Pyruvate sensing and transport in *Escherichia coli*

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Contents

Abbreviations

APC   amino acid-polyamine-organocation superfamily

APEC avian pathogenic *Escherichia coli*

ATP   adenosine- 5'-triphosphate

CA   catalytic and ATP binding domain

cAMP cyclic adenosine-5'-monophosphate

CM   cytoplasmic membrane

CP   cytoplasm

DHp   dimerization and histidine phosphotransfer domain

DRaCALA differential radial capillary action of ligand assay

GAF   protein domain in cGMP specific phosphodiesterases, adenylyl cyclases and FhlA

HAMP protein domain in histidine kinases, adenylyl cyclases,methyl accepting proteins and
      phosphatases

HK   histidine kinase

HTH   helix-turn-helix motif

MFS   major facilitator superfamily

OAA   oxaloacetate

OFA   oxalate/formate antiporter family

PAS   <u>P</u>er- <u>A</u>rnt- <u>S</u>im

PP   periplasm

REC   receiver domain

RR   response regulator

TCA   tricarboxyacid

TCS   two-component system

TM   transmembrane domain

UPEC uropathogenic *Escherichia coli*

wHTH winged helix-turn-helix motif

Publications and manuscripts originating from this thesis

Chapter 2:

Behr, S., **Kristoficova, I.**, Witting, M., Breland, E. J., Eberly, A. R., Sachs, C., Schmitt-Kopplin, P., Hadjifrangiskou, M., Jung, K. (2017). Identification of a high-affinity pyruvate receptor in *Escherichia coli.* Sci Rep, 7, 1388. http://doi.org/10.1038/s41598-017-01410-2

Chapter 3:

Kristoficova, I., Vilhena, C., Behr, S., Jung, K. (2017). BtsT - a novel and specific pyruvate/H$^+$ symporter in *Escherichia coli.* J Bacteriol, in press. http://doi.org/10.1128/JB.00599-17

Chapter 4:

Vilhena, C., Kaganovitch, E., Shin, JY., Grünberger, A., Behr, S., **Kristoficova, I.**, Brameyer, S., Kohlheyer, D., Jung, K. (2017). A single-cell view of the BtsSR/YpdAB pyruvate sensing network in *Escherichia coli* and its biological relevance. J Bacteriol, in press. http://doi.org/10.1128/JB.00536-17

Contributions to publications presented in this thesis

Chapter 2:

Stefan Behr, Michael Wittig, Philippe Schmitt-Kopplin, Maria Hadjifrangiskou and Kirsten Jung designed the experiments. Stefan Behr and Corinna Sachs performed in vivo expression studies. Ivica Kristoficova produced the proteins in membrane vesicles as well as right-side-out vesicles, and conducted protein-ligand interaction studies. Michael Wittig carried out the hydrophilic interaction liquid chromatography for determination of compounds concentration. Erin Breland and Allison R. Eberly performed the murine infections. Stefan Behr, Ivica Kristoficova, Maria Hadjifrangiskou and Kirsten Jung wrote the manuscript.

Chapter 3:

Ivica Kristoficova, Cláudia Vilhena, Stefan Behr and Kirsten Jung designed the experiments. Stefan Behr performed comparative genomic studies. Ivica Kristoficova carried out the transport measurements with intact cells and proteoliposomes. Ivica Kristoficova and Cláudia Vilhena conceptually developed experimental conditions for transport assays. Ivica Kristoficova and Kirsten Jung wrote the manuscript.

Chapter 4:

Cláudia Vilhena, Jae Yen Shin, Stefan Behr, and Kirsten Jung designed the experiments. Cláudia Vilhena performed all the experimental work presented on the publication. Cláudia Vilhena and Jae Yen Shin performed statistical analysis of the data. Ivica Kristoficova drew the model. Ivica Kristoficova and Cláudia Vilhena performed reporter assays for the elucidation of the crosstalk between the systems during the development of the manuscript. Eugen Kaganovitch, Alexander Grünberger and Dietrich Kohlheyer help conceptualizing microfluidic experiments during the development of the manuscript. Sophie Brameyer performed plasmid-based experiments during the development of the manuscript. Cláudia Vilhena,

Eugen Kaganovitch, Jae Yen Shin, Alexander Grünberger, Stefan Behr, Ivica Kristoficova, Sophie Brameyer, Dietrich Kohlheyer and Kirsten Jung wrote and corrected the manuscript.

We hereby confirm the above statements:

Ivica Kristoficova   Kirsten Jung

Summary

Two-component systems (TCS) represent a prevalent bacterial mechanism to respond to changing environmental conditions. They comprise an often membrane-integrated histidine kinase (HK) and a soluble response regulator (RR), which regulates target genes expression. The TCS YehU/YehT is found in all commensal and pathogenic representatives within the *Enterobacteriaceae*. In *Escherichia coli* the TCS YehU/YehT contributes to carbon scavenging before entry into stationary phase. The system belongs to the LytS/LytTR family and regulates the expression of *yjiY*, encoding a putative peptide transporter belonging to the CstA family and APC superfamily of secondary transporters. This thesis focuses on the functional characterization of the components involved in the TCS YehU/YehT.

In the first part, the chemical stimulus perceived by the TCS YehU/YehT has been investigated (Chapter 2). It was found out that activation of the TCS YehU/YehT is dependent on the concomitant nutrient starvation and the extracellular availability of pyruvate. Via hydrophilic liquid chromatography, it was demonstrated the extracellular pyruvate is generated from overflow metabolism. Furthermore, the extracellular binding of pyruvate to YehU in vitro was proved by a differential radial capillary action of ligand assay (DRaCALA) and it indeed showed that YehU is a high affinity sensor for extracellular pyruvate.

The second part of the study focused on the identification of the function of the transporter YjiY (Chapter 3). YjiY is composed of 18 transmembrane helices, and no representative of the CstA family has been functionally characterized thus far. Transport studies with intact cells provided first evidence that YjiY is a specific transporter for pyruvate. Furthermore, reconstitution of the purified YjiY into proteoliposomes revealed that YjiY is a pyruvate/H^+ symporter.

In the third part, we aimed at understanding the biological relevance of the TCS YehU/YehT (Chapter 4). A single-cell analysis of the *yjiY* activation demonstrated cell-to-cell variability that is influenced by external pyruvate availability upon nutrient limiting conditions. Depending on the demand of the individual cells, the TCS YehU/YehT as a part of a functional network with a TCS YpdA/YpdB, ensures an optimization of the physiological state of all cells within the population to withstand upcoming metabolic stress.

