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Membrane transporter engineering in industrial biotechnology and whole-cell biocatalysis

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Membrane transporter engineering in industrial biotechnology and whole-cell biocatalysis

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Abstract

Because they mainly do not involve chemical changes, membrane transporters have been rather a Cinderella subject in the biotechnology of small molecule production. We argue here that this has been a serious oversight: influx transporters contribute significantly to the flux towards product while efflux transporters have arguably an even more important role in ensuring the accumulation of product in fermentors in the much greater extracellular space. Programmes for improving biotechnological processes might therefore give greater consideration to transporters than may have been commonplace. Strategies for identifying important transporters include expression profiling, genome-wide knockout studies, stress-based selection and the use of inhibitors. In addition, modern methods of directed evolution and synthetic biology, especially those effecting changes in energy coupling, offer huge opportunities for increasing the flux towards extracellular product formation via transporter engineering.

Introduction

In any complex biochemical network, all steps contribute to the control of the flux through a particular pathway or even that catalysed by a specific enzyme (Heinrich and Rapoport, 1974, Kacser and Burns, 1973), but some steps exert a greater degree of control on the fluxes of interest than do others. While all steps do contribute to flux control, strategies for deciding how best to increase such fluxes (Kell and Westerhoff, 1986a, Kell and Westerhoff, 1986b) are therefore necessarily wise to pay special attention to these particular steps. In many cases of interest to the fermentation, biocatalysis and biotransformation communities, and especially for those systems involving xenobiotics, these steps include the cellular transporters that catalyse the influx of substrates and the efflux of products (and of any other potentially cytotoxic compounds). In other words, these steps are typically significantly rate-controlling. A quantitative measure of the extent of this rate or flux control is encapsulated in the flux-control coefficient (Box 1). With a relative density barely greater than 1, even at 100 $g.L^{-1}$ wet cell concentration (so that less than 10% of the total volume is intracellular), most of the volume of a fermentor is extracellular; thus to maximise volumetric productivity it will be desirable to ensure that cells excrete the products of interest (Krämer, 1994, Van Dyk, 2008).

But doesn't stuff just diffuse into and out of cells unaided?

There is a surprisingly widespread view in the pharmaceutical industry, starting with the relevant textbooks (e.g. (Gabrielsson and Hjorth, 2012, Rowland and Tozer, 2011)) and promulgated by some influential reviews (e.g. (Seeman, 1972)), that the main means by which most xenobiotics of interest (i.e. drugs) enter (and presumably exit from) biological cells is simply by diffusing passively across the lipid bilayer portion of cell membranes down their concentration gradients and according to their lipophilicity (log P or log D – see Glossary). If this were the case, we should have little to say in this review, but it is not (Dobson et al., 2009a, Dobson and Kell, 2008, Dobson et al., 2009b, Kell, 2013, Kell, 2015, Kell and Dobson, 2009, Kell et al., 2013, Kell et al., 2011, Kell and Goodacre, 2014, Kell and Oliver, 2014, Lanthaler et al., 2011); the main means by which small molecules cross biological cell membranes is via genetically encoded, proteinaceous transporter molecules, and this gives the cells, and the biotechnologist, important means by which to control and influence the process. The first thing to know, then, is qualitative (Kell, 2006, Kell and Knowles, 2006): which small molecules use which transporters? While we shall mainly consider microbes and fermentations, the principles we enunciate are general, and we recognise their role in the metabolic engineering of plants (Schroeder et al., 2013), where especially vacuolar (Martinoia et al., 2012), peroxisomal (Linka and Theodoulou, 2013), chloroplast (Rolland et al., 2012) and root (Zelazny and Vert, 2014) transporters can exert significant flux control. Fig 1 summarises this review in the form of a mind map (Buzan, 2002).

Classical strategies for detecting the roles of particular cell membrane transporters

Originally, the determination of which transporters accounted for the uptake of particular nutrients (or other compounds) used classical genetic techniques, often obtaining mutants in transporter genes by selecting for resistance to cytotoxic structural analogues of those nutrients. Thus canavanine (Fig 2) is a structural analogue of arginine that can be taken up by cells, including those of baker's yeast (Lanthaler et al., 2011) and humans (Swaffar et al., 1994), and is incorporated into proteins where it disrupts their function, thus proving cytotoxic (Grenson et al., 1966). Such cytotoxic molecules, that bear structural similarities to intermediary metabolites, are known as antimetabolites (Rhoads, 1955), and antimetabolite molecules such as analogues of folate, nucleobases and nucleosides (Fig 2) continue to play a major role in

cancer chemotherapy (e.g. (Parker, 2009, Tiwari, 2012)). In yeast the overwhelming bulk of canavanine uptake, and in mutants their resistance to it, is effected by the arginine transporter Can1p encoded by the gene *can1* (Whelan et al., 1979). Strains lacking this gene function are (depending upon the precise metric) more than 100-fold more resistant to the antimetabolite than is the wild type (Lanthaler et al., 2011), and of course the gene encoding the arginine transporter is explicitly named after (its ability to encode resistance to) this antimetabolite.

'Influx' and 'efflux' transporters

More generally, assessing the contributions of membrane proteins to the tolerance of stresses induced by fermentation or incubation conditions is also one important experimental approach to detecting 'efflux' transporters (e.g. (Kieboom et al., 1998a) and see later), a comment that leads us to note that thermodynamic principles mean that any transporter is theoretically reversible in its direction of operation (although for kinetic reasons connected with the Haldane relationship it may not appear to be). Thus 'influx' and 'efflux' transporters refer to their normal direction of operation *in vivo*, and this is determined both by the thermodynamics and the mechanistic details of any energy coupling involved (Fig 3). We might also comment that while our chief interest here is in identifying cases where transporters exert significant flux control, an increasing number of 3D protein structures for transporters are becoming available (e.g. (Booth et al., 2007, Forrest et al., 2011, Johnson et al., 2012)), and this is beginning to allow calculation of their molecular mechanisms from first principles, based on molecular dynamics (e.g. (Furuta et al., 2014, Heinzelmann and Kuyucak, 2014, Khalili-Araghi et al., 2009, Koldsø et al., 2013, Wang et al., 2010)). It will also, in time, assist in their rational redesign.

Genomics approaches to transporter identification

The more modern approach to detecting transporters, especially for systems biology-based strain improvement (Fig 4) is, of course, through genome sequencing (as in baker's yeast (Goffeau et al., 1996, Oliver et al., 1992)), where particular sequence motifs can more or less reliably identify transporters, even if not always their substrates (Oliver, 1996). The next step is to incorporate such transporters into genome-scale metabolic network reconstructions (e.g. (Feist et al., 2009, Lee et al., 2011, Palsson, 2006, Park et al., 2008, Sahoo et al., 2014, Thiele and Palsson, 2010a)). While this is most effectively done by domain experts (Thiele and Palsson, 2010b), recent advances in methods such as text mining for systems biology (Ananiadou et al., 2006, Ananiadou et al., 2010, Ananiadou et al., 2014), and other strategies (Büchel et al., 2013, Swainston et al., 2011), mean that it is becoming increasingly amenable to automation. (A list of 'predictive genome-scale metabolic network reconstructions' is maintained at http://systemsbiology.ucsd.edu/InSilicoOrganisms/OtherOrganisms.) Significantly, almost all free-living organisms so analysed are known to have genes encoding hundreds of transporters (Kell et al., 2011). Indeed, approximately one third of the reactions in the heavily curated yeast (Herrgård et al., 2008) and human (Swainston et al., 2013, Thiele et al., 2013) metabolic networks are represented by transporter reactions. Some useful online databases focussing on transporters are given in Table 1.

