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REVIEW ARTICLE

Metabolite secretion in microorganisms: the theory of metabolic overflow put to the test

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Abstract

Introduction Microbial cells secrete many metabolites during growth, including important intermediates of the central carbon metabolism. This has not been taken into account by researchers when modeling microbial metabolism for metabolic engineering and systems biology studies.

Materials and Methods The uptake of metabolites by microorganisms is well studied, but our knowledge of how and why they secrete different intracellular compounds is poor. The secretion of metabolites by microbial cells has traditionally been regarded as a consequence of intracellular metabolic overflow.

Conclusions Here, we provide evidence based on time-series metabolomics data that microbial cells eliminate some metabolites in response to environmental cues, independent of metabolic overflow. Moreover, we review the different mechanisms of metabolite secretion and explore how this knowledge can benefit metabolic modeling and engineering.

Keywords Microbial metabolism · Microorganisms · Active efflux · Secretion · Metabolic engineering · Metabolic modeling · Systems biology

1 Introduction

Microorganisms have been used for the production of industrially relevant compounds for many years. *Corynebacterium glutamicum* and its mutants have been employed for the large-scale industrial production of glutamate, lysine and other flavor active amino acids (Hermann and Krämer 1996; Krämer 1994, 2004). *Aspergillus niger* is utilized for the fermentative production of citric acid, accounting for more than 90% of total citrate production worldwide (Soccol et al. 2006). Another industrially significant metabolite, succinic acid, has been produced in large quantities by microorganisms including *Actinobacillus succinogenes, C. glutamicum, Escherichia coli* and some genetically modified yeasts (Liang et al. 2013; Agarwal et al. 2006). Consequently, numerous studies have focused on the characterization of metabolic pathways and the development of tools to manipulate these pathways in order to increase product yield (Hermann and Krämer 1996; Ljungdahl and Daignan-Fornier 2012; Wendisch et al. 2006). Metabolite secretion is useful for industrial production as it simplifies the downstream extraction and purification of metabolites of interest (Krämer 2004, 1996).

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The transport of substrates into the cell has been the subject of many studies (Düring-Olsen et al. 1999; Magasanik and Kaiser 2002; Nehls et al. 2001; Schweikhard and Ziegler 2012). Comparatively, very little is known about the mechanisms of secretion of intracellular metabolites, especially primary metabolites. The secretion of metabolites is an essential biochemical function and reflects the internal metabolic state of the cell in response to environmental conditions (Krämer 1994; Chubukov et al. 2014). This process allows the removal of cell metabolic by-products from the intracellular medium in order to maintain homeostasis.

Most metabolite secretions are believed to result from imbalanced intracellular metabolic pathways causing an overflow of pathway intermediates and secretion of these metabolites due to intracellular accumulation (Paczia et al. 2012; Reaves et al. 2013). Metabolic overflow is a phenomenon commonly observed in many microorganisms, usually when the rate of glycolysis exceeds a critical value (Valgepea et al. 2010; Vemuri et al. 2006a; Fu et al. 2014). Under fully aerobic and substrate-rich conditions, most microbial cells use inefficient metabolic routes and convert a substantial amount of accessible carbon to incompletely oxidized end-products such as ethanol, acetate and lactate (Molenaar et al. 2009; Tempest and Neijssel 1979). However, the concept of metabolic overflow cannot explain the secretion of many metabolites observed in time-series studies with parallel measurement of intracellular and extracellular metabolites (Krämer 1994; Carneiro et al. 2011, 2012; Han et al. 2013; Granucci et al. 2015). We often observe some metabolites being directly excreted to the extracellular medium without intracellular accumulation in response to environmental cues (Carneiro et al. 2011; Han et al. 2013; Granucci et al. 2015). This scenario is most obvious when toxic metabolites are actively secreted by the cell using different efflux pumps (Becker et al. 2013; Forsberg and Ljungdahl 2001; Segura et al. 2012). However, other metabolites can be secreted following intracellular metabolic overflow under one environmental condition while they are exported without intracellular accumulation under another. Both metabolic overflow and active efflux (secretion without a intracellular accumulation) processes are behind of metabolite secretion; however, their mechanisms of regulation, especially when not following metabolic overflow, are still unclear.

Here, we review the mechanisms of metabolite secretion and revisit the concept of metabolic overflow. By re-analyzing published time-series metabolomics data, we demonstrate that the secretion of many intracellular metabolites cannot always be explained by intracellular metabolic overflow. We argue that a yet-unknown cellular strategy for the regulation of intracellular metabolite levels may drive the active secretion of some intracellular metabolites. Finally, we discuss the impact of these secretion mechanisms on metabolic modeling.

2 Mechanisms of metabolite secretion by microorganisms

The study of microbial transport mechanisms has primarily focused on uptake systems (Shlykov et al. 2013). Efflux transport has received much less attention despite its immense relevance to industrial processes (Van Dyk 2008). The exceptions are the study of multidrug efflux pumps in bacteria and fungi due to their role in drug-acquired resistance (Poole 2004; Martín et al. 2005), Na^+/H^+ antiporter (Verkhovskaya et al. 2001; Kinclova-Zimmermannova and Sychrova 2007), metals transporters e.g. Cu^+/Ag^+ efflux pump (Franke et al. 2003) and the macromolecule secretion systems (Shlykov et al. 2013). The study of primary metabolite secretion mechanisms in microorganisms has been limited mostly to a handful of amino and non-amino organic acids of industrial interest (Krämer 1994; Van Dyk 2008; Velasco et al. 2004; Netik et al. 1997).

Efflux transport mechanisms are similar to those involved in metabolite uptake (Box 1). The secretion or efflux of intracellular metabolites to outside the cell occurs through active or passive mechanisms. Passive transport is not concentrative in nature and it is rather equilibrative of transmembrane thermodynamic activities (Kell and Oliver 2014). Passive secretion is carried out either through the cell membrane (lipoidal diffusion) or with the help of transporters (facilitated diffusion) (Fig. 1a) and it occurs for most non-charged molecules and depends on the hydrophobicity of the solute and the properties of the membrane (Konings et al. 1992). This is believed to be the mechanism of secretion for some small metabolites, particularly fermentation end-products such as alcohols, ketones and small organic acids (Ingram 1976; Walter and Gutknecht 1984). Organic acids are membrane permeable in the undissociated form; thus, diffusion depends on cytosol pH (Kell et al. 1981). Hydrophobic and branched-chain amino acids can also permeate the plasma membrane through passive diffusion (through the bilayer) (Fig. 1) (Driessen and Konings 1990). For example, proline overproducing strains of *E. coli* K12 or *Bacillus subtilis* generate a proline gradient of sufficient magnitude across the cytoplasmic membrane to support proline diffusion (Hoffmann et al. 2012; Rancourt et al. 1984). Although it is still a matter of debate if passive secretion/uptake occurs through the bilayer or not (Kell and Oliver 2014; Smith et al. 2014), there are enough literature evidence that support the existence of both passive lipoidal (through bilayer) and carrier mediated (facilitated) diffusion (Lepore et al. 2011; Nikaido 1993).