In summary, this thesis identified the TCS YehU/YehT to be involved in pyruvate sensing

and transport in order to optimize the physiological state within the whole population of *E. coli*. To indicate their role, the HK YehU, the RR YehT and the transporter YjiY were renamed to BtsS, BtsR and BtsT, for <u>B</u>renz<u>t</u>rauben<u>s</u>äure, respectively.

Zusammenfassung

Zwei-Komponenten-Systeme (TCS) stellen einen weit verbreiteten bakteriellen Signaltrans-duktion-mechanismus dar, um auf wechselnde Umweltbedingungen zu reagieren. TCSs be-stehen meinst aus einer Membran-integrierten Histidinkinase (HK) und einem löslichen Ant-wortregulator (response regulator, RR), der die Expression von Zielgenen reguliert. Das TCS YehU/YehT ist in allen kommensalen und pathogenen Vertretern innerhalb der *Enterob-acteriaceae* vorhanden. In *Escherichia coli* trägt das TCS YehU/YehT vor dem Eintritt in die stationäre Phase zur Aufnahme von Kohlenstoff verbindungen bei. Das System gehört zur LytS/LytTR-Familie und reguliert die Expression von *yjiY*, welches einen mutmaßlichen Peptidtransporter der CstA-Familie und der APC-Superfamilie von Sekundärtransportern kodiert. Diese Arbeit konzentriert sich auf die Aufklärung der Funktion der beteiligten Kom-ponenten des TCSs YehU/YehT.

Im ersten Teil wurde der durch das TCS YehU/YehT wahrgenommene chemische Reiz untersucht (Kapitel 2). Es wurde gezeigt, dass die Aktivierung des TCS YehU/YehT gleich-zeitig von Nährstoffmangel und der extrazellulären Verfügbarkeit von Pyruvat abhängig ist. Mittels hydrophiler Flüssigkeitschromatographie wurde gezeigt, dass das extrazelluläre Py-ruvat ein Produkt des Überflussmetabolismus ist. Darüber hinaus wurde die extrazelluläre Bindung von Pyruvat in vitro durch den Liganden-Assay DRaCALA (differential radial ca-pillary action of ligand assay) nachgewiesen, und es zeigte sich tatsächlich, dass YehU ein hochaffiner Sensor für die extrazelluläre Pyruvatkonzentration ist.

Der zweite Teil der Studie konzentrierte sich auf die Aufklärung der Funktion des Trans-porters YjiY (Kapitel 3). YjiY besteht aus 18 Transmembran-Helices, und bisher wurde noch kein Vertreter der CstA-Familie funktionell charakterisiert. Transportstudien mit intakten Zellen lieferten erste Hinweise darauf, dass YjiY ein spezifischer Transporter für Pyruvat ist. Darüber hinaus zeigte die Rekonstitution des gereinigten YjiY in Proteoliposomen, dass YjiY ein Pyruvat/H^+ Symporter ist.

Im dritten Teil wollten wir die biologische Relevanz des TCS YehU/YehT (Kapitel 4) verstehen. Eine Einzelzellanalyse der *yjiY*-Aktivierung zeigte eine Zell-zu-Zell-Variabilität, die von der externen Pyruvat-konzentration beeinflusst wird. Das TCS YehU / YehT sorgt als Teil eines funktionellen Netzwerks mit TCS YpdA/YpdB je nach Bedarf der einzelnen

Zellen für eine Optimierung des physiologischen Zustands aller Zellen in der Population, um dem anstehenden Stoffwechselstress standzuhalten.

Zusammenfassend wurde in dieser Arbeit festgestellt, dass TCS YehU/YehT am Detektion und Transport von Pyruvate beteiligt ist, um den physiologischen Zustand innerhalb der gesamten Population von *E. coli* zu optimieren. Um die Funktion der HK YehU, des RRs YehT und des Transporters YjiY im Namen zu veranhern, wurden diese Proteine in BtsS, BtsR und BtsT, für Brenztraubensäure, umbenannt.

Chapter 1

Introduction

Bacterial diversity is driven by an almost unlimited number of different environmental habitats where bacteria experience various fluctuations in physical and chemical parameters. In order to colonize, replicate and survive, bacteria need to respond to e.g. limitations of carbon and nitrogen or pH- and osmostress. Hence, bacteria evolved strategies for life-threatening situations by adjusting their metabolism to match the resource availability.

Prokaryotes are capable of persisting in diverse environments thanks to **signal transduction systems** (Boor, 2006) and fast cellular response mechanisms, e.g. acid (Haneburger *et al.*, 2012) or heat shock (Schumann, 2012). They as well depend on synchronizing processes, like biofilm formation (Njoroge and Sperandio, 2009), bioluminescence (Anetzberger *et al.*, 2012) and the expression of virulence genes (Rumbaugh *et al.*, 2009).

The simplest and the most predominant form of the bacterial signal transduction represent an one-component system (Ulrich *et al.*, 2005). One single protein contains both input and output domains. Another type of the signaling mechanism is an extracytoplasmic function σ factor with its cognate anti σ factor (Staron *et al.*, 2009). Last but not least, the signal transduction is achieved by **two-component systems** (TCS), which comprise an often membrane-integrated histidine kinase (HK) and a soluble response regulator (RR) (Jung *et al.*, 2012). The HK senses an intra- and/or extracellular stimulus and transfers the information to the cognate RR. Upon activation, the RR mediates the cellular reaction, mainly by adjusting a gene expression profile.

1.1 Two-component systems

TCSs are one of the most widespread transducers of extracellular stimuli in prokaryotes and lower eukaryotes. The number of HK/RR systems differs enormously from species to species, and correlates with the genome size (larger genome encode more TCSs) (Beier and Gross, 2006) as well as the number of different environmental and ecological niches (Alm *et al.*, 2006). Thereby, the number of HK/RR ranges from zero in *Mycoplasma genitalium*, an intracellular parasite living in constant environment, over 36/34 in *Bacillus subtilis* to 136/127 in *Myxococcus xanthus*, an extreme case of a bacterium inhibiting rapidly changing environments (Heermann and Jung, 2010; Capra and Laub, 2012). TCSs occur also in eukaryotes, as one TCS has been found in the yeast *Saccharomyces cerevisiae* and four in the plant *Arabidopsis thaliana* (Barrett and Hoch, 1998). Interestingly, TCSs are as a whole absent in the animal kingdom, as yet no gene encoding a HK or a RR is present in the genome of *Drosophila melanogaster*, *Homo sapiens* or any other animal cell (Wolanin *et al.*, 2002).

