CHAPTER FIVE

Molecular investigation of drug efflux pumps by deep mutational scanning

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Methods in Enzymology, Volume 724
ISSN 0076-6879, https://doi.org/10.1016/bs.mie.2025.09.007
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Abstract

Bacteria protect themselves from the noxious action of antibiotics and other toxic compounds by the expression of drug efflux pumps, which are highly efficient in extruding them out of the cell. Recent advances in structure determination using cryo-EM and structure predictions via AlphaFold have provided detailed insights into the architecture and conformational space of drug efflux machineries. In contrast, comprehensive functional data of high quality are scarce, although they are crucial to understand the enigmatic phenomenon of drug efflux at the molecular level. Recently, we have applied the well-established method of deep mutational scanning (DMS) to drug efflux pumps and thereby shed light on the molecular mechanism of drug transport mediated by the heterodimeric ABC exporter EfrCD of the opportunistic pathogen *Enterococcus faecalis*. In DMS, a single site-saturation library is generated by substituting a subset or all residues to the other 19 amino acids. The resulting library is subjected to a functional screen, where in the case of drug efflux pumps, variant activity in the presence of a transport substrate is reflected in the growth phenotype of the expressing cells. NGS is then used to quantify enrichment or depletion of variants relative to the wild-type transporter. Such experiments allow for the systematic assessment of thousands of variants in parallel, under identical conditions and across multiple substrates, thereby delivering comprehensive mutational landscapes of drug transport. In this chapter, we outline the technical details how DMS projects on drug efflux pumps are planned and executed.

1. Introduction

In recent years, the experimental determination of numerous high-resolution structures of bacterial drug efflux pumps has contributed to our mechanistic understanding of these transporters (Du et al., 2018). However, structural insights alone are insufficient to fully explain their function, particularly regarding their remarkable polyspecificity. To bridge this gap, functional assays play a key role by providing drug efflux activity data that are crucial to elucidate transport mechanisms. However, performing functional assays of bacterial drug efflux pumps pose several challenges. First, functional data should be of high quality and reproducible. *In vivo* studies often rely on measuring the growth of cells expressing transporter variants and therefore yield ambiguous results due to variability between biological and technical replicates (Meier et al., 2023). Second, the vast sequence space of these transporters makes it impractical to assess all possible mutations using conventional site-directed mutagenesis, where a limited number of positions are typically mutated to a restricted set of amino acids, in most cases alanine. This approach fails to capture the full mutational landscape and may fail to identify key functional residues as well as critical substitutions.

Deep mutational scanning (DMS) offers a powerful solution to these limitations. In a DMS experiment, selected positions are systematically randomized to all possible amino acids, ensuring unbiased coverage of the sequence space. The functional impact of these mutations is then assessed by subjecting the entire library of variants to a functional selection assay, followed by next-generation sequencing (NGS) of the libraries before and after selection (Fowler & Fields, 2014; Fowler et al., 2010). We established a DMS pipeline to investigate the bacterial multidrug efflux pump EfrCD from *Enterococcus faecalis*, a heterodimeric ATP-binding cassette (ABC) transporter composed of the two polypeptide chains, EfrC and EfrD (Meier et al., 2023) (Fig. 1). Each 'half-transporter' contains an N-terminal transmembrane domain (TMD) and a C-terminal nucleotide-binding domain (NBD). The TMDs form the large substrate-binding cavity and translocation pathway, while the more conserved NBDs are essential for ATP hydrolysis, which drives the transport process (Hürlimann et al., 2017; Meier et al., 2023). We systematically mutated each of the selected 79 residues in EfrCD to all other possible amino acids and assessed the functional consequences of over 1500 single mutations. A pool of *Lactococcus lactis* cells, each expressing a single transporter variant, was subjected to a growth-based selection in the

Fig. 1 Deep mutational scanning of EfrCD. **Library generation:** structure-based selection of 65 TMD and 14 NBD residues. A plasmid-based single site-saturation variant library containing 1501 EfrCD variants was generated. **Selection:** *L. lactis ΔlmrCD ΔlmrA* cells, each expressing one EfrCD variant, were grown in the presence of ethidium, Hoechst 33342 or daunorubicin at sub-minimal inhibitory concentration. **Next-generation sequencing:** plasmids were extracted from the input and selected culture, sheared into 150-bp fragments and sequenced in paired-end mode on Illumina NovaSeq 6000. Variants were counted in the input and selected libraries and variant scores were calculated with Enrich2 (Rubin et al., 2017) and visualized in a sequence-function heat map.

presence of ethidium, daunorubicin or Hoechst 33342 (hereafter referred to as Hoechst). By performing NGS of the input and selected library pools, variant scores were determined for each mutation to quantify their functional impact (Meier et al., 2023).

This high-throughput functional approach provided several advantages. First, it enabled the parallel analysis of hundreds of variants under identical experimental conditions, minimizing biological variability. Second, it captured a broad spectrum of mutational effects, including both gain- and loss-of-function variants – an aspect that is ignored in directed evolution studies based on gain-of-function screens. Moreover, the detection of subtle functional differences among variants was possible owing to the high sensitivity of NGS. Finally, DMS uncovered unexpected functional insights of EfrCD. Notably, the introduction of a single negative charge at specific TMD positions strongly enhanced ethidium efflux, while certain mutations led to Hoechst influx without affecting the efflux activity of ethidium and daunorubicin (Meier et al., 2023). These findings highlight the power of DMS in revealing intricate functional patterns that would likely remain undetected with conventional mutagenesis approaches.

2. Deep mutational scanning: Key steps and requirements

DMS involves three major steps (variant library generation, selection and NGS) and relies on a genotype-phenotype linkage. *In vivo* selection, typically based on fitness, has been widely applied in various systems, including bacteria (Stiffler et al., 2015; Sun & Palzkill, 2021), yeast (Melamed et al., 2013), viruses (Starr et al., 2020) and mammalian cells (Maes et al., 2023). This approach operates under the principle that functionally advantageous variants enhance cell survival and become enriched over time, whereas non-functional variants are gradually depleted. For the selection step to be meaningful, the investigated protein must meet specific criteria. Ideally, the drug efflux pump under study should exhibit strong activity, ensuring a clear and measurable phenotype. It is crucial to confirm that the observed phenotypic effects stem from the function of the studied pump rather than from endogenous transporters or off-target effects. To achieve this, it is recommended to use a bacterial strain in which major native efflux pumps have been knocked out, ensuring that observed phenotypes are directly attributable to the transporter variant being tested.

It is preferable to express the drug efflux pump from a plasmid under an inducible promoter, allowing precise control over expression levels and ensuring that all cells start from the same baseline condition.

3. Preliminary considerations

Using the bacterial ABC transporter EfrCD as a working model, here, we present a detailed protocol for performing DMS on a bacterial drug efflux pump. While the overall pipeline will remain the same, it is important to recognize that each transporter may require specific adjustments. Since all three major steps of DMS are interconnected, adjustments in one aspect of the workflow can impact the others. For example, the type of mutagenesis employed dictates the diversity of variants, while the sequencing strategy – whether using only short-read sequencing or combining short- and long-read approaches – can influence both plasmid design and codon choice. Designing and optimizing the experimental protocol is an iterative process which requires careful planning and a thorough quality assessment of the experimental design. While there is no single universal approach, this protocol has proven effective for our studies, and we aim to share our insights to help guide future DMS investigations of drug efflux pumps.

3.1 Selection of residues

For targeted mutagenesis, the first step is selecting residues for randomization. This can be guided by either structure-based selection – using experimentally determined structures or AlphaFold predictions (Jumper et al., 2021) – or sequence-based approaches. Including control positions with known or expected phenotypic outcomes is highly recommended, as they provide valuable reference points to validate the DMS results. These controls serve two key purposes: (i) variant scores and sequence-function patterns at these positions act as a sanity check to assess whether the obtained results are meaningful and (ii) the degree of enrichment or depletion in these variants helps to estimate the selection pressure, aiding in the comparison of datasets from different conditions. In our study, we focused on the large substrate-binding cavity, aiming to understand how different drugs interact with specific residues. Based on the cryo-EM structure of EfrCD (PDB ID 7OCY), we selected 65 TMD residues lining the cavity for randomization. Additionally, we included 14 conserved NBD residues as controls, expecting them to mainly show depletion due to

their critical role in ATP binding and hydrolysis (Hürlimann et al., 2016, 2017). We focused on variants harboring only a single mutation for two main reasons. First, combinatorial libraries containing at least two mutations result in an exponentially larger pool of variants, which can be affected by diversity bottlenecks and demand more extensive sequencing efforts. Second, our sequencing method involves shearing the gene prior to Illumina short-read sequencing and therefore, does not allow for the detection of variants with multiple mutations, especially when these mutations are situated far apart.