'Unexpected' transporters for the uptake of uncharged (nonelectrolyte) nutrients and other small molecules

Based on the pioneering studies of Overton (Overton, 1899) (for a more recent version see (Lieb and Stein, 1969)), who showed a close correlation between the logarithm of the rate of cellular uptake of non-electrolytes and their log P values, it had been widely assumed that small, uncharged molecules could permeate freely across the bilayer portion of biological membranes (even though it is well known that

glucose and other sugars do not (Madhavan et al., 2012)). However, it is now recognised that this is not at all the case, with transporters having been found (and required) for the uptake of many small, uncharged substances such as alkanes (Grant et al., 2014), ammonia (NH_3) (Lamoureux et al., 2010, Nakhoul et al., 2010, Wagner et al., 2011, Wang et al., 2013, Wang et al., 2010, Weiner and Hamm, 2007, Weiner and Verlander, 2011), carbon dioxide (CO_2) (Boron, 2010, Boron et al., 2011, Endeward et al., 2006, Kai and Kaldenhoff, 2014, Kaldenhoff et al., 2014, Maurel et al., 2008), ethanolamine (Penrod et al., 2004, Stojiljkovic et al., 1995), fatty acids (van den Berg, 2005, van den Berg et al., 2004), glycerol (Boury-Jamot et al., 2006, Fujimoto et al., 2006, Hara-Chikuma and Verkman, 2005, Ishibashi et al., 2011, Ishii et al., 2011, Morishita et al., 2004, Ohgusu et al., 2008), hydrogen peroxide (H_2O_2) (Bienert and Chaumont, 2014), hydroxyurea (Walker et al., 2011), urea (Bagnasco, 2005, Beckers et al., 2004, Fröhlich et al., 2004, Levin et al., 2009, Shayakul et al., 2013, Stewart, 2011, Strugatsky et al., 2013), and even water (Agre et al., 2002, Benga, 2012b, Day et al., 2013, Ishibashi et al., 2011, Öberg and Hedfalk, 2013). The last was a finding for which Peter Agre received the 2003 Nobel Prize (see (Agre, 2004) and (Benga, 2012a)). Given the catholic nature of aquaporin channels, it is not unreasonable to propose that they will also be found to transport NO and dioxygen too, which latter would potentially be of considerable biotechnological interest.

It has long been known that acetate enters cells mainly in its uncharged form (i.e. as acetic acid). This may be determined by osmotic swelling experiments (Kell et al., 1981), but these kinds of experiments do not say anything about the mechanism by which it enters (bilayer diffusion or transporter). However, it is now known in the important amino acid producer *C. glutamicum* that even the uptake of electroneutral acetic acid involves the use of a specific carrier (Jolkver et al., 2009).

Ethanol is another small nonelectrolyte of much biotechnological interest, and it is desirable to increase its export from producer cells (Dunlop et al., 2011). It is not yet quite certain which transporters are responsible for this, but the ABC transporter (Sá-Correia et al., 2009) Pdr18 (Teixeira et al., 2012) and the glyceroaquaporin Fps1 (Teixeira et al., 2009) possess properties that might be consistent with such a role (albeit other mechanisms may also be involved (Dikicioglu et al., 2014)). While we later discuss in more detail export (efflux) transporters of molecules not normally produced by the host, this section leads naturally to a discussion of those that are known to be involved in the secretion of metabolites that the host naturally produces.

Some useful case histories from classical fermentations

A notable example of the role of transporters in improving the yield of an important fermentation product (more than 2M tonnes p.a. (Sano, 2009)) comes from the history of the glutamate fermentation carried out using various coryneform bacteria, notably *Corynebacterium glutamicum* (Eggeling and Sahm, 2003, Sano, 2009, Tryfona and Bustard, 2004). Following the initial discovery of the fermentative production of glutamate (Kinoshita et al., 1957), various empirical findings in the 1960s and 1970s (Hirasawa et al., 2012, Vertès et al., 2013) showed that a variety of treatments, involving biotin limitation, or the addition of weak surfactants such as acetylated corn oil or Tween, or the use of certain auxotrophs, would enhance the efflux of glutamate in producer strains. Soon enough, however, it was recognised that this was not due to a general membrane-leakiness, because it was very selective for glutamate (and was even against a glutamate concentration gradient), but that it was due to a change in membrane tension that activated a mechanosensitive glutamate efflux pump encoded by a gene called NCgl1221 (a homologue of the *E. coli yggB* gene, now known as *mscS*, the mechanosensitive channel of small conductance) (Hashimoto et al., 2012, Hashimoto et al., 2010, Nakamura et al., 2007, Nakayama et al., 2012, Yamashita et al., 2013, Yao et al., 2009). Similar efflux pumps are now known to be involved in the export of product during a variety of other amino acid fermentations (Eggeling and Sahm, 2003, Mitsuhashi, 2014), such as those for lysine

(Bellmann et al., 2001, Bröer and Krämer, 1991a, Bröer and Krämer, 1991b, Kelle et al., 1996, Vrljic et al., 1996), isoleucine (Hermann and Krämer, 1996, Xie et al., 2012) threonine (Lee et al., 2007), methionine (Trötschel et al., 2005) and others (Van Dyk, 2008).

Why would a cell export its metabolites?

One may wonder (from an evolutionary perspective) why bacteria see fit to excrete important nutrients or metabolites, often at fast rates. While other more specific roles may be invoked (Van Dyk et al., 2004), Morbach and Krämer (Morbach and Krämer, 2002) rehearse what seems like the most persuasive general explanation, to the effect that soil bacteria (such as corynebacteria) that have experienced drought and are hit by a raindrop (a common stress (Ouyang and Li, 2013)) experience truly massive osmotic stresses or turgour pressures, that can only realistically be dealt with by a virtually instantaneous excretion of internal osmolytes catalysed by a mechanically sensitive, membrane-triggered osmoregulatory process (these also occur in plants (Kell and Glaser, 1993)). Such a role for the glutamate exporter (and one may suppose other such exporters) is consistent with the similar role of its *E. coli* homologue (Booth and Blount, 2012, Booth et al., 2007, Levina et al., 1999), and indeed since their initial discovery in bacteria (Martinac et al., 1987) a considerable number of such mechanosensitive exporters – whose role is indeed seen as being involved in regulating turgour pressure within acceptable bounds (Berrier et al., 1992) – are now known (Booth, 2014, Booth et al., 2007, Haswell et al., 2011), including seven (in two families) in *E. coli* (Pivetti et al., 2003).

Citric acid production

In a similar vein, the large-scale (well over 1M tonnes p.a. (Soccol et al., 2006)) fermentative production of citric acid by the fungus *Aspergillus niger* involves active export of the product from the producer strain using a proton symporting transporter (García and Torres, 2011, Netik et al., 1997); the same is true for citric acid production in yeasts (Anastassiadis and Rehm, 2005, Anastassiadis and Rehm, 2006).

Biomass production

In some fermentations, of course, the biomass itself is the product, and it is of interest to know what role transporters may play in controlling growth (rate) more generally. In one study, using a pHauxostat to select strains of the (already) fast-growing yeast *Kluyveromyces marxianus* for even faster growth, Westerhoff and colleagues (Groeneveld et al., 2009) evolved one that could grow up to 30% faster than the starting strain. This increase in growth rate, with a doubling time of 52 min (apparently the fastest reported for a eukaryote) was accompanied by an increase in surface area of some 40% at essentially constant volume, implying that membrane processes (such as substrate uptake) were most limiting to growth rate. Indeed 80% of the growth rate increase was ascribed to membrane processes (Groeneveld et al., 2009). Continuous selection is also an excellent strategy for selecting strains resistance to toxins such as solvents (Brown and Oliver, 1982, Lane et al., 1999), especially in turbidostats (Markx et al., 1991) in which growth rate can be measured online (Davey et al., 1996).