In the genome of *Escherichia coli*, the most investigated prokaryote, there are described 30 HKs and 30 RRs (**Fig. 1.1**). Recent years have seen an expansion of research about many of these TCSs however some still await characterization of their stimulus or their function. So far the well-characterized TCSs are e.g. a TCS CheA/CheY in the context of the bacterial chemotaxis (Thakor *et al.*, 2011), a TCS KdpD/KdpE with its importance in keeping potassium balance (Schramke *et al.*, 2017) and a TCS NarQ/NarP with its role in nitrate/nitrite sensing (Gushchin *et al.*, 2017).

Since TCSs are highly homologous between organisms (Grebe and Stock, 1999), the research of various TCSs are of a great importance. And so the basic principles of the signal transduction are providing us with further insights into bacterial virulence and pathogenicity (Gotoh *et al.*, 2010). For example, the TCS AgrA/AgrC is involved in regulation of *Staphylococcus aureus* virulence (Novick, 2003). As far as the future perspectives of TCSs are concerned, metabolic engineering would like to use TCSs to develop novel genetic networks (Salis *et al.*, 2009) and pharmacology to produce novel antimicrobial agents (Gotoh *et al.*, 2010).

Figure 1.1: An overview of all two-component systems in *E. coli*. The picture depicts all known histidine kinases, their cognate response regulators, activating stimuli and generated responses. Made by Kristoficova and Vilhena, 2017.

1.2   Signal transduction in histidine kinases

The major role of HKs is to recognize, integrate and amplify the external signals deriving from an environmental or cellular cue. Upon stimulus perception, a HK catalyzes an ATP-dependent autophosphorylation on a conserved histidine residue. Then, the HK transfers the phosphoryl group from His~P to a conserved aspartate residue of the RR.

HKs are prototypically composed of two structurally and functionally distinct parts: a N-terminal sensor domain and a conserved C-terminal transmitter domain (**Fig. 1.2**) (Grebe and Stock, 1999). The N-terminal sensor or so called the input domain is a very diverse part consisting of up to 13 transmembrane domains (TM) (Galperin, 2005). According to the site of the signal perception, HKs are divided into three major categories: (i) periplasmic sensing HKs in which the stimulus is perceived by the large extracellular loop flanked by two TMs, e.g. a C4 dicarboxylate sensor DcuS; (ii) transmembrane sensing HKs that are

usually composed of six TMs recognizing either a membrane interface (DesK) or create a hydrophobic pocket for signal binding (AgrC); (iii) cytoplasmic sensing HKs, both soluble and membrane integrated, responding to an intracellular signal (Krell *et al.*, 2010), (Mascher, 2014).
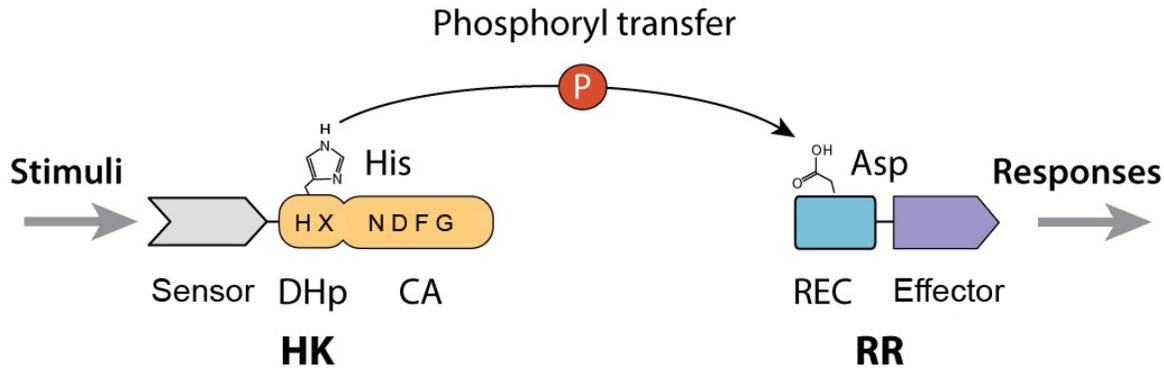
Figure 1.2: Signal transduction by two-component systems. Histidine kinase (HK) perceives the extra- or intracellular stimulus that results in the autophosphorylation of a conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartate residue of the response regulator (RR) which in turn mediates the cellular response. The picture summarizes the domain architecture of HK (sensor and transmitter (DHp and CA) domain) and RR (receiver (REC) and effector domain). Conserved amino residues are written in an one-letter-code. Figure adopted and modified from (Gao and Stock, 2009).

The C-terminal transmitter domain is composed of a <u>d</u>imerization and <u>h</u>istidine <u>p</u>hospho-transfer domain (DHp, PFAM: HisKA) and the <u>c</u>atalytic and <u>A</u>TP binding domain (CA, PFAM:HATPase_c) that are connected by a linker region (**Fig. 1.2**). The DHp domain is usually the site of homodimerization and harbors a conserved histidine residue that represents a phosphorylation site. The CA domain has a catalytic activity to autophosphorylate the HK using the γ-phosphate of ATP. Additionally, CA contains the highly conserved N, D, F and G boxes with corresponding amino acid residues, that are essential in ATP binding, catalysis and phosphotransfer (Stewart, 2010) (Wolanin *et al.*, 2002).

According to all six different amino acid motifs (H, X, N, D, F and G), the histidine kinases can be grouped in 11 families based on a comparative sequence analysis (Grebe and Stock, 1999). Some of these families contain a very distinct sequence similarity that clearly separates them from all other HKs, e.g. chemotaxis kinases. Other families do not show such clear

boundaries. Interestingly, the kinase subfamilies tend to group with regulator subfamilies; suggesting an independent co-evolution of different classes of His-Asp phosphorelay systems (Grebe and Stock, 1999).

A signal conversion in HKs is sometimes also achieved by additional domains, like HAMP, PAS, S-helices and GAF. They are located at the C-terminal part directly after the transmembrane region (Mascher, 2014). HAMP domain (present in histidine kinases, adenylyl cyclases, methyl accepting proteins and phosphatases) mediates the conversion of an extracellular stimulus into the cytoplasmic response by a conformational change (Barakat *et al.*, 2011). PAS (first discovered in proteins Per- Arnt- Sim) and GAF (present in cGMP specific phosphodiesterases, adenylyl cyclases and FhlA proteins) domains are additional sensor and interaction interfaces (Henry and Crosson, 2011) (Mascher, 2014). Last but not least, S-helices (from signaling helix) are preventing a constitutive activation of downstream signaling domains (Anantharaman *et al.*, 2006).