3.2 Mutagenesis strategy

The choice of method for generating variant libraries is critical, as it determines both the quality and the extent of coverage of the mutations. Several approaches are available, targeting either at the nucleotide level or the amino acid level (Wei & Li, 2023). Irrespective of the library generation method, it is important that the DMS library contains all possible amino acid substitutions at each selected position.

One common approach is to use NNK degenerate primers (N = A, T, G or C; K = T or G) in combination with overlap PCR for targeted mutagenesis. This method, though effective, by definition results in an uneven distribution of variants as the 20 amino acids are encoded by 32 NNK codons, effectively meaning that some amino acids such as methionine are only encoded by one codon, whereas other amino acids such as leucine are encoded by three different codons. Ideally, the starting library should feature mutations that are evenly distributed. An uneven distribution requires more extensive sequencing to obtain reliable statistical data. Moreover, with the NNK randomization approach, near-cognate sequencing errors may bleed into the reads of randomized codons at a noticeable frequency (see Section 3.4).

To address these challenges, we recommend using commercial providers like Twist Bioscience, which employ silicon-based DNA synthesis platforms to generate user-defined oligonucleotides. These oligonucleotides are then assembled into genes to create variant libraries that have two key advantages: (i) each amino acid is represented by one predetermined codon and (ii) the overall distribution of codons at each randomized position is balanced. In some instances, commercial providers might not deliver complete sub-libraries, i.e. the full set of 19 variants at a given position. If this occurs, NNK mutagenesis still proves to be an effective method for recovering the missing sub-libraries.

3.3 Plasmid construction

To generate the final expression plasmid for transporter variant expression in *L. lactis*, our workflow involves an initial PCR step to amplify the variant gene libraries, followed by two main cloning steps involving fragment exchange (FX) cloning (Geertsma & Dutzler, 2011) and vector backbone exchange (VBex) cloning (Geertsma & Poolman, 2007) (Fig. 2). Apart from the initial low-cycle PCR amplification used to increase the DNA yield from the ordered libraries, all subsequent steps – cloning the genes into the *E. coli* pREX propagation plasmid via FX cloning and transferring them from pREX to the final *L. lactis* pNZ expression plasmid using VBex – are performed exclusively by restriction digestion and ligation. This approach eliminates the need for further PCR amplification, thereby avoiding DNA shuffling and eliminating the risk of recombining mutations.

Both the pREX and the pNZ vectors are medium copy number plasmids. On both plasmids, the *efrCD* gene includes a C-terminal 3C protease cleavage site, GFP and a His_{10} tag, which enable downstream protein purification for functional assays. Notably, GFP technically allows for fluorescence-based applications such as FACS to determine protein

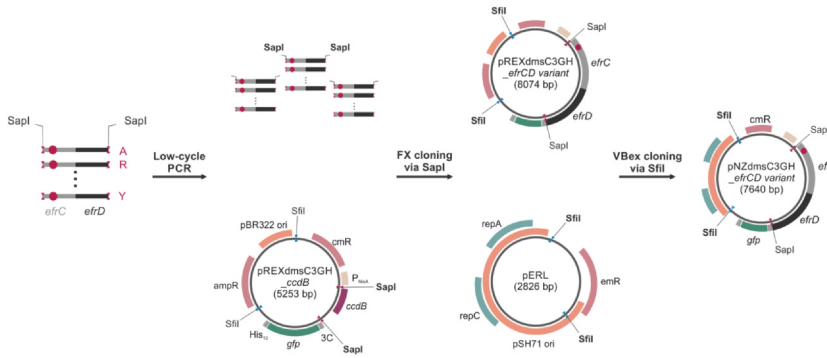

Fig. 2 Library generation. Commercially obtained variant gene sub-libraries flanked with *Sap*I restriction sites are PCR-amplified with a pre-determined low-cycle PCR. For FX cloning, the pREXdmsC3GH_*ccdB* plasmid and each amplified sub-library were digested with *Sap*I and ligated to generate individual pREXdmsC3GH_sub-library plasmids. Each sub-library was processed separately until this step. The sub-libraries are mixed equimolarly (not shown). For VBex cloning, the pREXdmsC3GH_*efrCD*_sub-library pools and the pERL vector are first digested with *Sfi*I, gel-purified and subsequently ligated to generate the pNZdmsC3GH plasmid pools harboring the *efrCD* variant genes.

variant abundances from the entire library pool, as well as *in-cell* GFP fluorescence measurements to quantify individual variant's expression level (see Section 5.2). Plasmids extracted from *L. lactis* are digested with *Sfi*I before shearing and sequencing. We found that extracting only the *efrCD* gene using restriction enzymes that cut precisely at the start and end of the gene (e.g., *Sap*I) led to low sequencing coverage at the 5′ region of the first and the 3′ region of the second half-transporter. By using the *Sfi*I cutting site, we retain approximately 2×1200 bp of flanking vector backbone on either side of the *efrCD* gene, which resolves the problem of having low sequence coverage at the beginning and the end of the transporter gene.

Our pipeline is based on Illumina short-read sequencing. More recently published DMS studies introduced NGS pipelines that combine short- and long-read sequencing by introducing barcodes to track individual variants (Grønbæk-Thygesen et al., 2024; Vanella et al., 2024; Wei & Li, 2023). Such strategies require a different plasmid design and additional cloning steps, which we do not discuss here.

3.4 Sequencing strategy

There are various sequencing platforms and approaches available for analyzing DMS libraries, each differing in sequencing depth, error rates, read length and other performance characteristics (Araya & Fowler, 2011; Satam et al., 2023). Given the large size of our *efrCD* gene (approx. 3.5 kb), we had the option of either employing long-read sequencing technologies such as Oxford Nanopore Technologies (ONT) and PacBio, or shearing the gene into smaller fragments suitable for short-read sequencing with Illumina. At the time we established our pipeline, long-read technologies were still limited by relatively high sequencing error rates and/or low throughput, which rendered them unsuitable for our study. As a result, we opted for Illumina short-read paired-end sequencing of sheared gene fragments (approx. 150 bp), thereby achieving an accuracy of around 99.9 %. While this approach provides reads with higher fidelity, it comes with its own set of challenges, primarily the loss of full-length gene context due to fragmentation.

This loss of context necessitates specific adaptations. First, a tagged wild-type variant must be introduced into the library. This is achieved by incorporating a synonymous codon change at a position not targeted for mutagenesis, enabling the identification of the wild-type transporter despite shearing. This tagged wild-type is spiked into the selection preculture and serves as a reference for enrichment or depletion calculations during data

analysis. To ensure its validity, the tagged wild-type transporter should be tested to confirm that its function and expression level does not deviate from the original wild-type transporter (see Sections 5.2 and 5.3). Second, despite the high accuracy and due to the overwhelming majority of sequenced fragments being wild-type, sequencing errors can be falsely interpreted as mutations. This mainly applies to near-cognate single-nucleotide changes. In our experiments, two independent NGS runs on wild-type *efrCD* gene yielded nearly identical sequencing error profiles, with C→A and G→T changes being the most prevalent (Meier et al., 2023). To mitigate the risk of misinterpreting sequencing errors as true mutations, we recommend codon-optimizing the wild-type drug efflux pump gene specifically at the positions selected for randomization. This involves determining two sets of codons: one set of 20 codons used to encode the selected wild-type amino acids on the gene sequence ('codon on wild-type gene') and a second set of 20 codons used for generating substitution variants ('codons for randomization'). The aim is to minimize the likelihood that sequencing errors would cause a codon from the wild-type set to be misread as a codon from the variant set. Typically, codon optimization would begin with selecting highly abundant codons in the expression host for both sets and evaluating the possibility for sequencing miscalls between each possible wild-type/variant codon pair. For each pair, an error score is calculated by multiplying the known error frequencies of each nucleotide change required to convert the wild-type codon into the variant codon. Based on previously determined error profiles (Meier et al., 2023), these potential misreads can be classified into four categories: high, medium, low or negligible. Codon pairs are accepted if the substitution requires two or three nucleotide changes, or a single nucleotide change that falls into the negligible error category. Codon pairs that involve single nucleotide changes falling into the high, medium or low error categories should be avoided whenever possible. If a codon pair is not suitable, alternative codons should be selected, prioritizing high-abundance options. In rare cases, low-abundance codons can be included only if they appear multiple times in the native efflux pump gene. The error-minimizing set of codons suitable for expression in *L. lactis* subsp. cremoris MG1363 is summarized in Table 1. Such a codon-optimized wild-type gene is then used as the template for generating the site-saturation variant library as well as for all single clone site-directed mutagenesis.