Secretion can also occur through specific transporters/carriers present in the cell membrane. Passive secretion occurring through channels or carriers is referred to as protein-mediated diffusion and this type of efflux can be observed when microbial cells react to hypo-osmotic stress. In this case, osmotic downshock releases a variety of metabolites such as proline, glutamate and glycine by stretching an activated channel (Lamark et al. 1992). Carriers are also involved in active secretion, a process where energy is used to drive metabolite efflux against its electrochemical gradient (Fig. 1b). The most-studied primary active efflux transporters utilize the hydrolysis of ATP. For instance, ABC exporters or ATP-binding cassette transporter proteins are widely used by cells to perform drug extrusion and also to excrete some primary metabolites (Lamark et al. 1992). Secondary active systems transport a second solute by harvesting energy obtained from the primary transport and can be classified as uniporters, symporters and antiporters (Saier Jr 2000; Forrest et al. 2011). These three basic mechanisms of transport represent the main mechanisms known for microbial efflux (Van Dyk 2008).

Table 1 summarizes some transporters known to secrete primary metabolites in *C. glutamicum, E. coli, S. cerevisiae* and *A. niger*; and a few of these are underlined below. Mechanisms for the secretion of amino acids are well studied in *C. glutamicum* and *E. coli* because of their industrial relevance (Wachi 2013; Eggeling and Sahm 2003; Kell et al. 2015). Consequently, important amino acid excretion systems have been characterized using these organisms as models. For example, *C. glutamicum* secretes L-lysine by secondary secretion in which cationic lysine is co-transported with two hydroxide ions (Krämer 1994). The transporter LysE identified in 1996 by Vrljic et al. is responsible for this transport as well as it appears to be involved in the secretion of arginine (Vrljic et al. 1996).

Despite the fact that approximately 2.1 million tons of monosodium glutamate is produced annually using *C. glutamicum* (Ajinomoto 2009), the secretion mechanism for L-glutamate has only recently been described as occurring via small-conductance mechanosensitive channels (Becker et al. 2013; Nakamura et al. 2007; Mitsuhashi 2014). This mechanism is triggered by alterations in cell membrane tension and prevents cell disruption by the fast release of small internal solutes (Becker et al. 2013; Bass et al. 2002). This explains why industrial techniques to influence the stability of the membrane of *C. glutamicum*, such as biotin limitation and the addition of some surfactants, lead to increased glutamate excretion (Burkovski and Krämer 2002). Moreover, it is now well known that the activation of mechanosensitive

Table 1 Primary metabolites and their respective secretion transporters in different microorganisms

Microorganism	Metabolite	Transporter	Mechanism	Reference
Corynebacterium glutamicum	L-Lys, L-Arg	LysE	Secondary active secretion	Vrljic et al. (1996), Stäbler et al. (2011)
	L-Thr, L-Ser	ThrE	Secondary active secretion	Eggeling and Sahm, (2003), Simic et al. (2001)
	L-Iso, L-Met, Leu, Val	BrnEF	Secondary active secretion	Nakamura et al. (2007), Trötschel et al. (2005)
	Glu	YggB/ NCgl1221	Protein-mediated diffusion	Nakamura et al. (2007), Becker et al. (2013)
	Succinate	SucE1	Secondary active secretion	Fukui et al. (2011)
Escherichia coli	Aromatic amino acids	YddG	Secondary active secretion	Airich et al. (2010)
	Thr/homoserine	RhtA(YbiF)	Secondary active secretion	Simic et al. (2001), Livshits et al. (2003)
	L-Glu	Yggb/MscS	Protein-mediated diffusion	Broda, (1968), Börngen et al. (2010)
	L-Cys and components of the cysteine pathway	YdeD	Secondary active secretion	Daßler et al. (2000)
	L-Cys, *O*-acetylserine	YfiK	Secondary active secretion	Franke et al. (2003
	Lactose and glucose	SetA, SetB	Secondary active secretion	Liu et al. (1999)
	γ-Hydroxybenzoate	AaeB	Secondary active secretion	Van Dyk et al. (2004)
Saccharomyces cerevisiae	Glycerol	FPS1	Protein-mediated diffusion	Beese-Sims et al. (2011), Geijer et al. (2012)
	Homoserine, Thr, Asp, Glu, Ala	Aqr1	Secondary active secretion	Velasco et al. (2004)
Aspergillus niger	Citric acid	ATP pump	Primary active secretion	Netik et al. (1997)

glutamate efflux pump (encoded by a gene called NCgl1221) occurs due to the change in membrane tension (Mitsuhashi 2014).

While aromatic amino acids are usually secreted by passive diffusion in *C. glutamicum* (Burkovski and Krämer 2002), an aromatic amino acid exporter has been characterized in *E. coli* by Airich et al. (2010), and it is assumed to be the major exporter for this class of amino acids. L-glutamate in *E. coli* seems to be secreted by the same channel mechanism (known as mscS) found in *C. glutamicum* (Becker et al. 2013; Wachi 2013). Similar excretion mechanisms are known to be involved in the secretion of many amino acids including lysine, isoleucine, threonine, methionine and others (Van Dyk 2008). In addition, sugar efflux transporters have been identified in *E. coli*. These can transport lactose and glucose as well as other sugar derivatives. However, it has been suggested that the genes encoding these are either poorly expressed or made redundant by other proteins with the same function in *E. coli* (Liu et al. 1999).

S. cerevisiae is used as a model eukaryote for genetics and cell biology. However, we know little about its primary metabolite secretion mechanisms, despite its widespread use in industrial fermentation processes. The latest comprehensive metabolite secretion study explores internal membrane transporter Aqr1 that works as an amino acid/H^+ antiporter (Velasco et al. 2004). This transporter is the major excretion system for homoserine and L-threonine, and increases the secretion of L-alanine, L-aspartate and L-glutamate when overexpressed. Unlike the bacterial transporters, *S. cerevisiae* does not secrete amino acids directly, but instead loads them into intracellular vesicles, which merge with the cytoplasmic membrane, releasing the amino acids to the extracellular environment (Velasco et al. 2004). In addition, *S. cerevisiae* has been widely used as an experimental model to explore the transport mechanisms of different drugs/drug resistance and in toxicogenomics studies (dos Santos and Sa-Correia 2011, 2015; dos Santos et al. 2014)

A. niger is another eukaryote commonly used in industrial fermentation, mostly for the production of citric acid. Citrate secretion has been demonstrated to occur actively through an ATP-dependent transporter (Netik et al. 1997), and also through passive diffusion during low extracellular pH (Mattey 1992), which is generally the case in industrial production. Many other secretion mechanisms for other metabolites in *A. niger* have been hypothesized, but most of them have not been demonstrated experimentally.