In this context, is noteworthy that Pir *et al*. (Pir et al., 2012), in a high-throughput screen of heterozygous deletants of diploid *Saccharomyces cerevisiae,* identified 145 transporter-encoding genes that exerted significant control over growth rate (so-called high-flux-control or HFC genes) in turbidostat culture. Ninety of these genes had a haploinsufficient (HI) phenotype, that is they reduced the maximum growth rate of yeast when present in only one copy in a diploid, while the remainder had a haploproficient (HP) phenotype, increasing the growth rate when in the heterozygous state. These HFC genes included those encoding plasma membrane transporters, but also genes specifying proteins involved in transporting ions and metabolites into sub-cellular organelles, especially the mitochondria and the vacuole. Amongst the HI

genes were those encoding plasma membrane transporters of metals (particularly iron and zinc), organic acids (including amino acids), ammonium, phosphate, sulphate, vitamins, sugars (including glucose) and sugar alcohols, and also the aquaporin gene, *AQY1.* This group of HI genes also includes 4 encoding drug efflux pumps. Given the discussion of efflux transporters, above, it would seem sensible for biotechnologists and synthetic biologists to pay attention not only to transporters of important nutrients but also those responsible for the efflux of potentially toxic products of metabolism, such as ethanol and other biofuels.

Transcriptome-based strategies for determining transporter-mediated activities

Virtually since its inception (Lockhart et al., 1996), it has been clear that genome-wide expression profiling at the level of the transcriptome provides one excellent strategy for identifying which gene products may be pertinent for particular biological processes. This applies equally to the role of transporters in biotechnology. Thus the availability of the *Penicillium chrysogenum* genome allowed van den Berg and colleagues (van den Berg et al., 2008) to compare the expression profiles of low- and high-producing strains, finding a considerable enhancement in transporter expression in the high-producers, again implying strongly that enhanced transporter expression could drive increased fluxes. While in general terms the expression of an individual gene does not necessarily correlate with the productivity of a fermentation, and certainly not over a wide range because of changes in the distribution of flux control (box 1), genome-wide trawls relating expression to activity can be highly beneficial, especially for metabolic networks. This is because metabolic transformations are subject to strict stoichiometric controls (no 'alchemy' is allowed).

Flux balance analysis

While the counsel of perfection in genome-scale metabolic modelling includes mechanistic details of every enzymatic step, that can then be turned into an ordinary differential equation (ODE) model that may be used to model or predict all the fluxes and concentrations of interest, we very rarely have sufficient of the kinetic parameters to do this (Almquist et al., 2014, Palsson, 2006, Smallbone and Mendes, 2013, Smallbone et al., 2013, Smallbone et al., 2010). However, the stoichiometric constraints alluded to above mean that the methods of flux balance analysis (Çakır et al., 2007, Curran et al., 2012, Gianchandani et al., 2010, Lakshmanan et al., 2014, Lee et al., 2006, Orth et al., 2010, Palsson, 2011, Palsson, 2015, Raman and Chandra, 2009, Smallbone and Simeonidis, 2008) (Box 2) may be used to attempt to predict the fluxes of interest. As part of a strategy to minimise the number of possible flux patterns that can explain the observable data (Smallbone and Simeonidis, 2008, Smallbone et al., 2007), we have found (Lee et al., 2012) that absolute transcriptomics provides a valuable surrogate for the flux through each step.

Fig 5 shows the distribution of expression levels for transporter and non-transporter genes determined in a recent study (Lee et al., 2012) of a yeast strain growing at 85% of its maximum growth rate (with the transporter nature of the genes as assessed by the present version of the yeast metabolic network); as judged by their median levels, as well as by 5000 permutations, there is a significantly lower level of expression ($p<0.0004$) of transporter genes (19.3 transcripts per cell) than of non-transporter genes (31.7). This is not inconsistent with the fact that as 2D structures cellular membranes possess a more limited amount of real estate for incorporating transporters and other membrane proteins than do the 3D intracellular spaces (Molenaar et al., 2009, Zhuang et al., 2011). The 'surfaceome', including SLCs, is also the most variable between different and differentiated cells (da Cunha et al., 2009).

Detecting relevant uptake transporter genes through genome-wide knockout analyses

Although individual genes were classically and typically discovered individually (see above), it is now possible to extend the analysis of transporter roles to the genomic level. The sole requirements are for a suitable variation in the extent of expression of different enzymes in different strains, most conveniently via single gene knockouts, and a means of selecting the phenotype of interest (Bochner, 2009) (e.g. growth selection) (Fig 6). Thus in the case of yeast, we were able (Lanthaler et al., 2011) to exploit the barcoded yeast deletion mutant collection (Giaever et al., 2002) to identify transporters for 18 out of 26 drugs tested. Most had multiple transporters, and for the eight where we could not detect which transporters were used, it is considered likely that this is because there were simply too many and removing just one did not provide sufficient selectivity.

That study (Lanthaler et al., 2011) used haploid strains (see Box 1) and a purpose-designed microarray chip, but nowadays it is recognised that deep sequencing is much more effective and reliable (Smith et al., 2009). Thus, in an exciting development, Superti-Furga and colleagues have used a near-haploid human cell line (KBM7) with a retroviral gene trap (Bürckstümmer et al., 2013, Carette et al., 2011) to detect that just a single transporter (called SLC35F2) is responsible for the uptake of the cytotoxic anticancer drug candidate sepantronium bromide (also known as YM155) into these cells (Winter et al., 2014). Clearly these kinds of methods may be applied to any system for which cells that have or have not taken up a particular drug may be discriminated and separated (e.g. by cell sorting (Davey and Kell, 1996)) and then identified genetically. It is worth stressing that this kind of experiment would not 'work', i.e. return any hits, if bilayer diffusion were the dominant mechanism of transmembrane transport. Put another way, it would indeed seem from such experiments that for drug transport into cells, phospholipid bilayer diffusion is negligible (Kell, 2015, Kell and Oliver, 2014).

Although in theory these kinds of knockout strategies could also be used to select strains with knockouts in efflux transporters (if such exist), via their greater sensitivity to a compound, positive selections (for resistance) are always more reliable. While it has already been noted the dilution of just one of the two copies of a gene is sufficient to produce a significant reduction in growth rate (Pir et al., 2012), it was also found that the removal of two genes, *PDR10* and *PDR12*, encoding ABC multidrug transporters actually enhanced growth rate. Thus further investigation of the substrate preferences of these apparently promiscuous efflux pumps might pay dividends, in both biotechnology and drug design.

Genes for efflux transporters

As well as the genes for efflux transporters described above, there is of course considerable interest in the recognition that a chief cause of antibiotic resistance, a huge continuing (Andersson and Hughes, 2010, D'Costa et al., 2011) and present problem (Laxminarayan et al., 2013), is the ability of microbes to pump out such molecules using 'multidrug resistance') (MDR) efflux transporters (e.g. (Fluman and Bibi, 2009, Grkovic et al., 2002, Li and Nikaido, 2009, Mazurkiewicz et al., 2005, Nasie et al., 2012, Nies et al., 2012, Nikaido, 2009, Nikaido and Pagès, 2012, Poole, 2012, Prasad and Rawal, 2014)); they are often of wide specificity e.g. for lipophilic compounds, and an increasing number of structures are becoming known (Dos Santos et al., 2014, Du et al., 2014, Nakashima et al., 2013, Tanaka et al., 2013). Efflux transporters are of wider significance in medicine because by removing toxins they lower the intracellular concentrations. This can be good in the case of genuine toxicants (e.g. (Wen et al., 2014)) but less so when they encode phenotypic resistance, e.g. to anticancer agents (e.g. (Callaghan et al., 2014, Ecker and Chiba, 2009, Nobili et al., 2012, Robey et al., 2007)). However, in biotechnology it is both desirable and possible to select for

strains that are particularly resistant to stresses, including stresses from organic solvents (Segura et al., 2012) and from high intra- and/or extra-cellular product titres.