Finally, as discussed in the previous section, the shearing-based approach fundamentally restricts us to analyze single substitutions. Variants

Table 1 Codon table showing the 20 codons selected to encode the wild-type residues and the 20 codons used for randomization.

Codon on wild-type gene	Amino acid	Codon for randomization
GCC	Ala	GCT
AGA	Arg	CGT
AAC	Asn	AAT
GAC	Asp	GAT
TGC	Cys	TGT
CAA	Gln	CAA
GAA	Glu	GAG
GGA	Gly	GGT
CAC	His	CAT
ATC	Ile	ATC
TTA	Leu	CTT
AAA	Lys	AAG
ATG	Met	ATG
TTC	Phe	TTT
CCG	Pro	CCT
TCA	Ser	AGT
ACA	Thr	ACA
TGG	Trp	TGG
TAC	Tyr	TAT
GTA	Val	GTT

with multiple substitutions – particularly those at distant positions – cannot be reconstructed from short reads, making combinatorial variant analysis infeasible with this method.

An advancement that makes the shearing strategy unnecessary is the use of DNA barcoding in combination with long-read sequencing technologies,

which now offer accuracies exceeding 99.5 % (Karst et al., 2021). In this method, unique barcodes are cloned with each variant and long-read sequencing is then used to link each barcode to its full-length variant gene sequence. Following the selection experiment, only the barcodes need to be sequenced via short-read platforms. However, implementing this approach requires a different plasmid design, additional cloning steps and an adapted NGS analysis pipeline. Since we did not test this strategy ourselves, we do not provide guidance here on its implementation.

3.5 Selection conditions

Prior to performing the main DMS experiment, it is necessary to optimize the selection conditions by determining both the inducer concentration for gene expression and the drug concentration (either a single concentration or a range) for selection. First, we performed titration experiments using different dilutions of the inducer nisin and monitored two parameters simultaneously: (i) EfrCD expression levels via GFP fluorescence, enabled by the C-terminal GFP fusion to the EfrD subunit and (ii) cellular growth, by performing cellular growth assays. The optimal inducer concentration was defined as the dilution yielding strong (ideally maximal) GFP fluorescence without negatively affecting cell growth (Fig. 3). GFP expression typically follows a bell-shaped curve: low inducer levels result in insufficient expression, while high levels can cause toxicity due to overexpression, leading to reduced fluorescence and

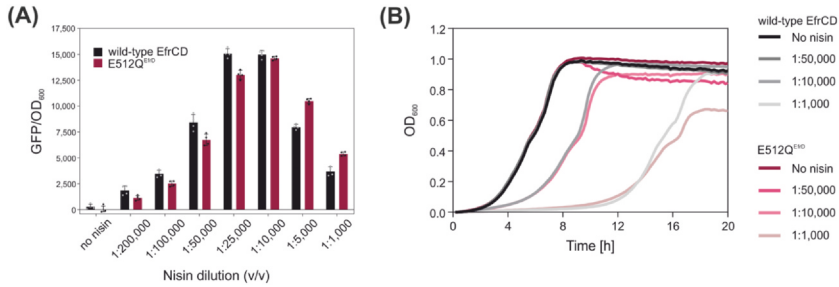

Fig. 3 Determination of the optimal inducer concentration. (A) Protein production determined via fluorescence of GFP fused to the C-terminus of the EfrD chain. The GFP standardized to OD_{600} is plotted for cells expressing the wild-type EfrCD (black bars) and the inactive $E512Q^{EfrD}$ variant (red bars) grown in the presence of different nisin dilutions. Data is represented as mean with standard deviations of technical quadruplicates. (B) Cellular growth assays of *L. lactis* Δ*lmrCD* Δ*lmrA* expressing the wild-type EfrCD (black curves) and the inactive $E512Q^{EfrD}$ variant (red curves) grown in the presence of different nisin dilutions. OD_{600} was measured in 10-min intervals for 20 h. The mean of technical duplicates is shown.

growth (Fig. 3A). Since the presence of drugs in the actual selection adds further burden, the chosen inducer concentration should yield sufficient expression, and at the same time only marginally affect growth (Fig. 3B).

Once the optimal inducer concentration was established, growth assays were conducted in the presence of varying drug concentrations to define the optimal range for the selection. The ideal concentration lies below the minimal inhibitory concentration, where the cells expressing wild-type EfrCD remain viable, but the inactive $E512Q^{EfrD}$ mutant fails to grow (Supplementary Fig. 4 of Meier et al., 2023).

We recommend generating and using the following variants as a test panel: the wild-type transporter, the tagged wild-type containing a synonymous codon mutation, a catalytically inactive mutant (e.g., $E512Q^{EfrD}$), a variant with enhanced efflux activity (e.g., $F255A^{EfrD}$) and a variant with reduced activity (e.g., $I239A^{EfrC}$). The wild-type and tagged wild-type should behave similarly, confirming that tagging does not affect function or expression. This careful optimization of selection conditions ensures that DMS produces robust and reproducible data.

4. Cultivation and genetic manipulation of *Lactococcus lactis*

L. lactis grows well under static, non-aerated conditions and does not require shaking. We cultivate cultures at 30 °C in standard plastic Falcon tubes or Schott bottles.

- **GM17-cm5 medium:** M17, 0.5 % glucose, 5 μg/mL chloramphenicol. **Note:** *Autoclave M17 broth and cool down, add glucose and chloramphenicol from the filtered stock solutions.*
- **GS-GM17 medium**: M17, 0.5 % glucose, 0.5 M sucrose, 1 % glycine, pH 7.0. **Note:** *Autoclave double concentrated (2x) M17 broth and cool down, add glucose, sucrose and glycine from the filtered stock solutions.*
- **Recovery medium:** M17, 0.5 M sucrose, 0.5 % glucose, 2 mM $MgCl_2$ and 0.2 mM $CaCl_2$. **Note:** *Autoclave double concentrated (2x) M17 broth, $MgCl_2$ and $CaCl_2$ stock solutions and add glucose, sucrose and glycine from the filtered stock solutions. Prepare the recovery medium freshly.*
- **M17-cm5 agar plates:** M17, 0.5 % glucose, 1.2 % (w/v) agar, 5 μg/mL chloramphenicol. **Note:** *Autoclave M17 broth and agar with a magnetic stir bar, add glucose from the filtered stock solution, and cool down before adding chloramphenicol and pouring the plates.*

4.1 Preparation of electrocompetent *Lactococcus lactis* cells

The following protocol is described for a 50 mL cell culture but can be scaled up or down as needed. If you plan to freeze the competent cells for later use, include 10 % ultra-pure glycerol in both wash and resuspension steps. If the cells are to be used immediately (e.g., for transformation during library generation), omit glycerol and use the freshly prepared cells within two hours. Work in a sterile environment and with sterilized consumables and reagents.