While here we mainly discussed primary metabolite secretion by industrially important microorganisms, it is noteworthy that metabolite secretion is also an important phenomenon for the ecosystems (Ponomarova and Patil 2015). In microbial communities, many microorganisms exhibit synergistic relationships and depends on each other to survive (Braga et al. 2016). For instance, metabolites secreted by one microbe are used as a nutritional source by another microbe and some harmful metabolites can be used by certain microbes, thus assisting other microbes within the community to survive (Schink 2002).

Microbial efflux systems have generally been studied in isolation, often in an artificial system with a focus on the secretion mechanism itself. This omits the complexity of the living organism. For full understanding, secretion mechanisms should be also investigated through a systems biology approach.

Box 1: Basis of metabolite secretion mechanism

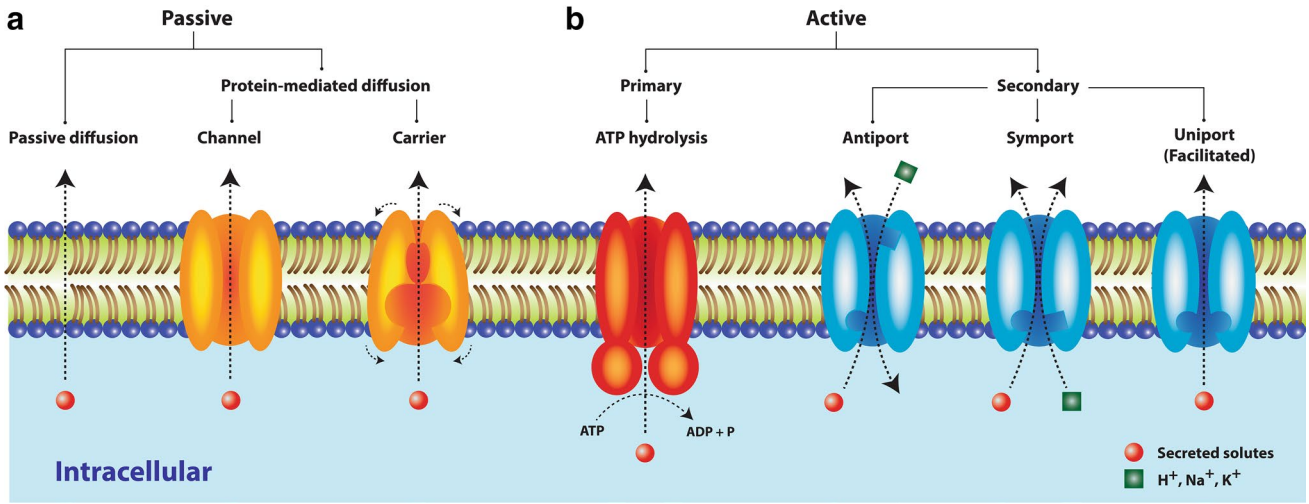

Fig. 1 Basic mechanisms for metabolite secretion, divided between passive and active. The figure shows passive diffusion through the membrane and protein-mediated diffusion by channel and carrier as a passive secretion (yellow). The primary (red) and secondary (blue) active secretion mechanisms are represented by the ATP pump and antiport, symport and uniport, respectively

The basis of transport mechanism is almost the same for uptake and efflux systems, but it occurs in opposite directions and can be an active or passive process. Passive secretion (Fig. 1a), when the metabolite is secreted in the direction of its chemical or electrochemical gradient, is carried out through the cell membrane or specific structures (lipoidal diffusion); the former is used by most non-charged molecules. Passive secretion can also occur through specific transporters also known as transport systems, porters, permease systems, and permeases; these refer to a protein or protein complex which catalyzes a vectorial reaction (Saier Jr et al. 2006). Transporters can be divided into two main categories: channels and carriers (Saier Jr et al. 2006). The Transporter Classification Database is a useful resource which comprehensively classifies transporters based on phylogenetic and functional information (Saier Jr et al. 2006) (http://www.tcdb.org/). Channels are proteins that create a narrow hydrophilic passage that allows a range of molecules to pass through, usually ions and other small molecules. In contrast, carriers are substrate-specific in their interaction and possess a lower transport rate. In carrier-type facilitated diffusion, the transport process occurs through conformational changes in the carrier where the solute binding site can be exposed to one side of the membrane or the other (Boudker and Verdon 2010; West 1997). Passive secretion occurring through channels or carriers is referred to as protein-mediated diffusion.

Unlike channels, carriers are also involved in active secretion (Fig. 1b), a process where additional energy is used to drive metabolite efflux against its gradient of concentration. Active secretion mechanisms are classified based on the type of energy used by the carrier. Two major classes are present in efflux systems: the primary active transporters, which require ATP hydrolysis, oxidation–reduction reactions or light as an energy source (not reversible); and secondary active transporters, which rely on the sum of transmembrane electrochemical gradients of all solutes, for example, protons, or sodium ions to drive the secretion of a metabolite (Saier Jr 2000). Secondary active systems harvest energy obtained from the transport of one solute down its electrochemical gradient to transport a second solute and might be reversible (Saier Jr 2000). This system is called secondary because, in the sequence of the events, the electrochemical gradient needs to be generated by primary mechanisms (Forrest et al. 2011). Well-known secondary mechanisms are uniport, symport and antiport systems. Uniport system transports a single metabolite down its electrochemical gradient, also called facilitated diffusion. Symport secretion uses the energy generated by the transport of a molecule to secrete, in the same direction, a second molecule; whereas antiport systems use downhill secretion energy as a driving force to transport a second molecule in the opposite direction.

3 Metabolic overflow

The concept of metabolic overflow was first explored by Herbert Crabtree in 1929 when describing the carbohydrate metabolism of tumor cells and it has been widely used to justify the secretion of intracellular metabolites. Crabtree observed the production of lactate by tumor cells under aerobic conditions and an excess of glucose (Crabtree 1929). This phenomenon of aerobic fermentation is now known as the Crabtree effect and is found in many different cell types and microorganisms, primarily during growth under high sugar concentrations (Pronk et al. 1996; Chumnanpuen et al. 2014). Metabolic overflow was first seen as a consequence of the Crabtree effect, and has been defined as a phenomenon which occurs during growth under high substrate concentration or during the transition from a substrate-limited to a substrate rich condition as well as during nutrient scarcity (Box 2, Fig. 2) (Neijssel and Tempest 1976; Pronk et al. 1996; Hagman et al. 2014). However, metabolic overflow goes beyond this and, in addition to the major fermentation products, includes the secretion of many primary metabolites. For instance, *Staphylococcus aureus* shows a strain-specific overflow metabolism by secreting isoleucine and a few other organic acids in culture medium used for infection related studies (Dörries and Lalk 2013). Often, metabolic overflow occurs when microbial growth is limited by an essential nutrient, or when the uptake of a substrate is not regulated efficiently (Burkovski and Krämer 2002). For example, *Bacillus subtilis* produces overflow metabolites including acetate, diacetyl and acetoin when growing in phosphate-limited chemostats (Sonenshein 2007). There is a trend of associating metabolic overflow with overall secretion of intracellular metabolites to the extracellular medium (Paczia et al. 2012; Dörries and Lalk 2013). For instance, the secretion of 30–40 primary metabolites by four different microorganisms (*S. cerevisiae, E. coli, Bacillus licheniformis* and *C. glutamicum*) has been referred to as a consequence of extended metabolic overflow (Paczia et al. 2012).