1.3   Signal transmission by response regulators

The major role of a RR is to transfer the signal received by the HK towards a downstream target, which is in the most cases a promoter sequence of a gene. Thereby, the response regulators usually acts as transcription factors with a DNA binding capability (Mizuno, 1997).

RR harbors two domains, a highly conserved receiver domain (REC) at the N-terminus and a variable effector domain at the C-terminus (**Fig. 1.2**). The REC typically contains the conserved aspartate residue able to acquire the phosphoryl group from the His$\sim$P of the HK. The Asp$\sim$P mediates a conformational change that subsequently effects the activity of the effector domain. In general, effector domains are very diverse. Most of them regulate the transcription of the genes (25 of 32 RR in *E. coli*) and these can be divided into three families represented by OmpR, NarL and NtrC (Stock *et al.*, 2000) (Mizuno, 1997). The OmpR family is the most abundant one with 14 members in *E. coli* that use a winged helix-turn-helix (wHTH) motif to bind DNA. The NarL family consisting of 7 members harbors a typical helix-turn-helix (HTH) motif. The third family, NtrC, has 4 members and its effector part is composed of both an ATPase domain and HTH motif.

1.4 LytS/LytTR-like two-component systems

A detailed understanding of the components, layout and mechanism of bacteria's TCS provides us with an opportunity for bioengineering or disturbing the bacterial pathogenicity. The main advantages of TCSs are a total absence in the animal kingdom, a relatively simple layout and a high degree of homology between organisms. We can therefore focus on constructing novel signaling circuits, e.g to improve production yields or stop the production of virulence factors.

In agreement with this, the search for systems regulating toxin production in several bacterial pathogens started with a genome sequencing. Soon after, an unusual response regulator has been found in *Staphylococcus aureus* and some other pathogens. The RR contains a non-HTH DNA binding domain (rather than HTH or wHTH motif typical in RRs) named as **LytTR** after RR LytT in *B. subtilis* and RR LytR in *S. aureus* (Nikolskaya and Galperin, 2002).

In several bacterial genomes, the gene coding for the LytTR-like RR is in an operon with a gene coding for **LytS**-like HK (Anantharaman and Aravind, 2003). These HKs harbor a typical 5 TM Lyt domain (called also Lyts-Yhck, PFAM: 5MTR-LYT), with the N-terminal helix located in a periplasm. In addition, the 5TM domain is combined with the C-terminal signaling domain, which very often is GAF. According to a bacterial sequence alignment, LytS-like HKs are part of the HPK8 family **Table 1.1**. They contain the conserved histidine residue in their H-box, however proline is found preceding the H-box rather to be 5 residues downstream of the box. The first residue after the H-box is phenylalanine instead of an acidic residue. In addition, N- and F- boxes are not conserved (Grebe and Stock, 1999).

Table 1.1: Selection of LytS-containing histidine kinases (HKs)

HK	Organism	Stimulus	RR	Reference
AlgZ	*Pseudomonas aeruginosa*	unknown	AlgR	(Yu *et al.*, 1997) (Okkotsu *et al.*, 2014)
LytS	*Staphylococcus aureus*, *Bacillus subtilis*	unknown	LytT	(Brunskill and Bayles, 1996)
YehU	*Escherichia coli*	Peptides or amino acids	YehT	(Kraxenberger *et al.*, 2012)
YesM	*Bacillus subtilis*	unknown	YesN	(Fabret *et al.*, 1994)
YpdA	*Escherichia coli*	Extracellular pyruvate	YpdB	(Fried *et al.*, 2013)
YwpD	*Bacillus subtilis*	unknown		

Actually, only around 2.7% proteins of all prokaryotic RRs contain the LytTR motif (Galperin, 2006). Even though this represents just around 1 to 2 proteins per genome, these RRs regulate virulence factors like bacteriocins, fimbriae, toxins and extracellular polysaccharides (**Table 1.2**).

Table 1.2: Selection of characterized LytTR-containing response regulators (RRs)

RR	Organism	Regulated process	Reference
AlgR	*Pseudomonas aeruginosa*	Alginate biosynthesis	(Deretic *et al.*, 1989)
AgrA	*Staphylococcus aureus*	Production of toxins and hemolysins	(Novick *et al.*, 1995)
CbaR	*Carnobacterium piscicola*	Carnobacteriocin A production	(Quadri *et al.*, 1997)
ComE	*Streptococcus pneumoniae*	Competence for genetic transformation	(Pestova *et al.*, 1996)
EntR	*Enterococcus faecium*	Enterocin A production	(O'Keeffe *et al.*, 1999)
FasA	*Streptococcus pyogenes*	Fibronectin-binding adhesin production	(Kreikemeyer *et al.*, 2001)
LytR	*Staphylococcus aureus*	Autolysis	(Brunskill and Bayles, 1996)
MrkE	*Klebsiella pneumoniae*	Expression of type 3 fimbriae	(Allen *et al.*, 1991)
PlnC	*Lactobacillus plantarum*	Plantaricin A production	(Diep *et al.*, 1996)
SppR	*Lactobacillus sakei*	Sakacin P production	(Huhne *et al.*, 1996)
VirR	*Clostridium perfringens*	Production of perfringolysin O, collagenase, hemagglutinin	(Shimizu *et al.*, 1994)
YpdB	*Escherichia coli*	Carbon starvation control	(Behr *et al.*, 2016)

All in all, LytS/LytTR TCSs play a significant role in bacterial virulence and represent a good target to decrease bacterial infections. For example, in *S. aureus* a modification of an autoinducing peptide (a stimulus for HK AgrC) resulted in inhibition of the LytTR-like RR AgrA virulent response (Lyon *et al.*, 2000). Unfortunately, still several LytS/LytTR TCSs are not well characterized and their perceived stimuli by HK are unknown.

1.5 TCS YehU/YehT in *Escherichia coli*

In Gram-negative bacteria, little is known about the LytS/LytTR-like two-component system YehU/YehT in *Escherichia coli*.

The *yehU* and *yehT* genes are clustered in an operon localized at 47.638 centisomes in *E. coli* MG1655 genome (**Fig. 1.3A**) (Kraxenberger *et al.*, 2012). The genes are 4 bp overlapping. Upstream of the coding region of *yehU* is *mlrA*, a regulator responsible of a curli production in *E. coli* (Brown *et al.*, 2001), *yohO* coding for a small unidentified protein (Hemm *et al.*, 2008) and an operon of *osmF-yehYXW* encoding a putative ABC transporter

(Checroun and Gutierrez, 2004). Downstream of *yehT* is *yehS*, *yehR*, *yehQ*, *yehP*, *yehM* of unknown function and *yehL* which defines a MoxR AAA$^+$ family (Snider *et al.*, 2006).

