenerate site catalytically active. Drug transport activity of the D492E<sup>EfrC</sup> mutant could not be distinguished from the wild-type transporter, both in cells and in ISOVs. The D492E<sup>EfrC</sup> mutant therefore appears to run in an ‘energy-saver’ mode, exhibiting wild-type transport activity at 5-fold reduced ATPase consumption.

The functional consequences of the Walker B mutations in the two NBSs are highly asymmetric; while they are comparatively well tolerated at the degenerate site, the equivalent mutation at the consensus site leads to a complete inactivation of the transporter. Nevertheless, a correctly positioned negative charge at the position of the noncanonical Walker B aspartate is of functional importance. Our results as well as previous studies [16] suggest that the noncanonical Walker B aspartate is not involved in ATP hydrolysis at the degenerate site. Therefore, the decreased ATPase activities observed for these mutants must be due to reduced ATP hydrolysis at the consensus site. The Walker B motif precedes the D-loop, which allosterically couples the two ATP-binding sites [11]. This coupling is likely compromised by mutations introduced at the degenerate site Walker B motif.

**Switch loop**

The switch loop histidine interacts with the Walker B glutamate and is part of the catalytic dyad at the consensus site. In EfrCD, the degenerate site contains a glutamine instead of the consensus histidine. In agreement with earlier studies on HlyB [23], the H543A<sup>EfrD</sup> substitution at the consensus site led to a

![Fig. 3. Transport activities of Walker B mutants. Ethidium (A), Hoechst 33342 (B), and BCECF (C) accumulation in *Lactococcus lactis* NZ9000 ΔlmrAΔlmrCD cells, and Hoechst 33342 accumulation in ISOVs (D) containing overexpressed wild-type (black), D492E<sup>EfrC</sup> (light blue), D492N<sup>EfrC</sup> (blue), or E512Q<sup>EfrD</sup> (gray) transporters.](image-url)
complete inactivation of transport and ATPase activity (Fig. 4, Table 1). The equivalent Q523A\(EfrC\) substitution at the degenerate site was comparatively well tolerated. The mutant was almost indistinguishable from the wild-type transporter in terms of ATPase activity, but had a clearly reduced transport activity, i.e., it is less efficient in coupling ATP hydrolysis with substrate transport. In the inward-facing TM287/288 structure (PDB: 4Q4A), this glutamine plays an important role in mediating cross-NBD hydrogen bonds, which are eliminated by the substitution to alanine. A weakened NBD–NBD interface of inward-facing EfrCD as a consequence of the Q523A\(EfrC\) mutation may therefore explain the observed coupling defect.

**ABC signature motif**

The ABC signature motif plays a key role in the dimerization of the NBDs as it binds to the phosphate groups of ATP in the closed NBD dimer. The degenerate site ABC signature motif of EfrCD has the sequence FSTGQ, whereas the equivalent sequence of the consensus site is FSGGQ. The T489G\(EfrD\) substitution at the degenerate site resulted in a mutant carrying two noncanonical ABC signature motifs. The mutant showed a mildly impaired transport activity for all three dyes tested, and had a 5.6-fold reduced \(v_{\text{max}}\).

Interestingly, the apparent affinity for ATP increased 2.4-fold, which is presumably caused by interactions of the hydroxyl group of the introduced threonine with the phosphates of ATP. In rat TAP1/2, the corresponding amino acid is a valine and it has been suggested that—in contrast to EfrCD—the noncanonical deviation causes a decrease of affinity, which permits the reopening of the degenerate site without the necessity to hydrolyze the bound ATP [18]. With regard to substrate transport, our data are in full agreement with a study on human TAP1/2, which showed increased peptide transport for a chimera carrying two consensus site ABC signature motifs and slightly reduced transport for a chimera containing two degenerate site ABC signature motifs [24].

The mutations in EfrCD lead to mildly asymmetric functional results. In general, alterations at the middle position of the ABC signature motif are comparatively well tolerated.