Metabolic overflow has been mostly studied in the model microorganisms *E. coli* and *S. cerevisiae* (Box 2, Fig. 2). In *E. coli*, the massive secretion of acetate is observed during aerobic growth under high glucose concentrations, and many other metabolites such as pyruvate and glucose-6-phosphate will overflow in smaller quantities (Paczia et al. 2012). On the other hand, *S. cerevisiae* produces ethanol and glycerol as major metabolic by-products when growing aerobically on high sugar concentration (Hagman et al. 2014). The production of glycolytic NADH exceeds the cellular capability for its oxidation and results in reduced conditions; consequently, *S. cerevisiae* reduces pyruvate to ethanol or dihydroxyacetone phosphate to glycerol to maintain the redox balance (Box 2). Depending on the carbon source and other substances available during growth, *C. glutamicum* is capable of producing various overflow metabolites such as pyruvate when grown on lactate (Cocaign-Bousquet and Lindley 1995), dihydroxyacetone and lactate when grown on fructose (Kiefer et al. 2004), and glutamate when growing on biotin limited media or in the presence of penicillin and other surfactants (Burkovski and Krämer 2002). The filamentous fungus *A. niger* is known to secrete massive amounts of citric acid when growing on high sucrose concentrations under limited nitrogen, phosphate and metal ion conditions (Mattey 1992). Citrate overflow in *A. niger* mainly occurs because of unobstructed metabolic flow through glycolysis that increases the production of acetyl-CoA and oxaloacetate, thus increasing the production and secretion of citrate from the condensation of these two TCA cycle precursors using citrate synthase found in the mitochondria (Kiefer et al. 2004; Legiša and Mattey 2007). Some studies point to the limited capacity of several citrate metabolizing enzymes, including aconitase and isocitrate dehydrogenase, as key factors responsible for citric acid accumulation in *A. niger* (Crabtree 1929). However, this has been controversial (Legiša and Mattey 2007; Kubicek 1987). The role of different trace metals has also been highlighted (Shankaranand and Lonsane 1994), and a recent hypothesis suggests a tricarboxylate transporter that directly competes with aconitase for citrate, pumping citrate outside the mitochondria and increasing the rate of citric acid secretion to the extracellular environment (Kubicek 1987).

These typical examples of metabolic overflow explain the secretion of the major fermentation products usually found at g/L scale in the spent culture media (e.g. ethanol, acetate, lactate, glycerol and citrate) (Box 2). However, the concept of overflow metabolism is often used to indiscriminately explain or interpret the secretion of all intracellular metabolites found in spent culture media (Mo et al. 2009). We believe this is not appropriate. Metabolic overflow is a reasonably well-characterized phenomenon and is likely to be involved in the secretion of many intracellular metabolites as well as major fermentation products. Nevertheless, metabolic overflow is not the only reason cells secrete intracellular metabolites. Time-series metabolomics experiments with concomitant measurement of intracellular and extracellular metabolites are the best way to study patterns of metabolite secretion.

Box 2: Metabolic overflow

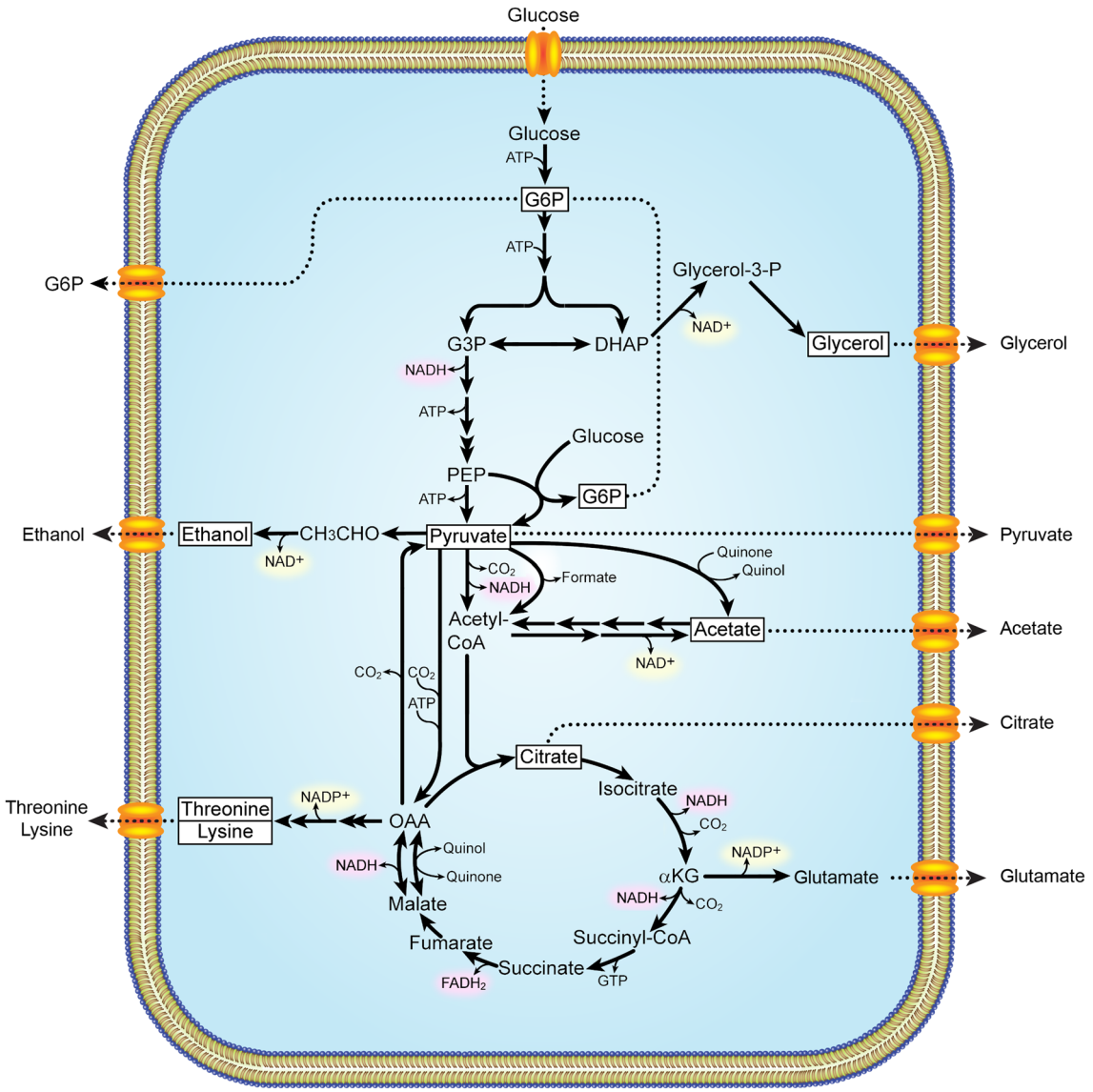

Fig. 2 Typical metabolic overflow of major fermentation intermediates in a microbial cell. Microorganisms secrete many metabolites outside the cell (highlighted in white) due to the intracellular accumulation (shown in boxes). The oxidation-reducing reactions of cofactors in different central carbon metabolic pathways are also highlighted (yellow/pink)