A
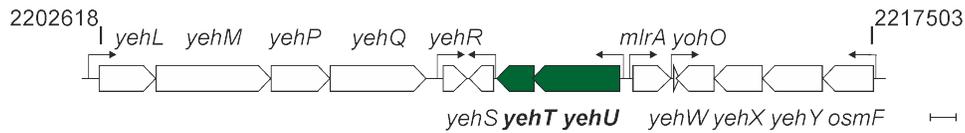
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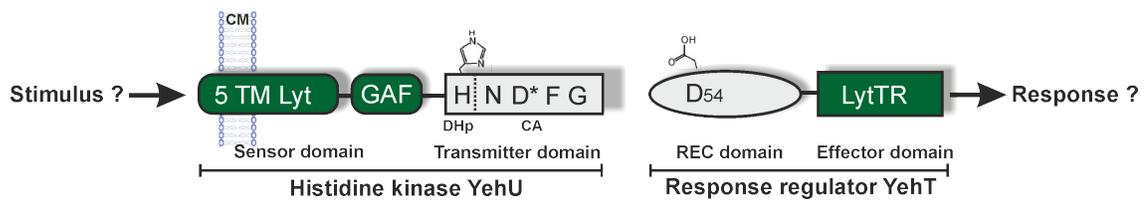
Figure 1.3: The YehU/YehT system in *E. coli*. (A) Chromosomal region between 47.48 and 47.77 centisomes around *yehUT operon* in *E. coli* MG1655. Adopted and modified from (Kraxenberger *et al.*, 2012). (B) Domain organization of YehU and YehT. HK YehU contains a 5TM Lyt domain, GAF domain and transmitter domain with conserved boxes (H, N, D*, F, G). D* box of YehU is not complete. YehT is composed of a receiver domain with the conserved aspartate residue and a LytTR effector domain. CM, cytoplasmic domain. The figure was provided by Stefan Behr, adopted and modified.

The HK YehU (561 amino acids, 62.1 kDa) contains the 5TM Lyt-like sensor domain, transmitter domain and an additional GAF domain (**Fig. 1.3B**). The bioinformatic analysis has revealed an additional transmembrane domain at the N-terminus. The RR YehT (289 amino acids, 27.4 kDa) contains a CheY-like receiver domain with the conserved aspartate residue at position 54 and LytTR effector domain (**Fig. 1.3B**). Up to now, the autophosphorylation of HK YehU or the transfer of the phosphoryl group to YehT was not experimentally detected (Yamamoto *et al.*, 2005).

The YehT RR activates the expression of *yjiY*, which is the only target regulated by the YehU/YehT TCS (Kraxenberger *et al.*, 2012). In *E. coli* MG1655 genome, *yjiY* is located at 98.87 centisomes disconnected from the *yehUT* operon. Interestingly, in other bacterial genomes (e.g. *Shewanella*, *Clostridium* and *Vibrio* species), the *yjiY* is colocalized with the *yehUT* operon. It was determined that YehT binds with the K_d of 75 nM to the *yjiY* promoter

within positions -112 and -13 upstream of the transcriptional start site of $yjiY$. Specifically, the binding occurs at two repeats of the sequence motif ACC(G/A)CT(C/T)A separated by a 13 bp spacer in the promoter of $yjiY$. The third repeat motif of the same sequence probably stabilize the YehT-DNA complex (Kraxenberger et $al.$, 2012).

In vivo reporter studies revealed that the activation $yjiY$ promoter occurs at the mid- to late- exponential phase in amino acid rich media, such as LB, peptone and casamino acids (**Fig. 1.4**). Furthermore, the $yjiY$ promoter activation was observed in minimal media containing amino acids, acetate, glucuronic acid, gluconic acid or pyruvate as a carbon source (Kraxenberger et $al.$, 2012). At the same time as activation of the $yjiY$ promoter is observed ($OD_{600} = 0.6$), the cells initiate production of $yjiY$ mRNAs (Behr et $al.$, 2014).
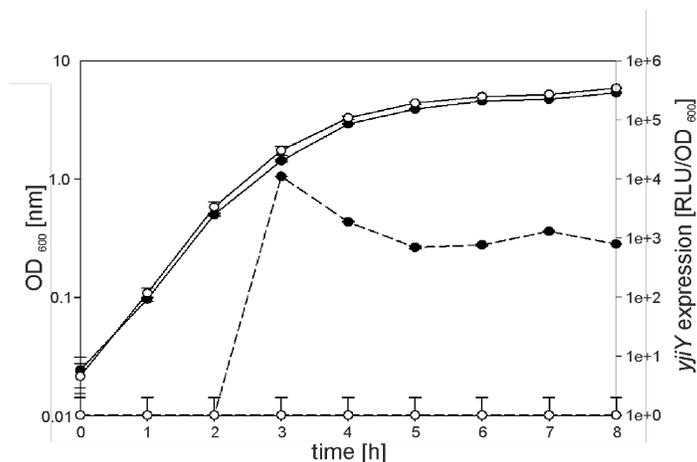
Figure 1.4: *In vivo* reporter assay of $yjiY$ promoter activation. *E. coli* MG1655 (●) and *E. coli* MG1655 $\Delta yehUT$ (○) transformed with pBBR $yjiY$-*lux* were grown in LB, and their growth and luminescence were recorded over time. Figure was adopted from (Kraxenberger et $al.$, 2012).

The expression of $yjiY$ requires cAMP and cAMP receptor protein (CRP). The CRP consensus sequence is upstream of the $yjiY$ promoter. The mutation of this motif or crp deletion reduced the induction of $yjiY$ (Kraxenberger et $al.$, 2012). Additionally, the carbon storage regulator A (CsrA), a protein blocking the ribosome binding to mRNA or influencing mRNA stability, is downregulating the expression of $yjiY$ at the posttranscriptional level (Kraxenberger et $al.$, 2012). The levels of $yjiY$ mRNA are dependent also on the 50S ribosomal protein L4 and the endonuclease RNase E (Singh et $al.$, 2009). Interaction between L4 protein and RNase E on *E. coli* transcripts leads to an increase of $yjiY$ mRNA.

1.6 Transporter YjiY of *Escherichia coli*

The target gene of the TCS YehU/YehT *yjiY* codes for a putative transporter with 16 to 18 predicted TMs of yet unknown function.