Indeed, it is precisely this kind of positive selection that can be used to our advantage in biotechnology. Thus by seeking tolerance to added compounds, efflux transporters have been found for alkanes (Ankarloo et al., 2010, Chen et al., 2013, Doshi et al., 2013, Fernandes et al., 2003, Foo and Leong, 2013, Ling et al., 2013, Nishida et al., 2013, Torres et al., 2011, Tsukagoshi and Aono, 2000)(and see (Grant et al., 2014)), arenes (Fillet et al., 2012, Heipieper et al., 2007, Isken and de Bont, 1996, Kieboom et al., 1998b, Sun et al., 2011), short-chain alcohols (Fisher et al., 2014, Foo et al., 2014), terpenoids (Foo and Leong, 2013, Jasiński et al., 2001, Yazaki, 2006), short-chain fatty acids (Gimenez et al., 2003, Islam et al., 2008, Moschen et al., 2012, Sá-Pessoa et al., 2013) and long-chain fatty acids (Khnykin et al., 2011, Lin and Khnykin, 2014, Villalba and Alvarez, 2014, Wu et al., 2006a, Wu et al., 2006b), while those for isoprene and isoprenoids are eagerly sought (Lohr et al., 2012). Unusual efflux transporters produced by microbes for specific purposes include one for FAD in *Shewanella oneidensis* (Kotloski and Gralnick, 2013), while virtually all free-living aerobes must and do secrete siderophores to permit them to effect iron uptake (see e.g. (de Carvalho et al., 2011, Hider and Kong, 2010, Kell, 2009, Kell, 2010).

Transporter-mediated osmotic stress engineering

If cells are to accumulate soluble products to high titres, there will always be the danger of significant osmotic stresses (as well as lowered water activities (Bhaganna et al., 2010, Nicolaou et al., 2010)). While these osmotic stresses can be relieved by the synthesis of so-called compatible solutes (e.g. (Brown, 1978, Hohmann et al., 2007)) such as betaine, another strategy includes their intracellular accumulation via uptake transporters (e.g. (Csonka, 1989, Farwick et al., 1995, Kempf and Bremer, 1998)). *Corynebacterium glutamicum* provides an excellent example (Ochrombel et al., 2011, Weinand et al., 2007). Note too that inducing the synthesis of such compatible solutes can also be of value in the production of soluble and functional recombinant proteins (Fahnert, 2004, Fahnert, 2012, Prasad et al., 2011).

Transporter engineering

Having established which transporters are important for the problem of interest, it is possible to improve them, typically by the methods of directed evolution (e.g. (Currin et al., 2015, Kell, 2012, Turner, 2009)). These involve varying the primary sequence of the protein, and selecting those with improved properties, in an iterative manner. The variation in primary sequence is done by various forms of mutation and recombination, nowadays including the methods of synthetic biology in which we control rather precisely which sequences are made by creating them at the DNA level by chemical synthesis (e.g. (Cameron et al., 2014, Church et al., 2014, Currin et al., 2014, Currin et al., 2015, Nielsen et al., 2014, Swainston et al., 2014, Way et al., 2014)). The question then arises as to what kind of objective function we might seek. This is most easily considered with regard to Fig 3. Thus we might wish to turn a concentrative uptake transporter into one that merely catalyses equilibration (i.e. efflux of product formed intracellularly). There is ample precedent for this loss of energy coupling, e.g. in mutants of the normally concentrative lac permease of *E. coli* (Brooker et al., 1989, Wilson et al., 1970) or of the mammalian intestinal di- and tripeptide transporter PepT1 (SLC15a1) (Meredith, 2009), and – for influx of substances normally pumped out – of drug uptake via uncoupled variants of the LmrP 'efflux' transporter in lactobacilli (Mazurkiewicz et al., 2004a, Mazurkiewicz et al., 2005, Mazurkiewicz et al., 2004b, Schaedler and van Veen, 2010). By contrast, Tirosh et al. were able (Tirosh et al., 2012) to change a multidrug monovalent 'efflux' antiporter into one that used divalent ions. Thus there seems little doubt that we should be as able to change the specificity (Madej et al., 2013), promiscuity (Khersonsky and Tawfik, 2010) or detailed molecular transport pathways (Yao et al., 2013) of transporters by directed evolution as easily (Daley et al., 2005) as we can do so for other proteins

(Chakraborty et al., 2013). Indeed, evidence for the selection of efflux transporters during the development of various amino acid fermentations was given above. Papers showing a gain-of-function of NCgl1221 to constitutive glutamate excretion (Becker et al., 2013, Nakayama et al., 2012) are of especial note, indicating the potential for transporter engineering.

E. coli contains a (possibly) surprising number of efflux pumps (one sixth of all its transporters (Daley et al., 2005)), even for sugars (Liu et al., 1999a, Liu et al., 1999b). Indeed, in *E. coli*, there are as many as 37 MDR transporters (Nishino and Yamaguchi, 2001), most commonly from the Major Facilitator Superfamily (Holdsworth and Law, 2012). Arguably, the main efflux transporters are acrB (Eicher et al., 2012, Pos, 2009), mdfA (Sigal et al., 2006), emrE (Nakashima et al., 2011, Schuldiner, 2009) and mtdM (Holdsworth and Law, 2012, Paul et al., 2014). Thus, and while n-alkanes are much less cytotoxic than are many other organic solvents (Salter and Kell, 1995), a particularly nice example of the directed evolution of a membrane protein for catalysing product efflux is the study of Foo and Leong (Foo and Leong, 2013), who evolved AcrB to drive improved efflux of the hydrocarbons n-octane and α-pinene from *E. coli*, using selection against the toxicity of n-octanol (that was also presumably excreted), while Fisher *et al*. did the same for shorter-chain alcohols (Fisher et al., 2014). Mutations in a number of other genes such as *lon*, *proV*, *soxS* and *marR* also act via AcrB to increase the solvent tolerance of *E. coli* (e.g. (Aono, 1998, Doukyu et al., 2012, Watanabe and Doukyu, 2012, Watanabe and Doukyu, 2014)). Multidrug resistance transporters have also been used to export dipeptides (Hayashi et al., 2010, Mitsuhashi, 2014) and arabinose (Koita and Rao, 2012) from *E. coli*, while NAD transporter engineering has been exploited to advantage in the whole-cell biocatalytic production of dihydroxyacetone (Zhou et al., 2013).

S. cerevisiae contains 28 members of the Major Facilitator Superfamily of multidrug efflux pumps and at least six members of the ATP-binding cassette (ABC) multidrug transporter family (Balakrishnan et al., 2012, Cherry et al., 2012, Goffeau et al., 1997). All of these efflux pumps reside in the plasma membrane, while Vmr1p is a vacuolar membrane protein. While the importance of the plasma membrane pumps in drug resistance (notably to azoles) in pathogenic yeasts is well recognised (Noël, 2012), any possible role in the efflux of diesel fuels from engineered yeast seems not to have been considered (Westfall and Gardner, 2011) or, at least, not published

Concluding remarks

In this short review, we have sought to summarise some of the evidence that membrane transporters represent rather underutilised yet excellent targets for the purposes of strain improvement in biotechnology. Some of the evidence comes from more classical fermentations where such changes 'emerged' from undirected (mutation and selection) strain improvement programmes, while more recently there are examples of more deterministic strategies based on metabolic engineering. We anticipate many major improvements in the future as the powerful techniques of directed evolution are brought to bear on selected membrane transporters, especially those catalysing concentrative efflux of the desired product. Much as with pharmaceutical drug transporters (Kell and Oliver, 2014), what we need now are good, predictive, quantitative structure-activity relationship (QSAR) models that will help determine the activity of any transporter (sequence) for any drug. Such models will bring us truly closer to the era of 'designer transporters for biotechnology'.

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Legends to Figures

Fig 1. A mind map setting out the contents of this review in an easy-to-read form.

Fig 2. Some antimetabolites that bear structural similarities to natural metabolites with which they compete for uptake transport (and intracellular activity).

Fig 3. An illustration of four kinds of transporters. V and W are transported in exchange for each other; if there is a concentration gradient of one it will drive the transport (antiport) of the other. X is transported out of the cell, potentially against its concentration gradient, by a transporter that couples its transport activity to ATP hydrolysis. Y enters and exits the cell by facilitated diffusion (it is a uniporter), while Z is taken up concentratively in symport with a sodium ion (that descends its own concentration gradient). The terms 'active' (concentrative) and 'passive' (equilibrative) are best used solely to describe the thermodynamics, with no mechanism being implied unless stated (Kell and Oliver, 2014). The membrane is drawn approximately to scale, with a typical *in vivo* ratio of protein:lipid (3:1 by mass). We do not show explicitly transporters (or pores) that are sensitive to osmotic pressure or turgour, but they are potentially very important in biotechnology, and we discuss them in the text. Note too that there can be a highly intimate interaction between specific lipids and transporter function (e.g. (Laganowsky et al., 2014, Naismith and Booth, 2012) such that changing the former may affect the latter.