Metabolic overflow is typically described as a phenomenon where microorganisms secrete metabolic intermediates (e.g. ethanol, glycerol and acetic acid) while growing on high substrate concentration (Fig. 2). It is usually observed in microorganisms when they use energy-inefficient metabolic routes by expressing their fermentative behaviour, even though they are capable of using energy efficient respiratory pathways (Molenaar et al. 2009). Most early studies clearly indicate that the extent of metabolic overflow and the nature of excreted metabolites depend on the carbon source used (Tempest and Neijssel 1979; Crabtree 1929; Neijssel and Tempest 1975; Holme 1957). Recent studies reveal that metabolic overflow mainly occurs when there is an imbalance between the fluxes of carbon uptake, energy production and biosynthesis (Molenaar et al. 2009; Holme 1957). Among the various parameters studied, redox balance seems to be the major force behind metabolic overflow (van Hoek and Merks 2012; Vemuri et al. 2006b) (Fig. 2).

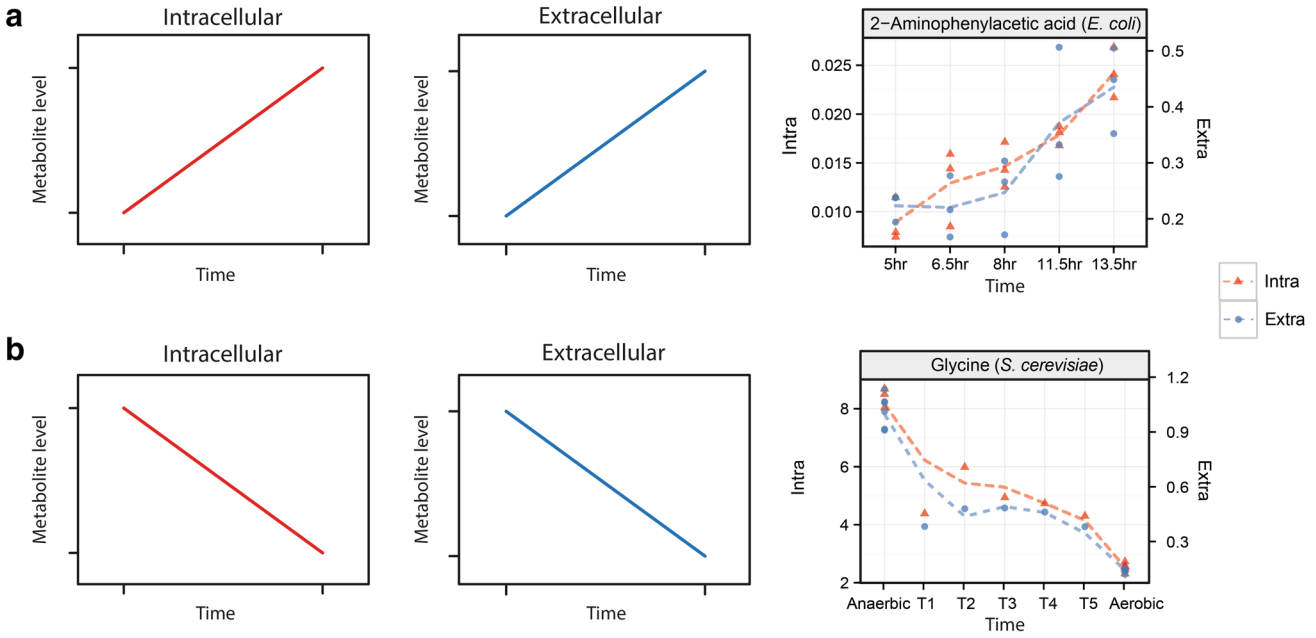

Fig. 3 Mirroring relationship patterns between intracellular and extracellular metabolite levels. **a** The pattern of a typical metabolic overflow where a metabolite level increases both intracellularly and extracellularly over time (e.g. 2-phenylacetic acid in *Escherichia coli* from Carneiro et al. 2012). **b** The pattern of concomitant decrease in level of a metabolite in both extracellular and intracellular samples (e.g. glycine in *Saccharomyces cerevisiae* from Granucci et al. 2015)

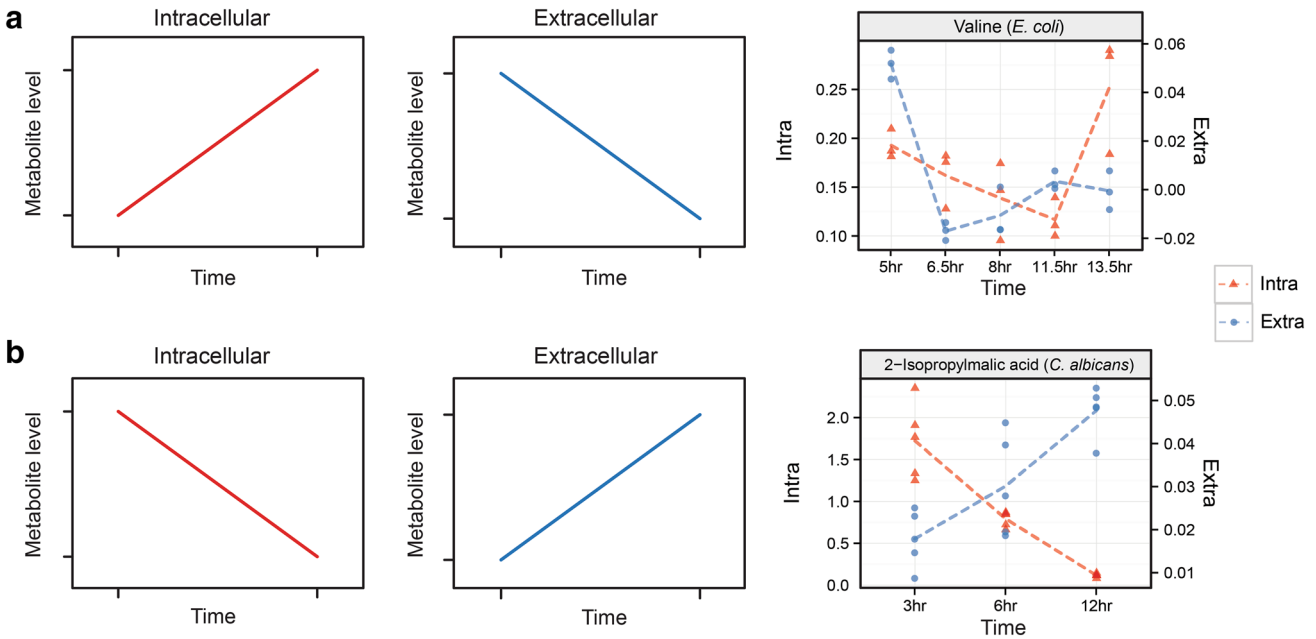

Fig. 4 Opposite patterns of level changing between intracellular and extracellular metabolites. **a** The increase in the intracellular level of a metabolite while its extracellular level decreases (e.g. valine in *Escherichia coli* from Carneiro et al. 2012). **b** A decrease of a metabolite level intracellularly with concomitant increase of its level extracellularly (e.g. 2-isopropylmalic acid in *Candida albicans* from Han et al. 2013)