1. Make an overnight culture by inoculating 5 mL of GS-GM17 medium with a single colony of *L. lactis* NZ9000 Δ*lmrCD* Δ*lmrA* grown on a M17-agar plate without antibiotics.
2. Next morning, inoculate 50 mL of GS-GM17 medium with 2.5 mL of the preculture (1:20 dilution) and grow at 30 °C to an OD_{600} of 0.4–0.6.
3. Cool the cells on ice for at least 30 min.
4. Harvest the cells by centrifugation (4000 *g*, 4 °C, 15 min).
5. Wash the cells twice with 15 mL of 0.5 M sucrose (if freezing, include 10 % ultra-pure glycerol).
6. Resuspend cells in 2 mL of 0.5 M sucrose (if freezing, include 10 % ultra-pure glycerol).
7. Aliquot the cells. Use them immediately or flash-freeze in liquid N_2 and store at −80 °C.

4.2 Plasmid extraction from *Lactococcus lactis* cells

The protocol outlines plasmid extraction from 2 mL of *L. lactis* cell culture. For larger volumes (e.g., the 14 mL for NGS library preparation), perform multiple parallel extractions per sample.

Prepare the following solutions and filter through a 0.22-μm filter:

- **Solution A:** 10 mM Tris/HCl, pH 8.0, 20 % sucrose, 10 mM K-EDTA, pH 8.0, 50 mM NaCl.
- **Solution B:** 200 mM NaOH, 1 % SDS.
- **Solution C:** 3 M K-acetate, 5 M acetic acid, pH 4.8.

1. Harvest 2 mL of culture (3500 *g*, 4 °C, 5 min). Resuspend the cell pellet in Solution A supplemented with 30 mg/mL lysozyme and incubate the mix at 55 °C for 20 min.
2. Add 400 μL of Solution B and invert carefully 3–4 times. **Note:** *Lysate will turn clear.*

3. Add 300 µL of Solution C and invert carefully 3–4 times. **Note:** *Lysate will turn cloudy.*
4. Spin at maximum speed in a tabletop centrifuge for 10 min. Carefully transfer 650 µL of the supernatant into a new 2-mL tube pre-filled with 1.35 mL of 100 % ethanol.
5. Freeze the mix at −20 °C for > 30 min.
6. Pellet the precipitated DNA at maximum speed in a tabletop centrifuge for 5 min at room temperature (RT).
7. Discard the supernatant and wash the pellet with 500 µL of 70 % ethanol stored at RT without resuspending.
8. Spin for 5 min at RT and carefully discard the supernatant. Dry-spin the tube for another 2 min and remove the remaining supernatant with a pipette tip.
9. Air-dry the pellet until it becomes transparent.
10. Resuspend the pellet in 200 µL of nuclease-free water (NF-H_2O) supplemented with 20 µg/mL RNase A and incubate for > 15 min at RT.
11. Column-purify the plasmids using the NucleoSpin™ Gel and PCR Clean-up Kit according to the manufacturer's protocol. To increase the yield, pre-heat the elution buffer to 70 °C and reload the eluate for a second elution.

4.3 Production of nisin

The nisin-controlled expression (NICE) system is widely used for expression of homologous and heterologous proteins in *L. lactis* (Kunji et al., 2003; Mierau & Kleerebezem, 2005). Nisin, a 34-amino acid peptide, is naturally produced and secreted by *L. lactis* NZ9700 strain. For induction, we use filtered nisin-containing culture supernatant at defined dilutions. Since nisin production can vary between batches, we recommend preparing a large batch at once to ensure consistency across experiments. Aliquot the supernatant into large volumes for long-term storage at −80 °C and small working aliquots for storage at −20 °C. Furthermore, in our experience, repeated freeze-thawing impairs nisin stability and lowers induction efficiency.

1. Make an overnight culture by inoculating 15 mL of GM17 medium with a single colony of *L. lactis* NZ9700 grown on a M17-agar plate without antibiotics.
2. Next morning, inoculate 500 mL of GM17 medium with 10 mL of the preculture (1:50 dilution) and grow at 30 °C, gently shaking at 90 rpm.

3. After 6 h, harvest the cells by centrifugation (4000 *g*, 4 °C, 20 min). Transfer the supernatant into a new bottle and keep it on ice.
4. Filter the supernatant through a 0.22-μm filter.
5. Aliquot the supernatant and store at −20 °C or −80 °C.

5. Preliminary test experiments

5.1 Cloning and site-directed mutagenesis of single variants

The plasmid maps and the scheme of the cloning steps are illustrated in Fig. 2. The vector pREXdmsC3GH was derived from pREXC3GH (Addgene, #47077) by introducing inverted *Sap*I recognition sites, enabling the excision of the *efrCD* gene through *Sap*I restriction digestion (Meier et al., 2023). To generate the FX-compatible transporter gene fragment, we PCR-amplified the *efrCD* gene with primers that introduce *Sap*I recognition sites. The resulting PCR product was gel-purified and used for FX cloning.

1. Pipette the following FX cloning reaction: 1 μL NEBuffer™ r3.1, 0.5 μL *Bsp*QI (5 U), 350 ng of gel-purified gene fragment, 150 ng of vector pREXdmsC3GH_*ccdB* (around 3:1 molar ratio) and top up to 10 μL with NF-H_2O. Incubate the reaction at 50 °C for 1 h followed by heat-inactivation at 80 °C for 20 min. **Note:** *BspQI and SapI are isoschizomers, with BspQI being the more cost-effective option.*
2. Cool down the reaction to 10 °C before adding 1.25 μL of 10 mM ATP and 1.25 μL T4 DNA ligase (6.25 U) to the tube. Continue with incubation at 25 °C for 1 h, heat-inactivate at 65 °C for 10 min and cool down to 10 °C.
3. Mix 10 μL of FX product with 50 μL chemically competent *E. coli* MC1061 cells and incubate on ice for 10 min. **Note:** *The commercially available cells are electrocompetent, but to reduce costs, they can be used to prepare chemically competent cells (e.g., using the RbCl method) for routine transformations, instead of purchasing new cells each time.*
4. Heat-shock at 42 °C for 45 s, incubate on ice for 10 min, add 600 μL of Luria broth (LB) and recover transformed cells at 37 °C for 30 min, shaking at 600 rpm.
5. Plate 100 μL of the recovered culture on LB-agar plate supplemented with 100 μg/mL ampicillin and incubate overnight at 37 °C.

6. On the next day, isolate the plasmid from a single colony and verify the construct by sequencing.
7. Use the sequence-verified wild-type construct (e.g., pREXdmsC3GH_*efrCD*) as template for QuikChange site-directed mutagenesis to generate the other variants. Pipette the reaction as follows: 10 μL Phusion HF buffer, 5 μL dNTPs (0.2 mM each), 2 μL of forward primer and 2 μL of reverse primer (400 nM each and both containing the desired missense codon in the center), 30–50 ng of template, 0.5 μL Phusion polymerase (1 U) and top up to 50 μL with NF-H_2O. Set up the PCR as follows: (1) initial denaturation, 3 min at 98 °C; (2) denaturation, 30 s at 98 °C; (3) annealing, 30 s at 58 °C; (4) elongation, 30 s/kb at 72 °C; repeat (2)-(4) for 20 cycles; (5) final extension, elongation time + 3 min at 72 °C.
8. To verify the presence of the PCR product, mix 10 μL of the PCR product with 2 μL of 6x DNA loading dye and run it on a 1 % agarose gel.
9. To get rid of the methylated DNA template, pipette the following digest reaction: 1 μL CutSmart™ buffer, 1 μL *Dpn*I (20 U) and 8 μL of the PCR product. Incubate the reaction at 37 °C for 1.5 h and heat-inactivate at 80 °C for 20 min.
10. Perform transformation, plating, plasmid isolation and sequence verification as described in **steps 3–6**. Use the generated and sequence-verified pREXdmsC3GH plasmids harboring the transporter variant genes for the VBex reaction.
11. For the VBex reaction, pipette 1 μL CutSmart™ buffer, 0.5 μL *Sfi*I (10 U), 112.5 ng of pERL, 300 ng of pREXdmsC3GH_*variant* plasmid and top up to 10 μL with NF-H_2O. Incubate the reaction at 50 °C for 1.5 h, heat-inactivate at 80 °C for 20 min and cool down to 10 °C. Add 1.25 μL of 10 mM ATP and 1.25 μL T4 DNA ligase (6.25 U) to the tube, ligate at 25 °C for 1.5 h and heat-inactivate at 65 °C for 20 min.
12. Column-purify the VBex product and elute in 15 μL NF-H_2O. **Note:** *We elute in water instead of the provided elution buffer to avoid arcing during electroporation.*
13. Mix 10 μL of purified VBex product with 40 μL of electrocompetent *L. lactis* cells (see Section 4.1). Transfer the mix into a pre-chilled electroporation cuvette and pulse the cells in the electroporator using 3.0 kV, 25 μF and 200 Ω. **Note:** *The typical time constant is around 4.5–5.5 ms.*
14. Immediately add 600 μL of recovery medium to the cells and transfer into a new sterile tube. Recover at 30 °C for 2 h. Spin down the cells,

decant the supernatant, resuspend in remaining medium and plate the cells on M17-cm5 agar plates. **Note:** *Colonies will be visible after 24–48 h.*