Fig 4. A modern strategy for transporter engineering in biotechnology requires first that we construct suitable metabolic networks from genomic and other data, then that we use variations in expression profiles and desirable phenotypic properties to identify qualitatively those transporters whose properties most need improving, and finally that we use the methods of intelligent directed evolution tom modify their properties and expression levels appropriately.

Fig 5. Expression profiles of 151 transporter and 6373 non-transporter transcripts in baker's yeast. Data are from (Lee et al., 2012). Note that fewer transport reactions in the model (327/1079, 30.3%) have associated genes (hence transcripts) than do all other metabolic reactions (1983/2255, 87.9%).

Fig 6. The principle of genome-wide identification of transporters for a toxic drug by evaluating the enrichment of survivors when the gene encoding the transporter for the cytotoxic drug is knocked out.

(NB The legend to the figure to be included in Box 1 is given in Box 1.)

Tables.

Table 1. Some databases with a focus on membrane transporters

Name	Focus/ organism(s)	URL	References
Bioparadigms SLC tables	Humans	http://www.bioparadigms.org/slc/intro.htm	(Hediger et al., 2013)
Caenorhabditis elegans Solute Transporter database	*C. elegans*	http://www.wormslc.org/	(Jäckel et al., 2010)
Drugbank	Humans/ drugs	http://drugbank.ca	(Law et al., 2014)
Human-intestinal transporter database	Humans/drugs	Not apparently directly online; data are downloadable from the paper's Supplementary Information.	(Sedykh et al., 2013)
Human transporter database	Humans	http://htd.cbi.pku.edu.cn	(Ye et al., 2014)
Transportal	Human/ drug transport	http://bts.ucsf.edu/fdatransportal/	(Morrissey et al., 2012)
TransportDB	Comparative genomics of transporters	http://membranetransport.org/	(Ren et al., 2007, Ren et al., 2004)
Transporter Classification Database TCDB	IUBMB-approved transporter classifications	http://www.tcdb.org/	(Saier et al., 2014, Saier et al., 2006)
Transporter database TP-search	Humans/ drug uptake	http://www.tp-search.jp	(Ozawa et al., 2004)
TransportTP	Transporter prediction	http://bioinfo3.noble.org/transporter/	(Li et al., 2009)
Yeast metabolome database	*S. cerevisiae*	http://www.ymdb.ca/	(Jewison et al., 2012)
Yeast Transport Protein database YTPdb	*S. cerevisiae*	http://ytpdb.biopark-it.be/ytpdb/	(Brohée et al., 2010, Van Belle and André, 2001 10873)
Yeti: Yeast transport	*S. cerevisiae*	http://genolevures.org/yeti.html	(De Hertogh et al.,

information			2002)

Box 1. Flux control in Metabolic Control Analysis

Imagine a metabolic network or pathway in which we vary the concentration of an enzyme E by an amount ΔE, with a concomitant change in the flux of interest ΔJ. (In the limit of small changes this becomes dJ/dE.) By normalising these changes to the flux and enzyme concentration at the operating point (J,E) we can obtain a dimensionless quantity C^J_E, the flux-control coefficient (of enzyme E on the flux of interest J), that describes in quantitative terms the extent to which that enzyme controls the flux. C^J_E is equivalent to a local sensitivity coefficient. If C^J_E is 0 then the enzyme exerts no flux control, while if it is 1 then it is then completely flux-controlling. Intermediate values are possible, and the flux-control summation theorem (Fell, 1996, Heinrich and Rapoport, 1974, Heinrich and Schuster, 1996, Kacser and Burns, 1973) proves that the sum of the flux-control coefficients for all enzymes on a particular flux is 1. This means that most enzymes have small flux-control coefficients (and, as in the figure, even a 50% knockdown typically has a limited effect on flux (Kacser and Burns, 1981), so to have major effects one should seek to use haploid organisms). Thus, in *C. pasteurianum* glycolysis the flux-control coefficient of the glucose transporter was under 0.2 (Walter et al., 1987); however, because of the involvement of branched pathways, that in *S. cerevisiae* exceeded 1 (Smallbone et al., 2013). A related concentration-control summation theorem shows that the sum of the concentration control coefficients = 0. Note that the flux-control coefficient is not constant – at a different operating point it would be higher or lower as flux control shifts among different parts of the network (Kell et al., 1989). For other pertinent reviews of metabolic control analysis, see (Cornish-Bowden and Cárdenas, 2000a, Cornish-Bowden and Cárdenas, 2000b, Fell, 1996, Fell, 1998, Heinrich and Schuster, 1996) The thesis in this review is that where the flux-control coefficients of transporters are determined, they will often be found to be larger than those of other enzymes, providing suitable suggestions for transporter engineering.

Box 2. Flux balance analysis

Flux balance analysis describes a series of techniques for estimating relative metabolic fluxes without the requirement to know any of the kinetics of the participating enzymes. All it requires is a knowledge of the stoichiometries of the participating reactions, the molecular identities of the reactants and products themselves, and an objective function that one is trying to maximise. Linear programming techniques can then be used to optimise the latter. The stochiometries, including mass, charge and energy balances, provide a very effective series of constraints to determine the possible fluxes (Covert and Palsson, 2003, Palsson, 2006, Price et al., 2004, Schellenberger et al., 2011); however, the objective function is more problematic. Typically biomass is used (i.e. the rate of biomass formation) (García Sánchez et al., 2012, Palsson, 2006, Schuetz et al., 2007), with biomass encoded as a 'molecule' with a non-integer empirical formula. This means that the problem is highly underdetermined, with a very great many possible flux distributions giving an equally good fit, and such an objective function hardly makes sense for non-growing cells producing a biotechnological product that is of interest. Thus if possible it is desirable to add further constraints, e.g. by confining specific fluxes to restricted ranges (Schuetz et al., 2012, Wilkinson et al., 2008) or finding flux distributions that best correlate with expression profiles (Lee et al., 2012, Shlomi et al., 2007).

Glossary

Term	Meaning
log D	The logarithm of the Distribution Coefficient, D. D is the ratio of the sum of the concentrations of all forms of a compound (ionised plus non-ionised) in each of two phases, typically 1-octanol and an equilibrated aqueous buffer, whose pH must be specified.
log P	The logarithm of the Partition Coefficient, P. P is a measure of the hydrophobicity of a molecule; log P is the logarithm (base 10) of the ratio of the concentration of a solute molecule in an organic solvent, usually 1-octanol (Young, 2014), to that of the non-ionised form of the same molecule in water