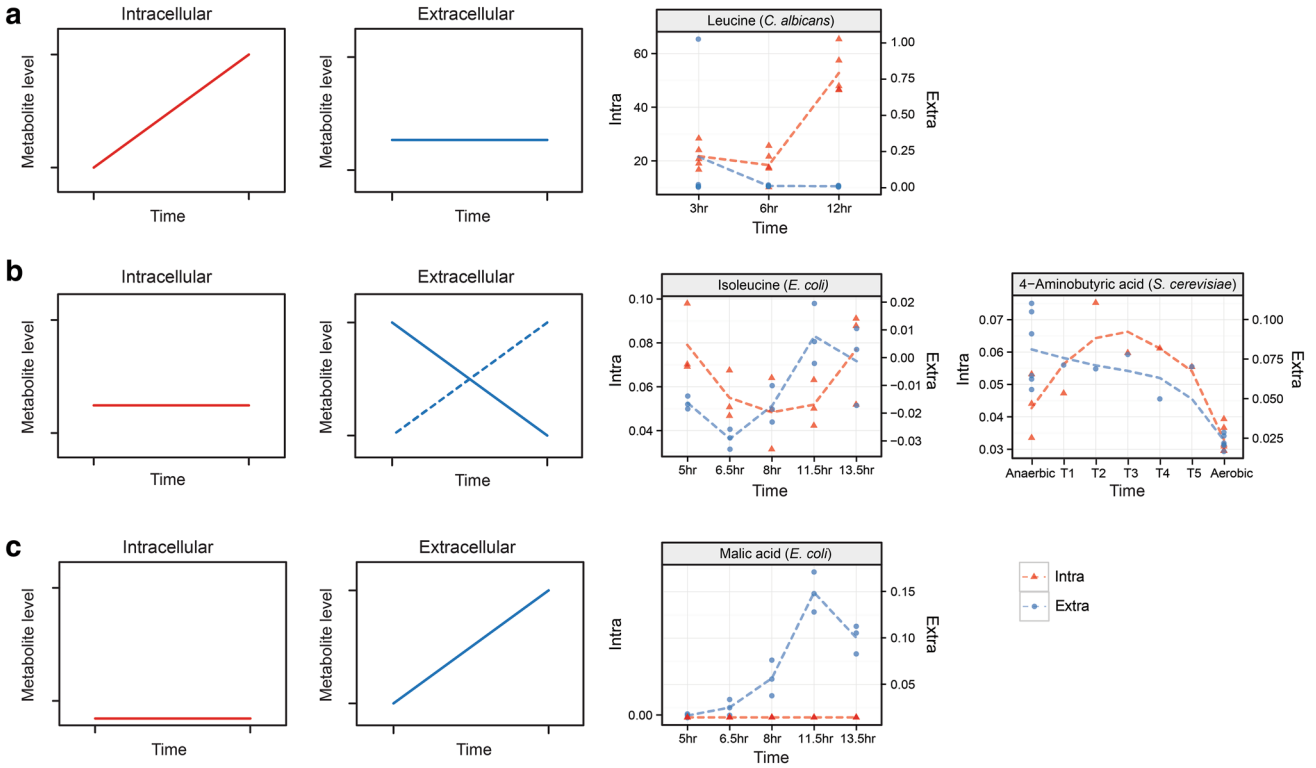

Fig. 5 Other relationship patterns between intracellular and extracellular metabolite levels. **a** The pattern when a metabolite level increases intracellularly with no significant change in its extracellular level (e.g. leucine levels in *Candida albicans* from Han et al. 2013). **b** The pattern when a metabolite level shows no significant change intracellularly, but its extracellular level either increases (dashed line) or decreases (solid line) (e.g. isoleucine in *Escherichia coli* from Carneiro et al. 2012 and 4-aminobutyric acid in *Saccharomyces cerevisiae* from; Granucci et al. 2015). **c** When a metabolite is detected only in the extracellular medium (e.g. malic acid in *Escherichia coli* from Carneiro et al. 2012)

4 Key relationship patterns between intra and extracellular metabolites based on time-series metabolomics experiments

Recent developments in metabolomics mean that comprehensive metabolite profiles of spent culture media are now possible. Exometabolomics, or metabolic footprinting (Allen et al. 2003), has been widely used to study microbial physiology and to characterize microbial phenotypes (Chumnanpuen et al. 2014; Kell et al. 2005; Villas-Bôas et al. 2008). A range of primary metabolites has been detected extracellularly from microbial growth in chemically defined media (reviewed in Pinu and Villas-Boas 2017). It is important to exclude the possibility that these metabolites are technical artefacts or result from cell damage or lysis. However, it has been convincingly demonstrated that the presence of these metabolites is the result of secretion (Paczia et al. 2012).

Recently, it has been proposed that extracellular metabolite profile data could be used to predict intracellular metabolic state (Fu et al. 2014; Mo et al. 2009; Aurich et al. 2014). It is noteworthy that most exometabolome studies are based on a single sampling point and/or lack the parallel measurements of intracellular metabolites (Paczia et al. 2012; Fu et al. 2014; Villas-Bôas et al. 2006). Therefore, we need to better characterize the relationship between intracellular and extracellular metabolite levels, the different ways they can be secreted, and the regulatory mechanisms involved in this process, before we can design accurate ways to predict the metabolic state of cells based on extracellular data alone. Time-series metabolomics data of both intracellular and extracellular metabolites can be a very useful dataset to be employed to achieve this goal; however, there are very few published studies in this format (Han et al. 2013; Carneiro et al. 2011; Granucci et al. 2015; Willemsen et al. 2015; Wiebe et al. 2008). Below, we highlight the different relationship patterns between intracellular and extracellular metabolites based on some published time-series metabolomics data (Supplementary Figs. 1–3).