**D-loop**

The D-loop owes its name to a highly conserved aspartate at the end of the consensus sequence SALD. In TM287/288, the D-loops are structurally asymmetric. The degenerate site D-loop strongly interacts with the opposite NBD and thereby prevents complete NBD separation of the inward-facing transporter. In contrast, the consensus site D-loop is highly flexible and only contacts the Walker A motif of the opposite

| Motif     | Mutation | NBS    | \(v_{\text{max}}\) (mmol Pi min\(^{-1}\)) | \(K_{\text{m}}\) (mM) | \(n\) |
|-----------|----------|--------|------------------------------------------|------------------------|-------|
| Wild-type | EfrCD    | Consensus | 6577 ± 194                       | 0.52 ± 0.04             | 1.78 ± 0.23 |
| A-loop    | Y338A\(EfrC\) | Degenerate | 64.5 ± 6.5                        | 1.95 ± 0.44             | 1.01 ± 0.10 |
|           | Y359A\(EfrC\) | Consensus | 88.3 ± 6.1                        | 1.91 ± 0.24             | 1.48 ± 0.16 |
| Walker A  | K369M\(EfrC\) | Degenerate | Inactive                             |                        |       |
|           | K389M\(EfrC\) | Consensus | Inactive                             |                        |       |
| Walker B  | D492E\(EfrC\) | Degenerate | 1231 ± 63                           | 1.38 ± 0.11             | 1.77 ± 0.16 |
|           | D492E\(EfrC\) | Consensus | 1241 ± 34                           | 1.33 ± 0.07             | 1.56 ± 0.09 |
| Switch loop | G523A\(EfrC\) | Degenerate | 5014 ± 66                           | 0.68 ± 0.02             | 1.79 ± 0.09 |
|           | H543A\(EfrC\) | Consensus | Inactive                             |                        |       |
| ABC signature | G489T\(EfrC\) | Consensus | 1171 ± 13                           | 0.22 ± 0.01             | 1.87 ± 0.14 |
|           | T489G\(EfrD\) | Degenerate | 3625 ± 84                           | 0.74 ± 0.36             | 1.97 ± 0.19 |
| D-loop    | D498A\(EfrC\) | Consensus | Inactive                             |                        |       |
|           | D518A\(EfrC\) | Degenerate | 182 ± 11                            | 1.69 ± 0.15             | 2.12 ± 0.29 |
| Q-loop    | O411A\(EfrC\) | Degenerate | 1940 ± 43                           | 1.27 ± 0.04             | 2.05 ± 0.11 |
|           | O431A\(EfrC\) | Consensus | 443 ± 12                            | 0.96 ± 0.04             | 2.11 ± 0.19 |

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when a nucleotide is bound to the degenerate site [11]. Introduction of the D-loop mutations into EfrCD had asymmetric functional consequences. The consensus site D-loop mutant D498AEfrC was completely inactive in terms of ATP hydrolysis and dye transport in cells and ISOVs (Fig. 6, Table 1). The degenerate site D-loop mutant D518AEfrD has a 36-fold reduced $v_{\text{max}}$ of ATP hydrolysis and its apparent $K_m$ is 1.69 mM, which is much increased compared to the wild-type transporter. Nevertheless, the D518AEfrD mutant still exhibited clearly measurable transport activity in cells and ISOVs. In summary, the degenerate site D-loop aspartate is important for the proper function of EfrCD, while the consensus site D-loop aspartate is essential. The asymmetric result of these equivalent mutations can be explained by structural differences between the D-loops as observed in TM287/288.