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Figure
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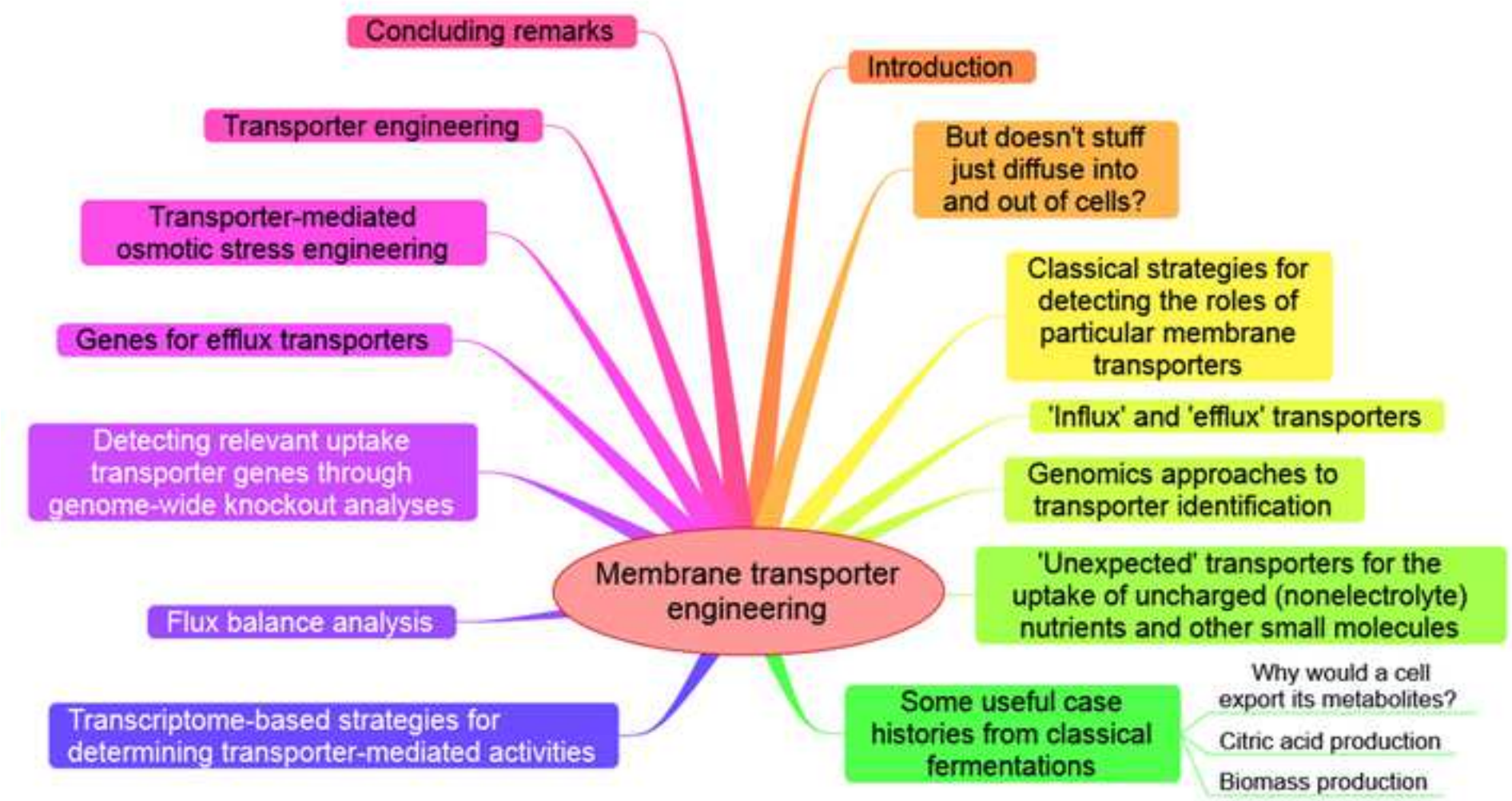

Figure 2
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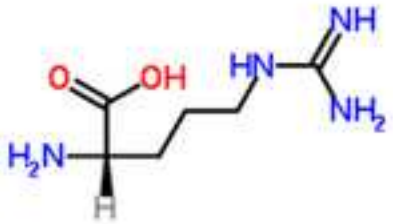
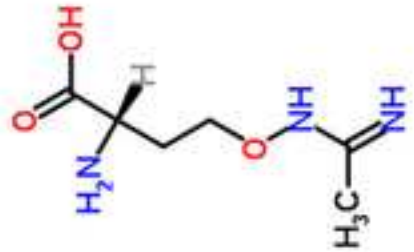
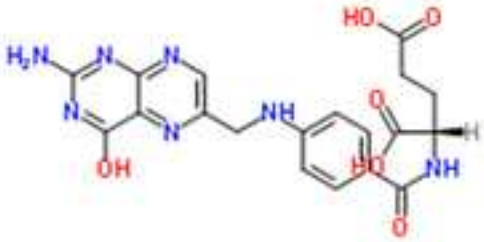