Based on the time-series metabolomics data of *Candida albicans* (Han et al. 2013), *E. coli* (Carneiro et al. 2011), and *S. cerevisiae* (Granucci et al. 2015) cultures, we confirmed the possibility of seven basic relationship patterns between intracellular and extracellular metabolite levels, which are summarized schematically in Figs. 3, 4, 5. Often

changes in the extracellular level of a metabolite mirror the changes happening inside the cell (Fig. 3). If the metabolite level increases both intracellularly and extracellularly, it characterizes a typical process of metabolic overflow (Fig. 3a). However, if a metabolite level decreases both intracellularly and extracellularly, it represents a reduction in its biosynthesis or increase in its intracellular turnover rate concomitantly with an increase in its uptake from the extracellular medium (Fig. 3b). On the other hand, the pattern of change in metabolite level may follow opposite directions when comparing intracellular and extracellular samples (Fig. 4). If a metabolite level increases intracellularly and decreases in the extracellular medium, it represents a typical case of metabolite uptake (Fig. 4a). But, when its level decreases intracellularly and increases in the extracellular medium, it gives strong evidence of metabolite efflux without a clear intracellular metabolic overflow (Fig. 4b). Nevertheless, frequently the level of a metabolite vary significantly inside the cell without affecting its extracellular level (Fig. 5a), suggesting an active regulation of its secretion mechanism. Conversely, the level of a metabolite may vary significantly in the extracellular medium without affecting its intracellular levels (Fig. 5b), which could result from the tight regulation of its intracellular level by the cell. The most puzzling scenario is when a metabolite produced inside the cell is detected only in the extracellular medium and its level decrease or even increase over time without it being found at detectable levels in intracellular samples (Fig. 5c). We speculate this could represent metabolites that are slowly uptaken and quickly metabolized inside the cells due to high concentration of enzymes and other metabolites (Fig. 5c dashed blue line), or they are secreted as a result of strong transporter affinity for the compounds over intracellular metabolic enzymes (Fig. 5c, full blue line). Alternatively, one cannot rule out the possibility of some metabolic reactions to take place outside the cells spontaneously or catalyzed by secreted or membrane-embedded enzymes. Nonetheless, these different patterns of metabolite secretion clearly illustrate that the change in extracellular media does not always reflect similar changes intracellularly. We also need to take into account to those out of equilibrium reactions that usually tend to have high flux control coefficient. To determine the effects of such pathway reactions on flows and on metabolite concentrations quantitatively, metabolic control analysis (MCA) has widely been used as a tool (Fell 1992; Kell and Westerhoff 1986; Moreno-Sanchez et al. 2008). Therefore, the combination of intra and extracellular metabolomics data and MCA could allow us to acquire in depth knowledge on overall metabolism of microbial cells.

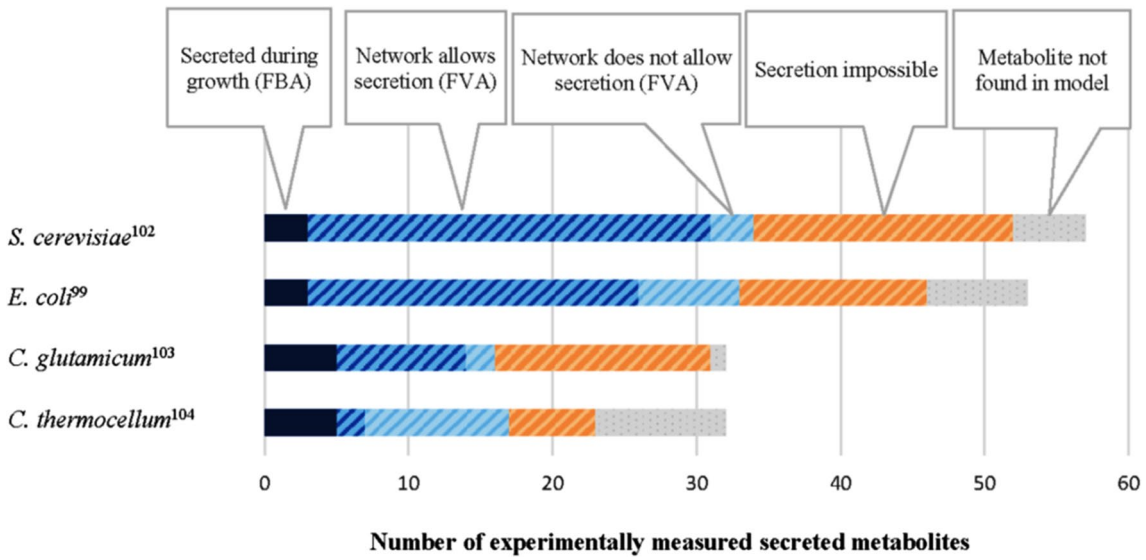

Fig. 6 Metabolite secretion in published *Saccharomyces cerevisiae* (Aung et al. 2013) *Escherichia coli* (Orth et al. 2011) *Corynebacterium glutamicum* (Shinfuku et al. 2009) and *Clostridium thermocellum* (Roberts et al. 2010) genome-scale models. Each bar represents all metabolites reported to have been secreted during growth according to quantitative time-dependent metabolomics analysis. Each bar segment illustrates the ability of the respective genome-scale model to secrete these metabolites. Metabolic models correctly identify the secretion of $<10\%$ of secreted metabolites during growth. Furthermore, a large proportion of metabolites are structurally unable to be secreted by the metabolic network; either flux variability analysis (FVA) indicates their secretion is never possible, or the network lacks a relevant transport reaction. Finally, some secreted metabolites do not exist in the metabolic network. The experimentally measured secreted metabolites assessed in this graph are from targeted metabolomics studies; untargeted studies suggest an even greater diversity of metabolite secretion

5 Impact on metabolic modeling and engineering

Metabolic engineering studies usually only consider the secretion of several compounds. However, these metabolomics studies show that hundreds of compounds are secreted into the culture broth during growth. As well as having a direct impact on metabolic engineering outcomes, their presence in the extracellular environment can provide insight into the metabolic state of a cell (Paczia et al. 2012; Mo et al. 2009; Aurich et al. 2014). Most simply, the secretion of a compound in response to intracellular accumulation suggests the existence of an imbalanced pathway, uncovering the potential metabolic engineering targets. Further insight can be gained when the mode of secretion does not conform to this classic metabolic overflow. For example, the secretion of amino acids may indicate an overabundance of nitrogen in the system, allowing the metabolic engineer to alter the metabolic network to reduce carbon leakage through amino acid secretion. Benzoic acid and hydroxybenzoic acid can be actively secreted independent of their intracellular concentration, likely because of their toxicity; this may inhibit cell growth in a bioreactor. The mechanisms behind the secretion of many metabolites require further study; with greater understanding, these processes can be more precisely identified and modulated in order to achieve a metabolic engineering goal.