**Q-loop**

The Q-loop contains an eponymous highly conserved glutamine residue. In high-resolution full-length ABC exporter structures, the oxygen atom of the glutamine side chain interacts with the catalytic magnesium ion and is in hydrogen bonding distance of the $\gamma$-phosphate of ATP [5,25–27]. The other residues of the Q-loop are at the base of a groove in the NBDs, which serve as docking site accommodating the coupling helices of the TMDs [28]. A recent study on ABCB1 has demonstrated that the Q-loop plays a role in coupling drug binding and transport events at the TMDs with the catalytic cycle at the NBDs [29]. By introducing the Q411AEfrC substitution, we disrupted this suggested cross-domain communication pathway in the degenerate site. The mutant was slightly impaired in transporting the dyes ethidium and Hoechst 33342.

| A | B | C | D |
|---|---|---|---|
| Ethidium | Hoechst 33342 | BCECF-AM | Hoechst 33342 (ISOVs) |
| EfrCD | E512QEfrD | H543AEfrD | Q523AEfrC |
| consensus | consensus | degenerate |

**Fig. 4.** Transport activities of switch loop mutants. Ethidium (A), Hoechst 33342 (B), and BCECF (C) accumulation in *Lactococcus lactis* NZ9000 _ΔmraΔlmrCD_ cells, and Hoechst 33342 accumulation in ISOVs (D) containing overexpressed H543AEfrD (green), Q523AEfrC (blue), wild-type (black), and E512QEfrD (gray) transporters.
and its $v_{\text{max}}$ was reduced by a factor of 3.4 as compared to wild-type EfrCD (Fig. 7, Table 1). The phenotype was more pronounced when the equivalent mutation Q431AEfrD was introduced into the consensus site, which led to a 15-fold reduced $v_{\text{max}}$ value and a clearly reduced capacity to transport all three dyes.

Both Q-loops proved to be of functional relevance in EfrCD. The consensus site Q-loop is in direct contact with the catalytic magnesium, which explains the observed loss of ATPase and transport activity. Interestingly, the equivalent mutation in the degenerate site as well resulted in a 15-fold reduced $v_{\text{max}}$ value and a clearly reduced capacity to transport all three dyes.

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Both A-loops of EfrCD contain a tyrosine residue which contributes to nucleotide binding by interacting with the adenine ring of ATP. To study its functional role, the tyrosines were replaced by alanines, thereby removing the aromatic ring needed for $\pi$-stacking. The corresponding mutants Y338A\textsuperscript{EfrC} and Y359A\textsuperscript{EfrD} in the degenerate and consensus site, respectively, had very similar functional properties.

**A-loop**

Both A-loops of EfrCD contain a tyrosine residue which contributes to nucleotide binding by interacting with the adenine ring of ATP. To study its functional role, the tyrosines were replaced by alanines, thereby removing the aromatic ring needed for $\pi$-stacking. The corresponding mutants Y338A\textsuperscript{EfrC} and Y359A\textsuperscript{EfrD} in the degenerate and consensus site, respectively, had very similar functional properties.
Their $v_{\text{max}}$ values for ATP hydrolysis were around 100-fold decreased with respect to the wild-type transporter (Table 1). In addition, the $K_m$ values are 1.95 mM and 1.91 mM for Y338AEfrC and Y359AEfrD, respectively, indicating a much decreased apparent ATPase affinity as a consequence of these mutations. Interestingly, the mutation in the degenerate site (Y338AEfrC) abrogated the positive co-operativity of ATP binding (Hill coefficient of 1.01 instead of 1.78 for wild-type EfrCD). Given the low ATPase activities of the mutants, the cellular transport activity remained surprisingly high for both A-loop mutants (Fig. 8), i.e., they were similar to the degenerate site Walker B mutant D492NEfrC. In contrast, Hoechst 33342 transport in ISOVs was heavily impaired, in particular for the degenerate site mutant Y338AEfrC. The A-loop mutants clearly show that nucleotide binding to both ATP-binding sites is of critical importance for EfrCD and that the mutations impair the function in a similar fashion regardless whether they were introduced at the degenerate or the consensus site.

**Walker A motif**

The Walker A motif contains a highly conserved lysine residue, which plays a role in ATP binding, NBD closure, and ATP hydrolysis by interacting with the $\beta$- and $\gamma$-phosphates of ATP. In EfrCD, both NBSs contain the conserved Walker A lysine and its positive charge was removed by substituting with methionine. The resulting single mutants K369MEfrC and K389MEfrD were catalytically inactive and did not exhibit any measurable transport activity in cells and ISOVs (Fig. 9, Table 1). This finding reinforced the observation made for the A-loop mutants that regardless whether the mutation was
introduced at the degenerate or the consensus site, the functional outcome was symmetric. From these results, it was concluded that nucleotide binding at the degenerate site is a strict requirement for NBD closure and transport activity of EfrCD.