15. Inoculate 5 mL GM17-cm5 medium with a single colony from the plate and incubate overnight at 30 °C.
16. Use 500 µL of the overnight culture to prepare a glycerol stock by mixing with 500 µL of sterilized 50 % ultra-pure glycerol and flash-freezing in liquid N_2. Use 2 mL of the same overnight culture to isolate the plasmid (see Section 4.2). Verify the full plasmid by sequencing or by test digestion with *Sfi*I followed by analysis of the correct bands on an agarose gel.

5.2 Determination of protein production levels via GFP fluorescence

Nisin, used as the inducer for gene expression, is produced in-house and subsequently titrated at different dilutions to cells expressing one of the test set variants (see Section 4.3). To account for background originating from *L. lactis* autofluorescence, cells producing wild-type EfrCD without a GFP tag (instead, it has an N-terminal His_{10}-3C cleavage and a $SGSG_5$ linker) were carried along. This assay not only serves to find the optimal inducer concentration but is also performed as a control experiment during selection to monitor overall expression levels of the entire library and to characterize individual variants identified as interesting candidates from the DMS analysis on a single clone level.

1. Prepare an overnight culture of each variant by inoculating 5 mL GM17-cm5 medium with the respective glycerol stock and incubating at 30 °C.
2. Inoculate 10 mL GM17-cm5 medium with 200 µL of the overnight culture and incubate at 30 °C until $OD_{600} > 0.5$ is reached.
3. Prepare a 96-deep-well plate by distributing 1 mL of GM17-cm5 medium supplemented with nisin into each well. For the titration experiment, we typically use a dilution range between 1:1'000 and 1:200'000. We typically perform four replicates per dilution.
4. Adjust the OD_{600} of each pre-culture in GM17-cm5 medium to 0.5.
5. Inoculate each well of the 96-deep-well plate with 10 µL of the OD_{600}-adjusted culture (starting OD_{600} is 0.005) and cover with plate seals. Incubate the plates overnight at 30 °C.

6. On the next day, harvest the cells by centrifugation (4000 *g*, 4 °C, 5 min). Carefully decant the supernatant.
7. Wash each pellet twice in 500 µL ice-cold PBS, pH 7.4 by gently pipetting up and down with a multichannel pipette. Harvest and resuspend in 500 µL ice-cold PBS, pH 7.4.
8. Place 105 µL ice-cold PBS, pH 7.4 in a white-frame 96-well plate, add 45 µL of resuspended cells and mix well shortly before measuring OD_{600} and GFP fluorescence (λ_{ex} 485 nm, λ_{em} 528 nm; 20 nm slit-width) in a plate reader. **Note:** *This dilution worked best in our hands. If the resuspended cell density is too high or too low, adjust the dilution accordingly.*
9. Standardize the fluorescence signal to the measured cell density by dividing GFP fluorescence signal by OD_{600}. The GFP/OD_{600} value of the cells producing the transporter devoid of GFP averaged over the replicates is subtracted from each GFP/OD_{600} value of cells producing GFP-tagged transporter variants (autofluorescence background subtraction).

5.3 Cellular growth assays

Cellular growth assays are performed (i) to determine the optimal inducer concentration that does not affect growth, (ii) to define the optimal drug concentration range, (iii) as a control experiment during selection to monitor the overall growth of the entire library and (iv) to characterize individual variants identified as interesting candidates from the DMS analysis on a single clone level. We recommend performing growth assays in parallel with GFP measurements by preparing a common preculture, which can then be used for both fluorescence and growth analyses. Note that these growth assays provide only a rough estimation and may not fully reflect the conditions during the selection experiments, as the entire library and the required upscaling can exhibit different growth dynamics compared to single clones and differences in material properties and surface adsorption can influence drug availability.

1. Prepare an overnight culture of each variant by inoculating 5 mL GM17-cm5 medium with the respective glycerol stock and incubating at 30 °C.
2. Inoculate 10 mL GM17-cm5 medium with 200 µL of the overnight culture and incubate at 30 °C until OD_{600} 0.3–0.4. Induce each culture with the pre-determined nisin dilution and incubate for 30 min. **Note:** *This short induction allows the cells to produce some transporters before being transferred into drug-containing medium, reducing the risk of immediate cell death upon drug exposure.*

3. Prepare the drug dilutions in GM17-cm5 medium supplemented with the pre-determined optimal nisin dilution. Distribute 150 µL of the prepared drug dilution into the respective wells of the microtiter plate. **Note:** *We prepare serial dilutions from a higher working stock concentration in tubes, in volumes sufficient for technical duplicates. For initial experiments, we typically use the following concentration ranges: 0.125–3 µM Hoechst, 4–32 µM ethidium and 2–64 µM daunorubicin. These ranges are adjusted in subsequent biological replicates based on the results of the first assay.*
4. Adjust the OD_{600} of each pre-culture in GM17-cm5 medium to 0.5.
5. Inoculate each well of the microtiter plate with 1.5 µL of the OD_{600}-adjusted culture (starting OD_{600} is 0.005). Cover the plate with a plate seal and ensure that the edges are perfectly sealed to avoid evaporation.
6. Measure OD_{600} using a microtiter plate spectrophotometer at 30 °C in 10-min intervals for 20 h, with 20 s of double-orbital shaking in-between the reads. For evaluation, subtract the background of the medium (determined at time point 0) from each measured time point.

6. Library generation

The site-saturation variant library was ordered from Twist Bioscience. The ordered gene fragments contain flanking *Sap*I sites (5' flanking sequence atatatGCTCTTCT; 3' flanking sequence GCAGGAAGAGCtatata). For each of the 79 targeted position in the EfrCD library, the 19 amino acid substitutions were introduced using the following codons: G:GGT, E:GAA, V:GTT, A:GCT, R:AGA, S:AGT, K:AAA, N:AAT, M:ATG, I:ATT, T:ACA, W:TGG, Y:TAT, L:TTA, F:TTT, C:TGC, Q:CAA, H:CAT, P:CCA and D:GAT. Note that this codon set was used before the implementation of a sequencing error-reducing codon scheme. Prior to ordering, we recommend consulting the provider regarding potential sequence optimizations (e.g., GC content or repeats) to ensure successful synthesis. The library was delivered in 96-well plate format, with each well containing a sub-library for one position (19 variants) and 50–100 ng of lyophilized DNA per well.

6.1 PCR amplification of the sub-libraries

An initial PCR amplification of each sub-library is performed to obtain sufficient material for downstream steps. We recommend testing two random sub-libraries first, using different cycle numbers (e.g., 10, 15, 20 and 30) to check for the presence of unspecific fragments. The cycle

number should be kept low to minimize the risk of introducing PCR-induced mutations. PCR amplification of all sub-libraries can be conveniently carried out in 96-well plate format.