A bacterial membrane is highly permeable for small molecules (e.g. water, N_2, O_2) and apolar solutes (e.g. glycerol). However, transporters in *E. coli* play an important role in metabolism due to impermeability of the bacterial membrane for polar solutes and charged molecules (e.g. ions and amino acids (Krämer, 1999)). They maintain balance between availability (influx) and loss (efflux) of the substrates. An energy-independent transport, a facilitated diffusion, is catalyzed by channels. Active transport is driven by cell energy and there are three types: primary (ATP), secondary (ions/solute gradients) and group translocation systems (phosphoryl group).

YjiY belongs to the amino acid-polyamine-organocation (APC) superfamily of secondary transporters (Wong *et al.*, 2012; Vastermark *et al.*, 2014). APC contains 18 families and most of their members exhibit a common 5+5 TMs topology (**Table 1.3**).

Table 1.3: Established families in the APC superfamily. Adopted from (Vastermark *et al.*, 2014).

TC#	Family name	Abbreviation	Topology
2.A.3	Amino Acid-Polyamine-Organocation	APC	5+5+2 TMs
2.A.15	Betaine/Carnitine/Choline Transporter	BCCT	2+5+5 TMs
2.A.18	Amino Acid/Auxin Permease	AAAP	11 TMs
2.A.21	Solute:Sodium Symporter	SSS	5+5+4 TMs
2.A.22	Neurotransmitter:Sodium Symporter	NSS	5+5+2 TMs
2.A.25	Alanine or Glycine:Cation Symporter	AGCS	11 TMs
2.A.26	Branched Chain Amino Acid:Cation Symporter	LIVCS	5+5+2 TMs
2.A.30	Cation-Chloride Cotransporter	CCC	12 TMs
2.A.31	Anion Exchanger	AE	7+7 TMs
2.A.39	Nucleobase:Cation Symporter-1	NCS1	5+5+2 TMs
2.A.40	Nucleobase:Cation Symporter-2	NCS2	7+7 TMs
2.A.42	Hydroxy/Aromatic Amino Acid Permease	HAAAP	11 TMs
2.A.46	Benzoate:H^+ Symporter	BenE	7+7 TMs
2.A.53	Sulfate Permease	SulP	8-13 TMs
2.A.55	Metal Ion (Mn^{2+}-iron) Transporter	NRAMP	5+5+2 TMs
2.A.72	K^+ Uptake Permease	KUP	5+5+2 TMs
2.A.114	Peptide Transporter Carbon Starvation CstA	CstA	13 - 18 TMs
2.A.120	Putative Amino Acid Permease	PAAP	5+5 TMs

APC is the second largest family of secondary transporters, being smaller only than the Major Facilitator Superfamily (MFS). Functionally characterized transporters of APC superfamily transport amino acids, peptides, inorganic anions or cations (Vastermark *et al.*, 2014).

Furthermore, YjiY belongs to the Peptide Transporter Carbon Starvation CstA family, consisting of proteins of various sizes and topologies. None of them has been biochemically analyzed. Characterized member of this family, the protein CstA, is postulated to be a peptide transporter. The evidence is derived from an observed lower growth rate on peptides of *cstA opp* double mutant compared to *opp* mutant (Schultz and Matin, 1991). In *Campylobacter jejuni*, the *cstA* mutant had a lower growth rate on peptides as nitrogen sources (Rasmussen *et al.*, 2013). In *Salmonella enterica*, the function as a peptide transporter is proposed to both CstA and YjiY (Garai *et al.*, 2015).

1.7 Role of the TCS YehU/YehT

It is worth to mention, that the TCS YehU/YehT is functionally interconnected with the LytS/LytTR-like TCS named YpdA/YpdB (Behr *et al.*, 2014). Both HKs and RRs share the same arrangement of structural domains and their amino acid sequences are over 30% identical (Behr *et al.*, 2017). YpdA/YpdB system responds to media supplemented with pyruvate (Fried *et al.*, 2013). The only target gene of the YpdA/YpdB TCS is *yhjX*, encoding the transporter YhjX, belonging to the the oxalate/formate antiporter (OFA) family and MFS. However, the function of YhjX is not yet elucidated.

It was proposed that these two TCSs, YehU/YehT and YpdA/YpdB, coordinate carbon scavenging and adjustments of bacterial metabolism in preparation for stationary phase (Behr *et al.*, 2014). The transporters YjiY and YhjX replenish carbon resources in order to circumvent the nutrient limitation, e.g. by taking up peptides/amino acids or other carbon sources from available resources. In order to get further insights in the role of both TCSs in *E. coli*, the stimuli of both histidine kinases and function of the regulated transporters need to be addressed. This thesis focuses primarily on the function of the TCS YehU/YehT and its target protein YjiY.

1.8 Aims of this thesis

Two-component systems in prokaryotes and lower eukaryotes have been investigated over 30 years. At the moment, many members in bacterial genomes have been identified, and their molecular details are being described. Despite all the new information, there is still a gap to be filled up. This project focuses on LytS/LytTR-like two component-system YehU/YehT in *E. coli*. The aim is to unravel the function of the TCS YehU/YehT and its target protein YjiY by *in vivo* and *in vitro* studies.

i **Characterization of stimulus perceived by HK YehU**

Based on the previous *in vivo* studies, it is suggested that YehU is an amino acid sensor. However, it is unclear, if the HK works as a direct sensor, and if so, which ligand is binding to it. After elucidating the stimulus identity by additional *in vivo* studies, a ligand binding assay will be carried out.

ii **Identification of the substrate transported by the CstA-like transporter YjiY**

The YehU/YehT system controls expression of *yjiY*, which codes for a transporter belonging to CstA family and APC superfamily of secondary transporters. It is proposed that YjiY is a peptide transporter, however without further characterization. Here, the substrate will be identified and then transport assays will be performed to determine the substrate specificity and mode of energization.

iii **Investigation of the biological relevance of the TCS YehU/YehT**

The promoter of the target gene *yjiY* will be analyzed at the single-cell level to obtain further insights into the biological significance of the TCS YehU/YehT. Then the different cellular fates (ranging from growing to dormant persister cells) will be analyzed.

Chapter 2

Identification of a high-affinity pyruvate receptor in *Escherichia coli*

Behr, S., **Kristoficova, I.**, Witting, M., Breland, E. J., Eberly, A. R., Sachs, C., Schmitt-Kopplin, P., Hadjifrangiskou, M., Jung, K.