Discussion

Many heterodimeric ABC exporters contain asymmetric ATP-binding sites in which one site carries a characteristic set of noncanonical substitutions. In Type I ABC exporters sharing a TMD architecture as described for TM287/288 [5], including the prominent human examples TAP1/2, SUR1, and CFTR (Fig. 2), the deviations occur in Walker B (aspartate or serine instead of glutamate), switch loop (glutamine or serine instead of histidine), and ABC signature (substitution of the central glycine by another amino acid), indicating that they play a dedicated function. Of note, there are asymmetric Type II ABC exporters with an inverse domain topology and different TMD architecture, such as Pdr5 [30], Cdr1 [31], and ABCG5/ABCG8 [32]. They contain a degenerate ATP-binding site as well, but the pattern and functional role of their noncanonical substitutions is different from classical Type I ABC exporters. In EfrCD and its closely related homologs, the deviations at the degenerate site lead to an inactivated catalytic dyad. This implies that the task of the consensus site is the one of a workhorse, which is responsible for ATP hydrolysis, while the functional role of the degenerate site is the one of a coachman. Thus, the degenerate site does not hydrolyze ATP, but rather senses nucleotide binding and regulates the events at the consensus site. Structure–function studies and DEER measurements on TM287/288 of EfrCD demonstrated that nucleotide binding at the degenerate site facilitates transport, while the functional role of the consensus site is to provide the energy for transport.
revealed two roles of nucleotide binding at the degenerate site \([11,12]\): (a) it prevents full NBD dimer separation by establishing additional cross-NBD hydrogen bonds in an allosteric fashion and (b) communicates across the NBD to the consensus site via the flexible consensus site D-loop to promote ATP hydrolysis.

Here, we studied key residues in the NBDs of EfrCD with unmatched completeness. Of note, all EfrCD mutants yielded similar amounts of protein and eluted as monodisperse peaks from size exclusion chromatography (not shown). Remarkably, all point mutants had a decreased ATPase activity compared to the wild-type transporter, and with the exception of \(T489G^{EfrD}\) and \(D492E^{EfrC}\), they were partially or fully defective in transport. Hence, the sequence motifs of both the degenerate and the consensus site form an intricate network, which operates in concert and requires all players for efficient ATP hydrolysis and substrate transport. To the best of our knowledge, A-loop and Q-loop mutants have never been investigated in other Type I ABC exporters containing a noncanonical NBS.

Mutations introduced into Walker B and switch loop, which vary between the two NBSs are comparatively well tolerated when introduced at the degenerate site, but inactivated the transporter when placed at the consensus site (Table 2). This finding is in accord with previous reports investigating the Walker B motifs of LmrCD \([16]\), CFTR \([19]\), TAP1/2 \([8]\), and TmrAB \([17]\), and the switch loop of TAP1/2 \([8,18]\). We also created EfrCD with two degenerate or two consensus ABC signature motifs, leading to a slight reduction or slight activation of transport activity, respectively. This finding was again in agreement with reports on TAP1/2 \([18,24]\).

**Fig. 8.** Transport activities of A-loop mutants. Ethidium (A), Hoechst 33342 (B), and BCECF (C) accumulation in *Lactococcus lactis* NZ9000 \(ΔlmrAΔlmrCD\) cells, and Hoechst 33342 accumulation in ISOVs (D) containing overexpressed \(Y359A^{EfrD}\) (green), \(Y338A^{EfrC}\) (blue), wild-type (black), and \(E512Q^{EfrD}\) (gray) transporters.
Substitutions of the A-loop tyrosine and the Walker A lysine, which are invariant in both NBSs, resulted in identical functional outcomes regardless where they were introduced. This finding stands in contrast to previous studies on TAP1/2 [33], MRP1 [10], and CFTR [19], in which Walker A mutants introduced at the degenerate site resulted only in a partial inactivation, while the equivalent mutations at the consensus site fully inactivated the transporters. At the consensus site, the Walker A lysine is involved in nucleotide binding and hydrolysis, which explains why the residue is crucial for function. At the degenerate site, however, the Walker A lysine plays only a role in nucleotide binding and NBD closure. Substitution of the Walker A lysine leads to a strong affinity decrease [7,10]. The residual nucleotide affinity at the degenerate site of MRP1 and CFTR may be sufficiently high for partial nucleotide binding, which then results in a partial transport activity, while in EfrCD, the affinity drop may be so severe that nucleotide binding vanishes completely. As an alternative explanation, Walker A