1. Spin the delivered 96-well plate at 2000 *g* for 1 min at RT. Carefully remove the foil and resuspend the DNA in 20 μL of 10 mM Tris/HCl, pH 8.5, to obtain a concentration of 2.5–5 ng/μL. **Note:** *After use, seal the plate and store at −20 °C for long-term storage.*
2. Prepare a PCR master mix for the number of sub-libraries plus excess. For one sub-library, pipette 10 μL Phusion HF buffer, 5 μL dNTPs (0.2 mM each), 2 μL forward and 2 μL reverse primer (400 nM each and PAGE-purified), 0.5 μL Phusion polymerase (1 U) and 28.5 μL NF-H_2O.
3. Transfer 2 μL of each sub-library into the corresponding well of a PCR plate. Add 48 μL of the master mix to each well, seal the plate with a PCR-compatible foil and run the reaction mixture in a thermocycler using the following program: (1) initial denaturation, 3 min at 98 °C; (2) denaturation, 30 s at 98 °C; (3) annealing, 30 s at 58 °C; (4) elongation, 30 s/kb at 72 °C; repeat (2)-(4) for 12 cycles; (5) final extension, elongation time + 3 min at 72 °C. **Note**: *The test PCR on two EfrCD sub-libraries showed that 12 cycles yield sufficient product without excessive short fragments.*
4. Column-purify each PCR product and elute in 30 μL elution buffer. Analyze 5 μL of the purified product on a 1 % agarose gel. **Note:** *If strong unspecific bands (typically being shorter than the full-length transporter) are present, gel-extract and purify the correct product. The efrCD gene is around 3.5 kb long.*

6.2 FX cloning and *E. coli* transformation

FX cloning can be carried out in a 96-well format, with restriction digest and ligation in the same well, as described in Section 5.1. If transformation efficiency is low, we recommend digesting each sub-library and the pREXdmsC3GH backbone separately, column-purifying the fragments followed by ligation.

1. Prepare a master mix for the number of sub-libraries plus excess. For one sub-library, pipette 1 μL NEBuffer™ r3.1, 0.5 μL *Bsp*QI (5 U), 350 ng of amplified and purified sub-library, 150 ng of the vector pREXdmsC3GH_*ccdB* (around 3:1 molar ratio) and top up to 10 μL with NF-H_2O. Incubate the reaction at 50 °C for 1.5 h followed by heat-inactivation at 80 °C for 20 min.

2. Cool down the reaction to 10 °C before adding 1.25 μL of 10 mM ATP and 1.25 μL T4 DNA ligase (6.25 U) to the tube. Continue with incubation at 25 °C for 1.5 h, heat-inactivate at 65 °C for 10 min and cool down to 10 °C.
3. Thaw and add 50 μL of chemically competent *E. coli* MC1061 to each well. Incubate the plate on ice for 15 min, heat-shock at 42 °C for 40 s and place it on ice for 5 min.
4. Distribute 600 μL of pre-warmed LB medium into each well of a 96-deep-well plate. Add the transformation mixture to the respective well, seal with a gas-permeable foil and incubate at 37 °C for 30 min, shaking at 300 rpm.
5. Mix 6 μL of recovered cells with 60 μL of LB medium and plate each mix on LB-agar plates supplemented with 100 μg/mL ampicillin. Incubate the plates overnight at 37 °C to allow colony formation.
6. Inoculate 10 mL of LB medium containing 100 μg/mL ampicillin in individual 50-mL Erlenmeyer flasks with the remaining recovered culture from each well. Incubate overnight at 37 °C, shaking at 160 rpm.
7. On the next day, count the colonies on each plate and calculate the transformation efficiency for each sub-library by multiplying number of colonies on plate with 100 (dilution factor). **Note:** *We recommend proceeding if the recovery culture yields more than 2000 colonies, i.e. 20 colonies on the plate corresponding to a 100-fold oversampling of each variant in a sub-library.*
8. Split the 10 mL overnight culture into 2 × 5 mL and harvest the cells (6000 *g*, 4 °C, 10 min). Store one pellet at −20 °C as backup. Extract the plasmid from the other pellet and elute in 50 μL elution buffer.
9. Measure the concentration of the purified plasmids using a Nanodrop.

6.3 Sub-library mixing and VBex cloning

Until this stage, PCR amplification and FX cloning were performed separately for each sub-library, while the VBex cloning proceeds with pooled sub-libraries. Before pooling, we recommend performing a test digest of 600 ng of each individual sub-libraries with *Sfi*I and analyzing the products on an agarose gel to ensure complete digest and absence of prominent unspecific fragments. Once verified, the sub-libraries are pooled for VBex cloning. To generate three biological replicates, the pooling is performed three times independently. This is done separately for each half-transporter, resulting in a total of six pools. Although VBex cloning allows restriction digest and ligation in a single reaction tube, we recommend a

cleaner approach due to the low electroporation efficiency in *L. lactis*. The pooled sub-libraries and the pERL backbone are digested separately, gel-purified and then ligated before electroporation.

1. For the first half-transporter, prepare three independent mixtures by pipetting 500 ng of each corresponding sub-library in the pREXdmsC3GH vector in three separate tubes (one for each replicate). Repeat the same for the second half-transporter. This results in three biological replicates for each half-transporter (six tubes in total).
2. Measure the concentration of each pool using a Nanodrop.
3. Digest a total of 20 μg pERL in 4 × 50 μL reactions to obtain sufficient backbone material. In parallel, digest 10 μg of each pREX sub-library pool in 2 × 50 μL reactions. For one digest reaction, pipette 5 μL CutSmart™ buffer, 3 μL *Sfi*I (60 U), 5 μg of pERL or pREX sub-library pool and top up to 50 μL with NF-H_2O. Incubate the reaction at 50 °C for 2 h and heat-inactivate at 80 °C for 20 min. Run the digests on a 1 % agarose gel and gel-purify the backbone (1.7 kb) or the fragment containing the genes (5.9 kb).
4. Prepare 5 ligation reactions per replicate for each half-transporter library (5 × 3 × 2 = 30 reactions). For each reaction, pipette 2 μL T4 ligase buffer, 2.5 μL T4 DNA ligase, 300 ng of library fragment, 100 ng of pERL backbone and top up to 20 μL with NF-H_2O. Incubate the reaction at 25 °C for 1.5 h and heat-inactivate at 65 °C for 20 min. **Note:** *We perform 5 reactions for each sample as the transformation efficiency is generally low in L. lactis cells and to have a reasonable oversampling of each variant.*
5. Column-purify the ligation product and elute in 15 μL pre-heated NF-H_2O. Re-load the eluate on the column and elute to obtain a higher yield. **Note:** *This step desalts the ligation product and avoids arcing during electroporation.*
6. Mix the entire purified ligation product with freshly prepared 200 μL of electrocompetent *L. lactis* Δ*lmrCD* Δ*lmrA* cells (see Section 4.1). Transfer the mix into a pre-chilled electroporation cuvette. Pulse the cells in the electroporator with 3.0 kV, 25 μF and 200 Ω.
7. Immediately add 1 mL of cold recovery medium to the cuvette, transfer the mix to a 5-mL tube, add another 2 mL of recovery medium (stored at RT; total 3 mL recovery) and recover at 30 °C for 2 h. **Note:** *We noted cellular batch variations, which can confound DMS experiments. To*

avoid this problem, we recommend to transform pNZdmsC3GH plasmids harboring the wild-type, tagged wild-type (essential) and the E512Q^{EfrD} genes into the very same batch of competent cells as the DMS library itself. This ensures that observed differences in the test experiment during selection stem from the variants and not from the host strain.

8. Plate 30 μL of the recovered cells (1/100 of the total recovery) on M17-cm5 agar plates and incubate at 30 °C for > 24 h.
9. Transfer each of the remaining recovered culture into 17 mL GM17-cm5 and incubate overnight at 30 °C.
10. Count the colonies on the next day. To calculate oversampling for each replicate of a half-transporter, multiply the number of colonies on the plate with 100 (dilution factor), sum across all 5 ligation/transformation reactions and divide by number of variants in that pool. **Note:** *Based on our experience, a 20-fold oversampling per variant of a half-transporter is sufficient. However, higher oversampling is recommended to ensure library diversity.*
11. Harvest the culture (4000 *g*, 4 °C, 15 min), discard the supernatant and resuspend the pellet in 6 mL M17 supplemented with 25 % ultra-pure glycerol by gentle pipetting.
12. Pool all cell suspensions (corresponding to the 5 ligation/transformation reactions) of each replicate of a half-transporter (in case of EfrCD, this results in 6 suspensions in total: 3 biological replicates of the EfrC library and 3 biological replicates of the EfrD library). Prepare at least 10 glycerol stocks for each library (60 in total), each of 500 μL volume. Flash-freeze in liquid N_2 and store at −80 °C. **Note:** *These glycerol stocks will be used as input material for the selection.*
13. **Recommended:** Pick a few single colonies from the plates, extract the plasmids and sequence to assess the plasmid quality (e.g., correct insert length, absence of unintended mutations).
14. **Recommended:** Perform NGS of the input library at this stage to assess the variant distribution before proceeding with large-scale selection experiments. To this end, grow an overnight culture of each pool by inoculating 50 mL GM17-cm5 medium with one entire glycerol stock (i.e. 500 μL) and extract the plasmids from 10 mL of this culture. In parallel, prepare the mixed DMS libraries in the pREXdmsC3GH vector (see Section 6.3 step 1) to assess whether there were diversity bottlenecks during the cloning into the pNZ vector in *L. lactis*. Proceed with NGS as outlined in Section 8.