SCIENTIFIC REP⚙RTS

Identification of a High-Affinity Pyruvate Receptor in *Escherichia coli*

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Two-component systems are crucial for signal perception and modulation of bacterial behavior. Nevertheless, to date, very few ligands have been identified that directly interact with histidine kinases. The histidine kinase/response regulator system YehU/YehT of *Escherichia coli* is part of a nutrient-sensing network. Here we demonstrate that this system senses the onset of nutrient limitation in amino acid rich media and responds to extracellular pyruvate. Binding of radiolabeled pyruvate was found for full-length YehU in right-side-out membrane vesicles as well as for a truncated, membrane-integrated variant, confirming that YehU is a high-affinity receptor for extracellular pyruvate. Therefore we propose to rename YehU/YehT as BtsS/BtsR, after "**B**renz**t**rauben**s**äure", the name given to pyruvic acid when it was first synthesized. The function of BtsS/BtsR was also assessed in a clinically relevant uropathogenic *E. coli* strain. Quantitative transcriptional analysis revealed BtsS/BtsR importance during acute and chronic urinary-tract infections.

Exponential growth of bacteria in complex, nutrient-rich media usually ends when at least one nutrient has been used up. We recently reported that the histidine kinase/response regulator system YehU/YehT of *E. coli*, belongs to the LytS/LytTR family and presumably plays a role in tuning bacterial exploitation of available carbon sources[1]. Strikingly, the YehU/YehT system is the most widespread representative of its family found in γ-proteobacteria – and many LytS/LytTR-type systems regulate crucial host-specific mechanisms during infection of human or plant hosts by members of this bacterial clade[2]. This system is conserved in non-pathogenic as well as pathogenic *E. coli*.

Our previous studies on YehU/YehT in *E. coli* identified *yjiY* as its sole target gene[3] (Fig. 1). This gene codes for the putative carbon starvation transporter YjiY, which is homologous (61.1% identity) to CstA[4] and was found to be expressed in cells that were grown in complex media containing a high content of amino acids, such as LB or CAA (casamino acids), as well as in minimal medium supplemented with certain carbon sources, such as gluconic or glucuronic acid. Studies in *E. coli* revealed that YehT-mediated *yjiY* transcription is also regulated by the cAMP/CRP complex[3] (Fig. 1), and down-regulated in the presence of energetically favorable carbon sources like glucose. Furthermore, YjiY is subject to translational control via the Csr regulatory circuit[5] (Fig. 1), which synchronizes the output of *E. coli* central carbohydrate metabolism (glycolysis versus gluconeogenesis) with YjiY production[1,6]. Finally, *yjiY* transcription is under positive feedback regulation by a second two-component system, YpdA/YpdB, and its gene product YhjX[1] (Fig. 1).

Here, we performed a comprehensive *in vivo* characterization of *yjiY* expression in order to identify the primary stimulus sensed by the histidine kinase YehU. We found that the YehU/YehT system responds to depletion of nutrients specifically serine and the concomitant presence of extracellular pyruvate. Biochemical studies revealed that pyruvate binds specifically to the extracellular side of the membrane-spanning domain of YehU. We therefore renamed the system BtsS/BtsR, for "**B**renz**t**rauben**s**äure", the original name given by Jöns Jakob

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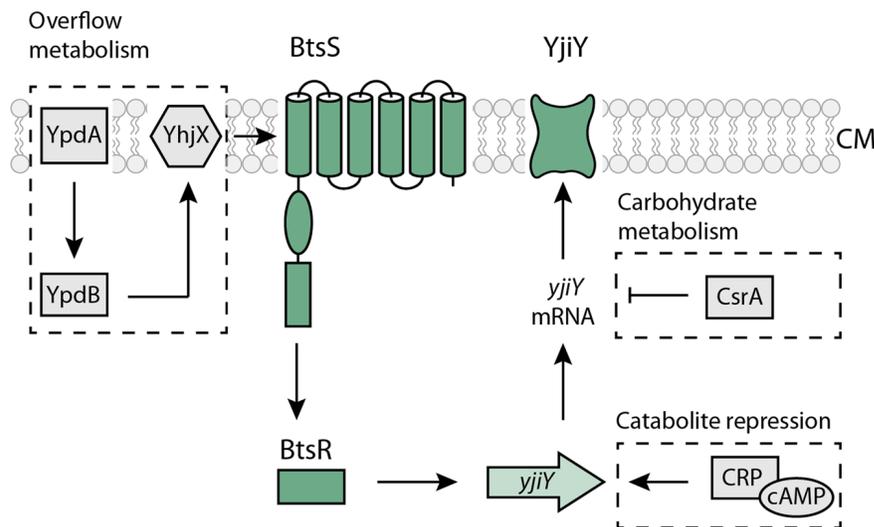
Figure 1. Schematic depiction of the BtsS/BtsR (YehU/YehT) system in *Escherichia coli*. The scheme summarizes the regulatory network associated with signal transduction by the BtsS/BtsR two-component system, the influence of two-component system YpdA/YpdB and the global regulators CsrA and CRP. Membrane proteins are integrated in the cytoplasmic membrane (CM). Activating (↑) and inhibitory (⊥) effects are indicated. See text for details.

Berzelius to the compound when he first synthesized pyruvic acid in 1835[7]. Finally, we found that the BtsS/BtsR system of uropathogenic *E. coli* may contribute to acute urinary tract infection.

Results

Elucidation of the stimulus for BtsS/BtsR (YehU/YehT) by *in vivo yjiY* expression analyses. It was previously shown that cultivation of *E. coli* in amino acid-rich media leads to activation of the BtsS/BtsR system and transient expression of the target gene *yjiY* in the late-exponential growth phase[3]. To identify a potential quorum sensing-like molecule, we used a combination of chemical fractionation of the medium and measurements of reporter strain activity. For this purpose, we cultivated *E. coli* MG1655 Δ*yjiY*/pBBR *yjiY-lux* in M9-minimal medium with gluconic acid as sole carbon source[3]. Shortly before the induction of *yjiY* we removed the cells and fractionated the supernatant by high pressure liquid chromatography. All fractions were analyzed for their potential to induce *yjiY* using reporter strain *E. coli* KX1468 pBBR *yjiY-lux* grown in minimal medium and succinate as C-source. After several rounds of fractionation/freeze-drying we found that the fraction with the highest induction potential contained a high concentration of gluconic acid, the initial carbon source (data not shown). This result ruled out the possibility that *E. coli* produces and senses a quorum sensing-like molecule. Then we quantified *yjiY* expression as a function of nutrient levels. For this purpose, we cultivated the reporter strain *E. coli* MG1655/pBBR *yjiY-lux* in LB medium with decreasing amounts of nutrients (1.0x, 0.5x, 0.4x, 0.3x LB, 0.2x LB and 0.1x LB), keeping the osmolarity of the medium constant. The growth rates (μ) of *E. coli* cells decreased with the dilution of LB medium, and exponential growth ceased at different time points (Table S1). Strikingly, expression of *yjiY* always began shortly before the onset of stationary phase (Fig. S1), and *E. coli* cells grown in 0.1x LB did not express *yjiY*. These results suggested that BtsS/BtsR somehow responds to nutrient limitation. We reasoned that supplying the relevant nutrient(s) in excess should suppress or postpone *yjiY* induction. Therefore, the reporter strain was grown in LB media supplemented with an excess of each individual L-amino acid (Fig. 2A). Particularly, the addition of L-serine delayed the expression of *yjiY* by almost two doubling periods (Fig. 2A). Subsequently, we tested different concentrations of L-serine in the reporter assay and found a concentration-dependent delay in *yjiY* expression, accompanied by a decrease in peak expression levels (Fig. 2B). The addition of serine does not influence the growth of *E. coli* and does not delay the onset of stationary phase (Fig. 2C). Although L-serine is not a preferred carbon source for *E. coli* and high external concentrations are actually toxic to the organism, it is the first amino acid to be consumed when mixtures of amino acids are available[8]. These data suggest that BtsS/BtsR responds to depletion of nutrients, specifically serine.