Table 2. Data summary. sign., signature.

| Motif     | Degenerate | Consensus | Functional outcome |
|-----------|------------|-----------|--------------------|
| Wild-type | None       | +++       | None               | +++                |
| A-loop    | Y→A        | +/+       | Y→A                | +/+                |
| Walker A  | K→M        | -         | K→M                | -                  |
| Walker B  | D→E/N      | ++        | E→Q                | -                  |
| Switch loop | Q→A       | +         | H→A                | -                  |
| ABC sign. | T→G        | +++       | G→T                | ++                 |
| D-loop    | D→A        | +         | D→A                | -                  |
| Q-loop    | Q→A        | +         | Q→A                | +                  |

Fig. 9. Transport activities of Walker A mutants. Ethidium (A), Hoechst 33342 (B), and BCECF (C) accumulation in Lactococcus lactis NZ9000 ΔlmrAΔlmrCD cells, and Hoechst 33342 accumulation in ISOVs (D) containing overexpressed K389MEfrD (green), K369MEfrC (blue), wild-type (black), and E512QfrD (gray) transporters.
The influx of dyes into the mutated at the consensus site D-loop did not facilitate dependent passive facilitator [34]. Of note, EfrCD mutant turned the active transporter into a nucleotide-active in terms of transport and the consensus site 2, in which the degenerate site mutant remained fully determined, while it was found to be even increased for the equivalent mutation at the degenerate site [11]. The EfrCD data also differ from a D-loop study on TAP1/2, in which the degenerate site mutant turned the active transporter into a nucleotide-dependent passive facilitator [34]. Of note, EfrCD mutated at the consensus site D-loop did not facilitate the influx of dyes into the L. lactis cell. Facilitated dye influx was previously described for uncoupled mutants of the major facilitator family drug transporter LmrP in the same organism [35].

By comparing basal ATPase activity and transport activity, we observed differences in coupling efficiencies. The A-loop mutants, degenerate site Walker B mutants and the consensus site ABC signature mutant, exhibited strongly reduced ATPase activities, but retained comparatively high transport activities. Hence, they seem to couple ATP hydrolysis more efficiently with substrate transport than the wild-type protein and appear to run in an ‘energy-saver’ mode. In contrast, the switch loop mutant Q523AEfrC retained a high ATPase activity while being severely impaired in transport, indicating a coupling defect. Interestingly, the apparent ATP affinity of most ‘energy-saver’ mutants was found to be decreased and their transport rates likely become strongly attenuated if the ATP concentration drops in the cytoplasm. Unfortunately, we were unable to measure EfrCD-mediated ATPase activities directly in cell-derived ISOVs, which would have permitted the determination of the ATPase activity in the native membrane. Therefore, conclusions regarding the coupling need to be taken with a grain of salt.

Overall, we observed asymmetric results for mutations introduced at motifs that differ among the two NBs. Mutations placed at the noncanonical NBS were consistently better tolerated. Asymmetric functional results were as well obtained for mutations of the D-loop and the Q-loop, which play a role in interdomain communication. While the D-loop exhibits distinctive structural differences among the two NBs, it remains to be shown whether this is also the case for the Q-loop, for example, in the still missing outward-facing structure of a heterodimeric ABC exporter. Finally, when mutating motifs which are invariant among the two NBs, such as Walker A and A-loop, the observed loss of function was identical regardless where the mutations were introduced.