7. Selection experiments

Prior to the selection experiments, the inducer concentration and the optimal drug range have to be determined (see Section 5). On the day of the actual DMS selection experiment, three assays are carried out in parallel: (i) The DMS selection of the libraries in the presence and absence of drugs, which are later processed by NGS, (ii) continuous growth curve determination in microtiter plates to assess the growth of the DMS libraries side by side with cells expressing only the wild-type transporter, the tagged wild-type transporter or the inactive $E512Q^{EfrD}$ variant, (iii) assessment of expression levels via GFP of the DMS libraries, the wild-type transporter, the tagged wild-type transporter or the inactive $E512Q^{EfrD}$ variant, which requires also the inclusion of the GFP-less transporter to determine autofluorescence for background subtraction.

Note that drug titrations to determine optimal concentrations are performed in microtiter plates for convenience, whereas large-scale selections are carried out in Falcon tubes. Since drug availability can differ between formats, likely due to varying adsorption to plastic surfaces, we recommend using a range of drug concentrations during the DMS selection step. On the day after selection, we evaluate the cell density of the cultures grown in the drug-containing medium to decide which conditions to process further for NGS analysis. In parallel to the drugs, we also perform selections in absence of any drug but in the presence of the inducer to account for variant-induced growth bias ('no-drug control').

1. Prepare overnight cultures of each of the six library pools (3 replicates × 2 half-transporters) by inoculating 50 mL GM17-cm5 medium with an entire glycerol stock (500 μL). Prepare 5 mL overnight culture of the wild-type transporter (GFP-tagged and GFP-less), the tagged wild-type transporter or the inactive $E512Q^{EfrD}$ variant for the control experiments. Grow all overnight cultures at 30 °C.
2. On the next day, inoculate for each of the three biological replicates 100 mL GM17-cm5 medium with the following overnight cultures: 1 mL of the first half-transporter library, 1 mL of the second half-transporter library and 100 μL of the tagged wild-type (corresponds to 5 % of the total library). **Note:** *We grow the cultures in two 50-mL screw-cap tubes for convenience.*
3. For the controls, inoculate 10 mL GM17-cm5 medium with 200 μL of the respective overnight culture.

4. Incubate all cultures at 30 °C until OD_{600} is 0.3–0.4. Induce the cultures with the pre-determined nisin dilution (see Section 5.2) and continue growth at 30 °C for another 30 min.
5. Meanwhile, prepare drug dilutions sufficient for the DMS selection and the growth assays in microtiter plates:
   a. For DMS selection, distribute 3 × 14 mL of the drug-containing medium for the three replicates in screw-cap tubes.
   b. For the growth assay, prepare the microtiter plates in technical duplicates.
6. In parallel, prepare the 96-deep-well plate for GFP measurements by adding 1 mL GM17-cm5 medium containing the pre-determined nisin dilution to each well in technical quadruplicates.
7. Measure the OD_{600} of the library cultures induced in step 4 and use a fraction thereof to adjust them to OD_{600} 0.5 for further inoculations.
8. Harvest the remaining induced culture (4000 *g*, 4 °C, 15 min) and store the pellet at −20 °C until further use. **Note:** *This will be the input library.*
9. For the actual DMS experiment, inoculate the 14 mL of drug-containing or drug-free ('no-drug control') medium with 140 µL of the OD_{600}-adjusted culture (starting OD_{600} is 0.005) and incubate overnight at 30 °C.
10. For the growth assay, inoculate each well containing 150 µL of nisin- and drug-containing medium with 1.5 µL of the respective OD_{600}-adjusted library replicate or one of the control variants. Continuously measure the OD_{600} for 20 h as described in Section 5.3.
11. For the GFP measurements, inoculate each well with 10 µL of the respective OD_{600}-adjusted culture. Incubate the plate overnight at 30 °C.
12. On the next day, measure the final OD_{600} of the selected cultures in microtiter plates. Dilute the cultures 1:20 or 1:50 in the respective drug-containing or drug-free medium to stay within the linear range. If the cell density is low, transfer 150 µL directly without dilution. **Note:** *If a range of drug concentrations was tested, we recommend selecting the condition for NGS where growth is moderately impaired, i.e., reduced compared to the 'no-drug control' but not completely inhibited. Nevertheless, cultures from other concentrations can be harvested and stored at −20 °C as backup in case one later decides to perform NGS on additional conditions.*

13. Harvest the selected cultures and store the pellets at −20 °C.
14. Analyze the growth assay results as described in step 6 of Section 5.3. **Note:** *Libraries may grow similarly or differently from the wild-type, while the inactive variant serves as a control to confirm sufficient drug pressure.*
15. Perform the GFP measurements as described in Section 5.2. **Note:** *The results provide a glimpse at the overall transporter production in the library compared to control variants. In our case, the libraries often show a lower GFP/OD_{600} signal, likely due to many variants being poorly expressed.*

8. Library preparation for NGS

While sequencing is outsourced to a core facility, the preparation of input and selected libraries for NGS is performed in-house and described in detail below.

1. Extract plasmids from 14 mL of input or selected cultures using the extraction protocol detailed in Section 4.2 to obtain sufficient material for downstream digestion.
2. Digest at least 3 μg of the extracted plasmid pools. Pipette the following reaction: 5 μL CutSmart™ buffer, 2.5 μL *Sfi*I (50 U), 3 μg plasmid pool and top up to 50 μL with NF-H_2O. Incubate the reaction at 50 °C for 2.5 h, heat-inactivate at 80 °C for 20 min.
3. Column-purify the digest reaction and elute in 30 μL of pre-heated elution buffer. Re-load the eluate on the column and elute to obtain a higher yield.
4. Run 400 ng of digested material on an agarose gel to verify the presence of the gene-containing fragment and the backbone, and the absence of any unexpected bands (typically being shorter than the full-length transporter). **Note:** *If strong unexpected bands are present, we recommend to digest a larger amount of the plasmid pools (7 μg) and to extract the gene-containing fragment from a preparative DNA gel.*
5. For shearing, use 500 ng of the column-purified fragment (alternatively use gel-purified fragment, see note in step 4). If needed, dilute with NE buffer to a final volume of 55 μL in low-binding tubes.
6. Prepare the Covaris instrument by degassing and cooling the water to 4 °C. Set the parameters as follows to obtain fragment lengths of

around 150 bp: treatment time 280 s, peak power 175.0, duty factor 10.0, cycles/burst 200, average power 17.5. Ensure that the water level is at position 6 during the run.