L-(+)-arginine



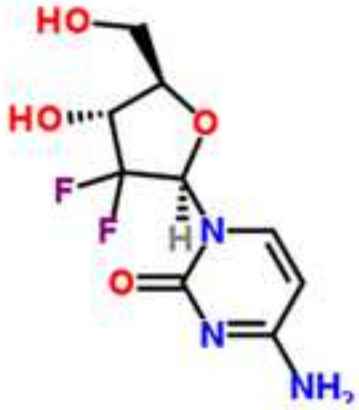
canavanine



folate

methotrexate

cytidine



gemcitabine

Figure 3
Click here to download high resolution image

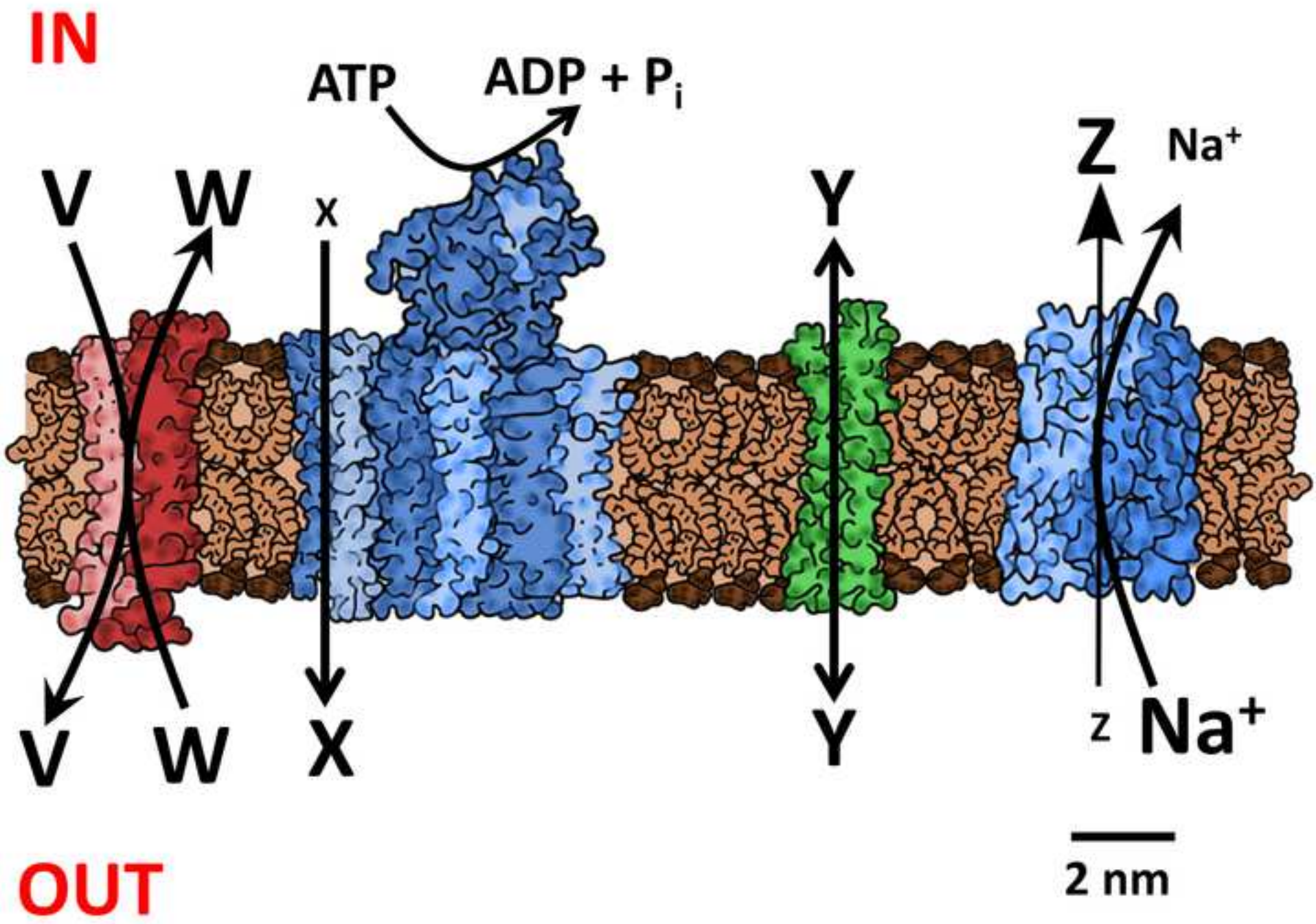


Figure 4
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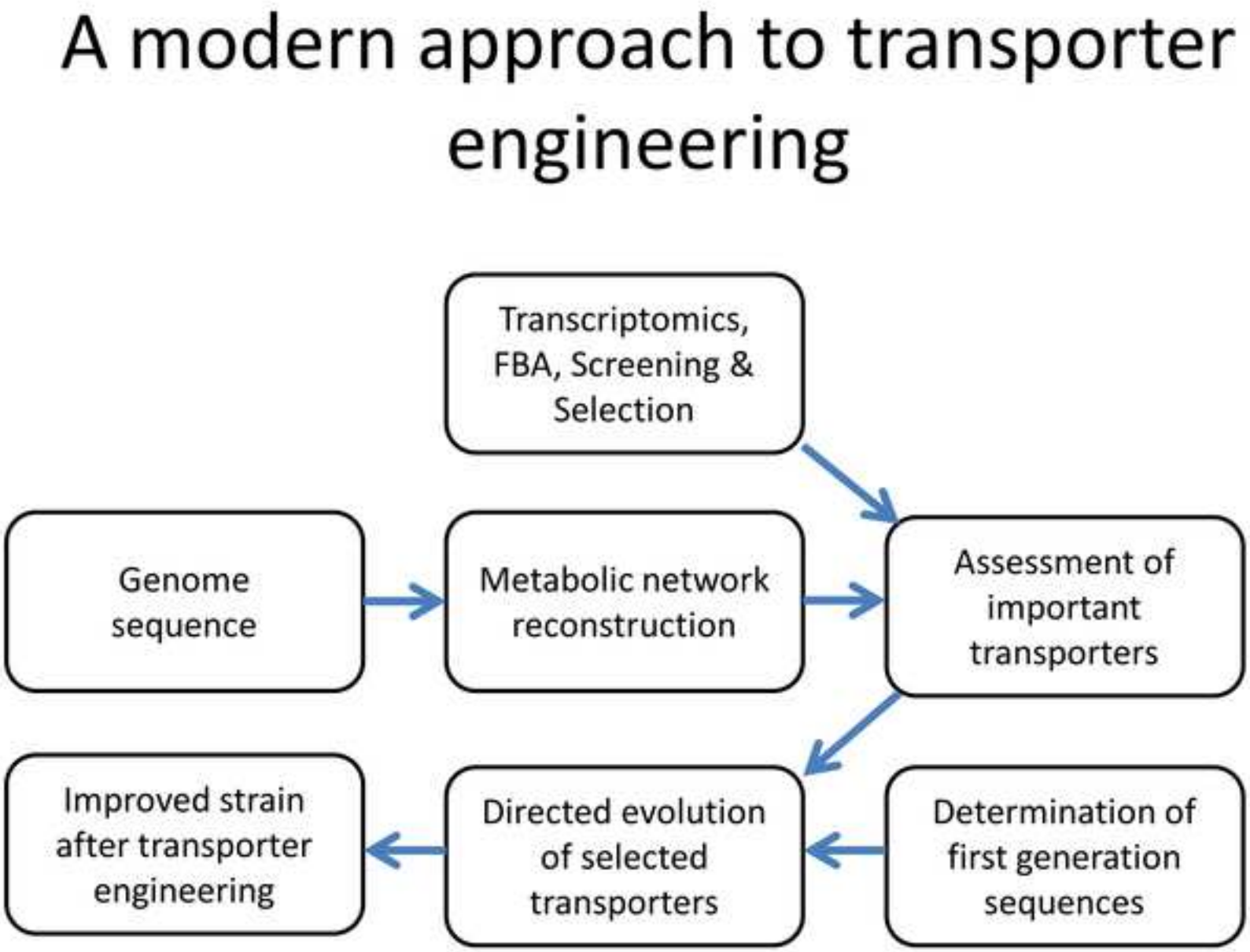

Figure 5
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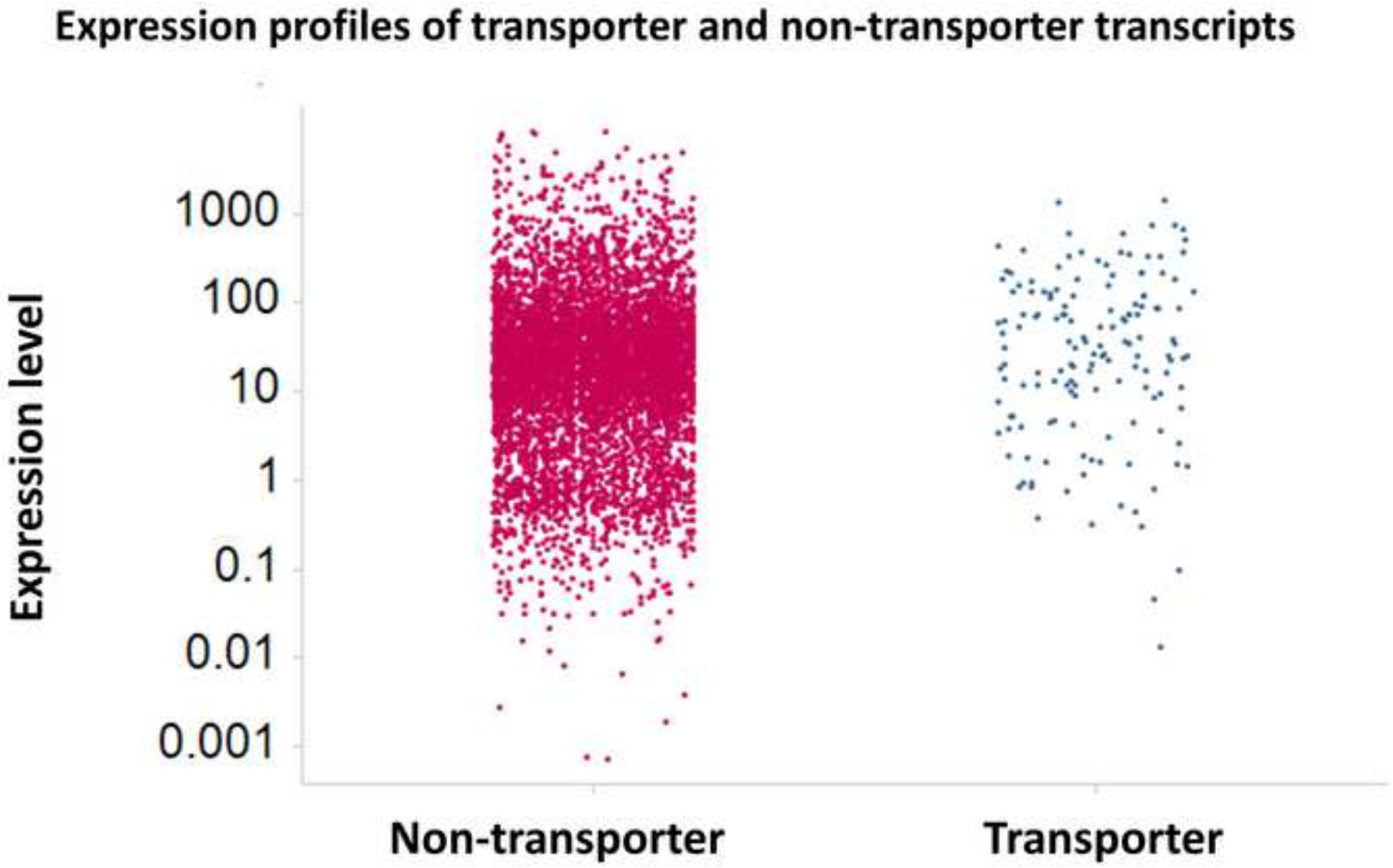