This in-depth study provides novel insights into the functional mechanism of the large family of heterodimeric ABC exporters and will pave the way for further studies on transporters of high medical relevance involved in multidrug resistance and hereditary diseases.

**Experimental procedures**

**Bacterial strains, growth media, and chemicals**

*Escherichia coli* MC1061 was grown in Luria broth (LB) at 37 °C. *Lactococcus lactis* NZ9000 *AlmrAAlmrCD* was grown in M17 (Oxoid, Pratteln, Switzerland) supplemented with 0.5% glucose at 30 °C. The concentrations of antibiotics were as follows: ampicillin, 100–120 μg·mL⁻¹ (*E. coli*); chloramphenicol, 20–25 μg·mL⁻¹ (*E. coli*) or 5 μg·mL⁻¹ (*L. lactis*); and erythromycin, 5 μg·mL⁻¹ (*L. lactis*). All chemicals and antibiotics were purchased from Sigma-Aldrich (Buchs, Switzerland).

**Cloning, expression, and purification of transporters in and from *L. lactis***

The enterococcal ORF *efrCD* was amplified from the genomic DNA of *E. faecalis* V583 and cloned into the pREXNH3 shuttle vector as described previously [20,36]. The mutants were generated by QuikChange site-directed mutagenesis (primer sequences are given in Table 3 and were obtained from Microsynth, Balgach, Switzerland). Wild-type and mutant *efrCD* were then subcloned into the *L. lactis* expression vector pNZ8048 via vector-backbone exchange (VBEx) cloning [37]. EfrCD was expressed in *L. lactis* and purified using β-DDM as described previously [20].

**Transport assay of fluorescent dyes in cells**

*Lactococcus lactis* NZ9000 *AlmrAAlmrCD* cells harboring plasmids encoding wild-type or mutant EfrCD were grown in M17 containing 5 μg·mL⁻¹ chloramphenicol and 0.5% glucose at 30 °C. Expression was induced at an OD₆₅₀ of 0.4–0.6 with a nisin containing culture supernatant of *L. lactis* NZ9700 for 1 h [1 : 1000 (v/v)]. Cells were washed and resuspended using fluorescence buffer (50 mM Kᵢ, pH 7.0, 5 mM MgSO₄), adjusted to an OD₆₅₀ of 0.5 and
energized by adding 0.5% glucose. Nigericin and valinomycin (1 μM each) were added prior to the addition of 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) to avoid changes of BCECF fluorescence as a result of pH changes in the cytoplasm. The accumulation of 5 μM ethidium, 0.5 μM Hoechst 33342, or 0.2 μM BCECF-AM was followed at 25°C for 600 s using a Fluorescence Spectrometer LS-55 (Perkin Elmer, Schwerzenbach, Switzerland). Excitation and emission wavelengths and slit widths were set at 520 nm, 10 nm and 595 nm, 15 nm for ethidium, 355 nm, 5 nm and 457 nm, 5 nm for BCECF, and 352 nm, 2.5 nm and 457 nm, 5 nm for BCECF-AM, respectively. Transport assays for each mutant were conducted four times with two independent cell batches. For clarity of discussion, one representative dataset is shown.

**Hoechst 33342 accumulation in inside-out membrane vesicles (ISOVs)**

Inside-out membrane vesicles were prepared using a Microfluidizer (Microfluidics) at 30 000 psi as described previously [20] and collected by ultracentrifugation. The protein concentration in ISOVs was determined by the Micro bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce, Reinach, Switzerland) and BSA as standard. ISOVs containing 400 μg protein were added to 2 mL of 50 mM KPi pH 7.0 and 10 mM MgSO4. About 0.5 μM Hoechst 33342 was added 10 s after starting the measurement. Transport was initiated by adding 5 mM ATP after 180 s. Hoechst 33342 fluorescence was monitored for 600 s at 25°C. Excitation and emission wavelengths (slit widths) were set at 355 nm (5 nm) and 457 nm (5 nm), respectively. Transport assays were conducted four times with two independent ISOV batches and one representative dataset is shown.