7. Transfer 50 μL of the samples into the Covaris shearing tubes, place and secure them in the metal rack.
8. After one round of sonication, remove the tubes from the rack, shortly spin down and sonicate a second time using the same settings. Carefully transfer the sheared fragments to new low-binding tubes.
9. Analyze the fragment size on a TapeStation system using D1000 screen tapes. Mix 3 μL of D1000 sample buffer and 1 μL of sheared DNA and vortex for 1 min. Read out the concentration (ng/μL) in the region from 50–600 bp. **Note:** *The length of the fragments should be around 150–170 bp.*
10. Perform library preparation and adaptor ligation using the TruSeq® Nano DNA Library prep kit, following the manufacturer's protocol starting with the cleanup of sheared fragments (last section of 'Fragment DNA') and including the section 'Enrich DNA Fragments'. Always use low-binding tubes. Start with 50 ng of each sheared library in a 50 μL volume; if dilution is needed, use the resuspension buffer (RSB) provided in the kit. **Note:** *The only modification to the protocol is during the size selection step (section 'Remove Large DNA Fragments') after end repair. For one sample, a ratio of 135 μL sample purification beads (SPB) to 25 μL NF-H_2O yielded optimal removal of fragments larger than 150 bp.*
11. After adaptor ligation, analyze the libraries on a TapeStation system (see step 9). The expected fragment size is around 300–350 bp. Pool the libraries equimolarly such that the final DNA concentration is 10 nM in a volume of 60 μL. **Note:** *Occasionally, additional bands are present (particularly at around 150 bp). If this is the case, pool the libraries equimolarly using 4 nM per sample if pooling > 12 samples, or 10 nM if ≤ 12. Purify the pooled library following the "Clean Up Amplified DNA" section of the kit's protocol to retain DNA fragments with a size of 300–350 bp. Adjust the SPB volume according to the total volume of your pool. Analyze the library pool again on a TapeStation system (see step 9) and directly proceed with step 12.*
12. Sequence the pooled libraries on an Illumina platform. **Note:** *We used the NovaSeq 6000 and NovaSeq X Plus (1 lane, 10B flow cell) with 150 bp paired-end reads. For our library and gene size, we aimed for a minimum sequencing depth of 40 million reads per library.*

9. Data analysis

Since data analysis workflows vary across laboratories, we outline here the essential steps from raw sequencing reads to sequence-function heat maps. First, adapter sequences resulting from short insert sizes are removed using Cutadapt (v2.3). The trimmed reads are then aligned to the wild-type *efrCD* DNA sequence using the Burrows-Wheeler Aligner (BWA) (v0.7.17-r118). A custom Python script (deposited on https://github.com/giameier/DMS_ABC) was used to further filter the aligned reads, retaining only those with a Phred quality score > 30, no insertions or deletions, and fully overlapping read pairs. Variant calling was restricted to sequences containing a single amino acid substitution with perfect overlap. Variant scores were calculated using Enrich2, applying wild-type normalization (counts of the tagged wild-type codon) and computing variant enrichment as natural log ratios (Rubin et al., 2017).

Table 2 List of strains, enzymes, reagents, consumables and selected machines are listed below. Unless otherwise stated, we use typical laboratory consumables.

Name	Provider, catalogue number	Comment
Strains		
E. coli MC1061	Lucigen, 60514-1	Cloning strain
L. lactis NZ9700		Production of nisin
L. lactis NZ9000 Δ*lmrCD* Δ*lmrA*		Expression strain
Enzymes		
Lysozyme from chicken egg white	Merck, L4919	
RNase A (DNase-, protease-free; 10 mg/mL)	Thermo Scientific, EN0531	
*Bsp*QI in NEBuffer™ r3.1	NEB, R0712	Isoschizomer of *Sap*I
T4 DNA ligase	Thermo Scientific, EL0011	
Phusion™ Hot Start II DNA Polymerase	Thermo Scientific, F549	
*Dpn*I in CutSmart™	NEB, R0176	
*Sfi*I in CutSmart™	NEB, R0123	

Reagents

M17 broth	Formedium, M170105	Oxoid, CM0817B is no longer available
Glucose	Merck, D9434	20 % (w/v) stock, sterile-filtered
Sucrose	Merck, 84100	1.2 M stock, sterile-filtered
Chloramphenicol	Carl Roth, 3886.1	5 mg/mL stock in EtOH, sterile-filtered
Glycine	AppliChem, A1067	15 % (w/v) stock, pH 7.0
Magnesium chloride	Merck, M8266	1 M stock
Calcium chloride dihydrate	AppliChem, A4689	0.1 M stock
Ultra-pure glycerol, puriss. p.a.	Merck, 49770	50 % (v/v) stock
Tris/HCl (Trizma base)	Merck, 93350	1 M stock, pH 8.0 with HCl
K-EDTA	Merck, EDS	0.5 M stock, pH 8.0 with KOH
NaCl	Merck, 71380	1 M stock
SDS	Merck, 75746	
NaOH	Carl Roth, P031.1	
K-acetate	Merck, P1190	5 M stock
Glacial acetic acid	Carl Roth, 3738.4	
Ethanol, puriss. p.a.	Honeywell, 02860	Pure and 70 % stock
Nuclease-free water	Invitrogen, 10977035	
Adenosine 5′-triphosphate (ATP) disodium salt hydrate	Merck, A3377	
Ampicillin sodium salt	Carl Roth, HP62.1	100 mg/mL stock in H_2O

(continued)

Table 2 List of strains, enzymes, reagents, consumables and selected machines are listed below. Unless otherwise stated, we use typical laboratory consumables. (*cont'd*)

Name	Provider, catalogue number	Comment
dNTPs	Thermo Scientific, R0181	
bisBenzimide H 33342 trihydrochloride (Hoechst 33342)	Merck, B2261	10 mM stock in H_2O
Daunorubicin hydrochloride	Merck, 30450	10 mM stock in H_2O
Ethidium bromide solution	Merck, E1510	10 mM stock in H_2O
D1000 Sample Buffer	Agilent, 5067−5602	For TapeStation
Consumables		
NucleoSpin™ Gel and PCR Clean-up Kit	Macherey-Nagel, 740609	Column-based purification
Steritop Threaded Bottle Top Filter, 0.22 μm	Millipore, SCGPS05RE	
QIAprep Spin Miniprep Kit	Qiagen, 27106	Plasmid isolation from *E.coli*
Gene Pulser/MicroPulser Electroporation Cuvettes, 0.2 cm gap	Biorad, 1652086	
Deep 96-well plate PP 2.2 mL	TreffLab, 2.9971.01	For GFP expression growth
Adhesive plate seals	Abgene, AB−0580	
Isoplate 96-well, white frame clear well	PerkinElmer, 6005040	For GFP expression measurement
Microplate, 96-well, PS, F-bottom, clear	Greiner, 655101	For growth assays and end OD_{600} measurements
PCR Plate, 96-well, non-skirted	Thermo Scientific, AB0600	

MicroAmp™ Optical adhesive film, PCR compatible	Thermo Scientific, 4311971	
Gas-permeable foil	Macherey-Nagel, 740675	
5-mL tubes	Eppendorf, 0030119401	
Screw cap 15-mL tubes	Sarstedt, 62.554.002	
Screw cap 50-mL tubes	Sarstedt, 62.547.004	
RNase-free/low binding 1.7-mL microtubes	Sorenson, 39640T	
microTUBE AFA Fiber Pre-slit Snap-Cap 6 × 16	Covaris, 520045	For DNA shearing
D1000 ScreenTape	Agilent, 5067−5582	For TapeStation
TruSeq DNA Nano Low Throughput Library Prep Kit (24 Samples)	Illumina, 20015964	
IDT for Illumina – TruSeq DNA UD Indexes v2 (96 Indexes, 96 Samples)	Illumina, 20040870	
Machines		
Electroporator Gene Pulser Xcell	BioRad, 1652662	
NanoDrop™ 2000c Spectrophotometer	Thermo Scientific, ND-2000C	
Plate reader	BioTek, Cytation 5	For GFP expression and growth assays
Incubators with orbital shaking platform	Kühner, ISF1-X	
Tabletop centrifuge	Eppendorf, 5424R	For 1.5-/2.0-mL tubes

(continued)

Table 2 List of strains, enzymes, reagents, consumables and selected machines are listed below. Unless otherwise stated, we use typical laboratory consumables. (*cont'd*)

Name	Provider, catalogue number	Comment
Centrifuge	Eppendorf, 5804R	For 15-/50-mL tubes
Heraeus Multifuge 4 KR	Thermo Scientific, 75004461	For 96-well plates
E220 Focused-ultrasonicator Rack 96 Place microTUBE	Covaris Covaris, 500282	
4200 TapeStation system	Agilent, G2991BA	

Reagents and material

List of strains, enzymes, reagents, consumables and selected machines are listed below. Unless otherwise stated, we use typical laboratory consumables Table 2.

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