Figure 6
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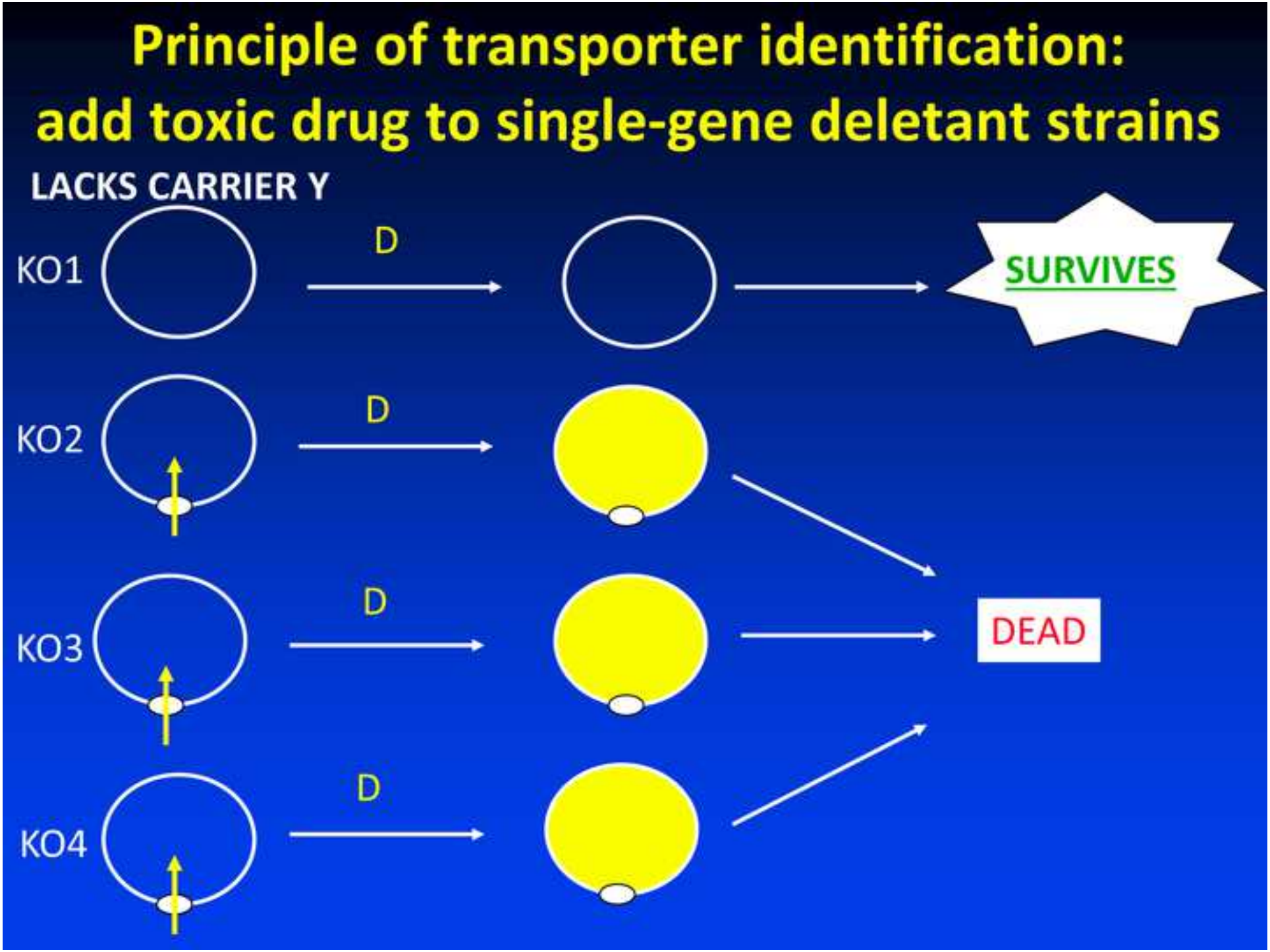
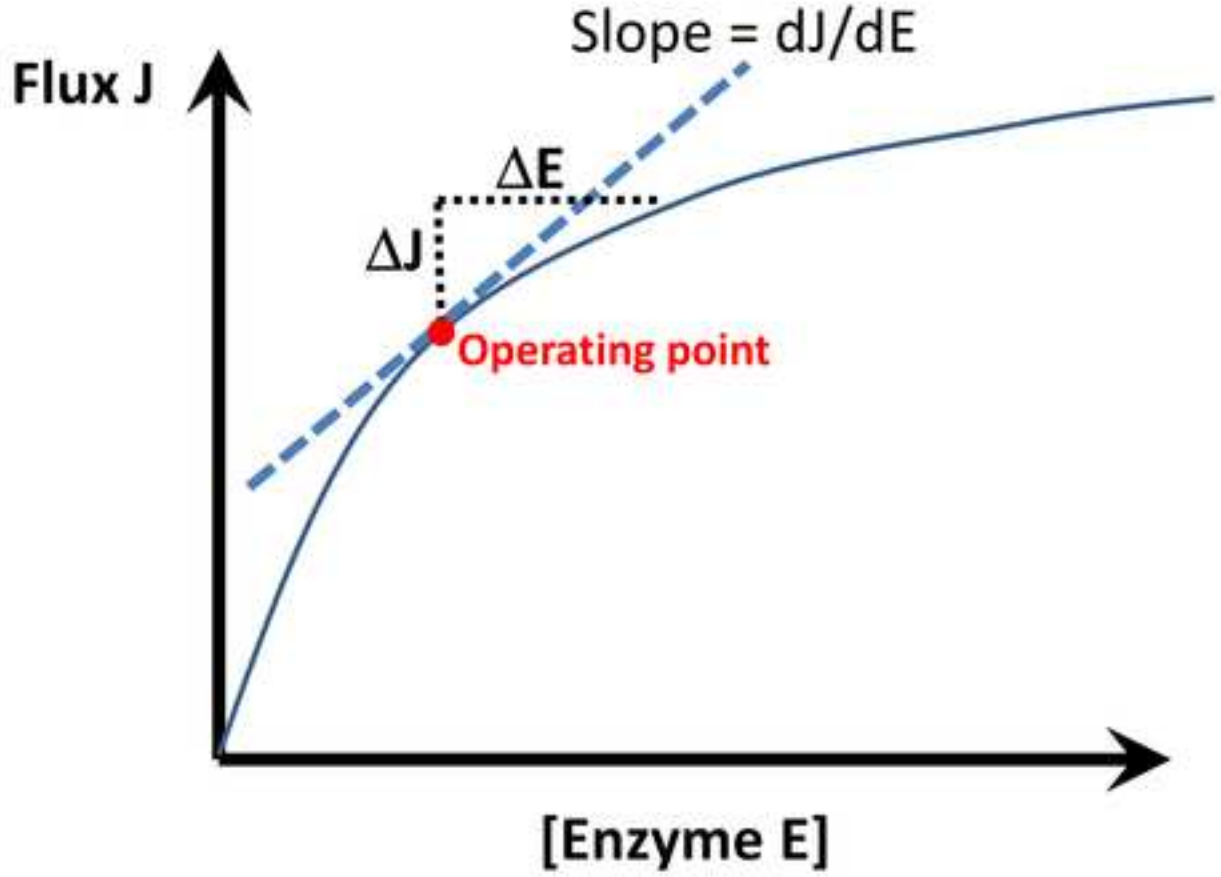


Figure for box 1
Click here to download high resolution image



Flux-control coefficient $C^J_E = (dJ/J)/(dE/E)$

Highlights

- Transporters are a relatively undervalued set of proteins contributing to the control of biotechnological fluxes
- We highlight the evidence for this
- This is true for both ‘influx’ and ‘efflux’ transporters
- Transporter engineering offers many opportunities to overcome flux control
- The methods of Synthetic Biology offer particular promise for transporter engineering

There is no specific cover letter, so this is a placeholder