**ATPase activity assay**

ATPase activity was determined using detergent-purified protein in 50 mM K-HEPES pH 7.0, 10 mM MgSO4, and 0.03% β-DDM containing increasing concentrations of ATP (0.1–8 mM) for 15 min at 30°C. Hydrolyzed inorganic phosphate was detected colorimetrically using a mala- chite green-molybdate solution as described previously [11]. The data were fitted using the Hill equation (SIGMAPLOT 11.0; Scientific Solutions, Pully, Switzerland).

### Table 3. Primer sequences for mutant generation using QuikChange site-directed mutagenesis.

| Primer sequences for mutant generation using QuikChange site-directed mutagenesis. |
|---------------------------------------------------------------|
| **EfrC_Y338A** | FW: CCACGTATCTTCCGTTGtgcTCTCTGGGGAGT |
| RV: CATCCCGAGGgCAAGCAATACGAGT |
| **EfrC_K369M** | FW: GGCCGTGTTGAtgcTCTCATATTG |
| RV: GCCAAAGCtACAGGCAACG |
| **EfrC_Q411A** | FW: GTTTCCTTTGGTGTGGcAAGACCAATCTG |
| RV: CAGGATGTCTTTGcCAACCAAGG |
| **EfrC_G469T** | FW: GAGTATACACATTTCgAGGCAAAAAGAC |
| RV: CGGTGTTTTTGGCCgTAAGCT |
| **EfrC_D492E** | FW: GATTTAGATTGAgAGCACCAGCG |
| RV: CGCGCTGGTGCTATATAT |
| **EfrC_D492N** | FW: GATTTAGATTGaAGCACCAGCG |
| RV: CGCGCTGGTGCTATATAT |
| **EfrC_D498A** | FW: GCGCGTTGcTGCTCGTTCGGAG |
| RV: CTCCGAACGACAgCCAACG |
| **EfrC_QS23A** | FW: CGATCGTTATGCGcAAAAATTTCTCCTGG |
| RV: CAACAGAAGAAAAATTGGTCGAGCAATAACGATC |
| **EfrC_K369A** | FW: CATGTTGTTTTGGCgcTAAATCCAG |
| RV: CTGGATTAgcGCCAAAACACATG |
| **EfrC_K389M** | FW: GGCTCCAGGAAtgcACAGCATTG |
| RV: CATAAACCTTTCGcAAGGTCAT |
| **EfrC_Q431A** | FW: GCCATGTGGTTGGcAGAGCCTCGTACTAAACG |
| RV: GAAAATAAGCAGCGACTCtgcCAAAACACATG |
| **EfrC_T489G** | FW: GAATACCCCTTTTGGTgGAGGCAAAAAGC |
| RV: GCTTTTGGCACCcACTGAAAAGTTATTC |
| **EfrC_E512Q** | FW: GAATTATTAATTTTGGATcAAGCAACAAG |
| RV: CTTTGTTGCTGTagAGAAATATTAGAAATAC |
| **EfrC_D518A** | FW: GCAACAGGATATGTTGcTCAGTAAACAGAAAGT |
| RV: TAGCTTCTGTTAAGTAgCAACATTACTTGTTG |
| **EfrC_H543A** | FW: GTTTTTGTGATTGCGcTGTTTGGAAAC |
| RV: CGTTTTCCAAACGAgcGCCAAATCACAACAAAC |
\[ Y = \frac{v_{\text{max}} \cdot X^n}{K_m + X^n} \]

In which \( v_{\text{max}} \) corresponds to the maximum rate of ATP hydrolysis, \( K_m \) to the apparent ATP affinity, and \( n \) to the Hill coefficient.

**Conflict of interest**

The authors declare no conflict of interest.

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**Author contributions**

MAS conceived and coordinated the study. MH introduced LMH into the project and supervised the project at the beginning. LMH designed, performed, and analyzed all experiments and made all figures. MAS wrote the first draft of the manuscript, which was completed together with LMH and MH. All authors approved the final version of the manuscript.

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