Published genome-scale models do not correctly represent metabolite secretion during simulated growth. Metabolic engineers are increasingly utilizing these constraint-based models, which predict metabolic pathway fluxes (Mccloskey et al. 2013). These models link genotype to phenotype and allow microbial strain designs to be tested *in silico* by applying a computational method to a reconstructed metabolic network. Figure 6 summarizes the observed exometabolomes of *S. cerevisiae, E. coli, C. glutamicum* and *Corynebacterium thermocellum* under standard conditions and compares them to the predicted genome-scale model exometabolomes using flux balance analysis (FBA), a technique which predicts steady state metabolite flow based on a biological goal, usually the maximization of growth rate (Orth et al. 2011). In all cases, the model under-represents the diversity of metabolite secretion. For example, while *E. coli* model iJO1366 predicts the secretion of six metabolites, targeted analysis has quantified 34 secreted metabolites (Paczia et al. 2012). In another study, 39 metabolites were secreted and over 200 GC–MS peaks were detected during metabolic footprinting analysis, at least half of which would represent genuine extracellular metabolites based on their unique mass spectra, retention times and experiments with isotope labelled derivatizing reagents (Carneiro et al. 2011). This discrepancy between simulation and reality is because, by definition, only growth-coupled metabolite secretion will contribute to the FBA maximise biomass optimal solution recommended by most authors (Lee et al. 2012; van Berlo et al. 2011). Models can capture the overflow of common products such as acetate, ethanol and lactate because these secretions are linked to growth during simulation, often driven by the redox and energy balance. However, the secretion mechanisms of many primary metabolites under normal growth conditions have not been elucidated and must be investigated to determine if they can be predicted by FBA.

Regardless of the growth simulation method used, models are structurally unable to secrete many metabolites that have been observed extracellularly (Fig. 6). For example, glyceraldehyde-3-phosphate, dihydroxyacetone phosphate and phosphoenolpyruvate are secreted by *E. coli* (Paczia et al. 2012), but cannot be secreted by model iJO1366 under any condition because it lacks the relevant transport reactions (Orth et al. 2010). This omission is common across published models; none of the 93 models reviewed have the ability to secrete phosphoenolpyruvate, likely because there is no annotated transporter for this compound in any of the major reaction databases. Furthermore, some secreted compounds do not exist at all in the metabolic network reconstruction. For example, itaconate, malonate, 2-phenylglycine and benzoate can be secreted by *E. coli*, but reactions to form these are not defined in any of the relevant metabolic reconstructions or public databases (including KEGG and EcoCyc) (Carneiro et al. 2011). The solution is to incorporate reactions which account for these secretions (Mo et al. 2009). However, in some cases these enzymes or transporters do not exist in reaction databases and require elucidation. Transporters and enzymes can act in a non-specific way, catalyzing reactions beyond those they were characterized for (Notebaart et al. 2014; Guzmán et al. 2015). Techniques to identify and incorporate enzyme promiscuity into genome-scale reconstructions may resolve some of these knowledge gaps (Guzmán et al. 2015).

Incorrect metabolite secretions predicted by genome-scale models make up a small proportion of total metabolic flux and the overall carbon balance and their exclusion has little impact on model fitness under standard training conditions. For *E. coli*, these secretions represent $< 1\%$ of substrate carbon yield, with a remaining 6% gap in the experimental carbon balance assumed to be systematic error (Paczia et al. 2012). Model fitness can be calculated using mean relative error to assess predicted secretions and growth rates against experimental measurements (Perez-Garcia et al. 2014). When using this method to calculate model fitness for *E. coli* model iJO1366, the exclusion of these secretions reduces accuracy by 2.13% under normal training conditions. While the output of carbon is incorrectly distributed and leads to a systematic error in predictions, this introduced error is less than overall measurement error and can

be considered a valid compromise when carrying out simulations using growth as an objective function.

However, the ultimate measure of the accuracy of a model is its ability to predict the flux distribution throughout the metabolic network, and recent research indicates that minor secretions can influence the predicted distribution of fluxes within the cell. Secretions can be measured and applied as constraints to a metabolic model to predict internal metabolic states. For example, Mo et al. (2009) apply secretion constraints to a yeast genome-scale model and use a flux sampling approach to successfully predict intracellular metabolic flux distributions. While researchers had previously done this in a limited way, this recent work takes into account a greater number of observed secretions by manually adding and constraining additional exchange reactions (Mo et al. 2009; Aurich et al. 2014). This work demonstrates that the incorporation of these secretions allows intracellular flux states to be predicted more accurately, despite the fact that they represent a small fraction of the overall flux. This technique is interesting because the exometabolome can be readily measured and applied to identify control points and characterize flux in different microbial strains (Aurich et al. 2014). To uncover the full power of this technique, the full exometabolomic profile can be included; however, for this to be done, transporters need to be incorporated into metabolic models in order to capture the full diversity of secretions.

Furthermore, in several metabolic engineering scenarios, the correct representation of metabolic secretion becomes important. One example is the use of genome-scale models to predict metabolic perturbations that increase product yield. Algorithms are used to assess different combinations of gene knockouts and their impact on both growth rate and product formation. If metabolite secretions are not correctly represented, inferior metabolic perturbation strategies may be recommended. For example, the elimination of a competing pathway in vivo may result in a metabolic bottleneck and the leakage of a metabolite previously secreted at low levels. Without the ability to represent this metabolite's secretion, the model will not accurately predict the outcome. Finally, this knowledge can be applied to current models by using data on metabolite secretion to define reference states when using algorithms that predict genetic perturbation. In this instance, techniques such as minimization of metabolic adjustment (MoMA), which find a solution near to the flux distribution of the unperturbed states, are better able to predict suboptimal growth associated with the secretion of metabolites (Segrè et al. 2002).

6 Conclusions

The most accepted theory for the secretion of metabolites is the concept of metabolic overflow (Paczia et al. 2012; Mo et al. 2009). However, time series metabolomics data reviewed here clearly indicate that intracellular metabolic overflow cannot explain the secretion of all intracellular metabolites found in spent culture media. It is not yet possible to propose any species-specific or metabolite-specific rules for secretion. Therefore, more time-series metabolomics studies, mainly involving phylogenetically close species, are necessary to understand better the relationship between intracellular and extracellular metabolite levels. Moreover, a special attention also need to be paid on developing more methods for acquiring quantitative data and also on better experimental design. We also recommend that the metabolic engineering community takes into consideration both the large number of secretions which can occur during the different phases of microbial growth and the unique mechanisms through which these can occur, often not conforming to the standard theory of metabolic overflow. As well as having a direct impact on metabolic engineering outcomes, their presence in the extracellular environment can provide insight into the metabolic state of a cell. Many mechanisms of secretion still require elucidation before secretion can be predicted by metabolic models. However, the presence of a metabolite in the exometabolome indicates that relevant reactions should be included in the network reconstruction, and these can be constrained using intracellular and extracellular metabolomics data to predict intracellular flux states. Future study should focus on identifying these mechanisms, and their fit within the framework of predictive constraint-based modeling techniques, to allow predictions to be made. Within these scenarios transport fluxes can be quantified using ^{13}C-labelling studies.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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