Changes in extracellular serine and pyruvate concentrations during growth of *E. coli*. When *E. coli* is grown in amino acid-rich media, 50.7% of L-serine is converted directly to pyruvate, whereas 36.3% is used for glycine synthesis, 6.5% for cell biomass, and the remainder for other metabolites[9]. Its central role in pyruvate supply provides one explanation for the importance of L-serine in growing *E. coli*[10]. We therefore monitored the changes in extracellular serine and pyruvate concentrations during growth in LB medium, and found that extracellular levels of serine decreased at a constant rate (Fig. 3). The starting concentration of serine in the medium (approximately 200 μM) was completely exhausted after 120 min of growth at the late-exponential growth phase. At the same time, the abundance of extracellular pyruvate peaked (approx. 500 μM) and shortly after *yjiY* expression reached its maximum level (Fig. 3). It was previously shown that the external pyruvate derives from overflow
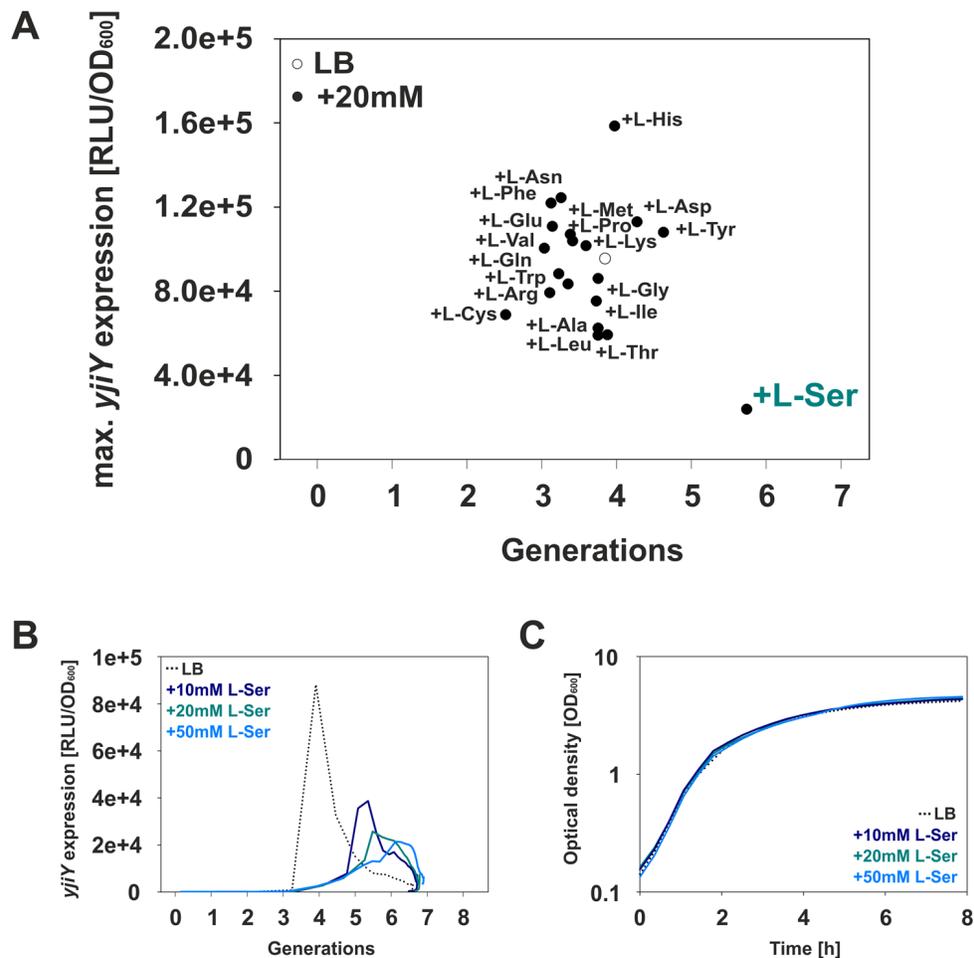
Figure 2. Effects of an excess of individual amino acids on the expression of *yjiY*. (**A**) *E. coli* MG1655/pBBR *yjiY-lux* was grown in LB medium supplemented with one of the indicated amino acids (at 20 mM), and growth and luminescence were monitored over time. Maximal *yjiY* expression values are depicted. The open circle provides a benchmark and indicates *yjiY* expression in *E. coli* grown in LB medium. (**B**) Expression of *yjiY* in LB (dotted line) supplemented with increasing L-serine concentrations. (**C**) Corresponding *E. coli* growth curves in LB media supplemented with increasing L-serine concentrations. Experiments were performed at least three times (standard deviation <10%), and results of a representative experiment are shown.

metabolism in *E. coli* during growth in amino acid-rich media[11], which was confirmed by monitoring the intra-cellular concentrations of serine and pyruvate (Fig. S2).

These data reveal that induction of BtsS-dependent *yjiY* expression coincides with the decline of serine in the medium and an extracellular accumulation of pyruvate (Fig. 3).

Extracellular pyruvate triggers *yjiY* expression under nutrient limitation. In the next experiment we tested the influence of serine, pyruvate and related metabolites on *yjiY* expression in *E. coli* cells growing in low-nutrient environment. Since *E. coli* harbors a second two-component system, YpdA/YpdB, which responds to high concentrations of extracellular pyruvate (the threshold concentration that leads to induction was determined to be 600 μM) and positively regulates the BtsS/BtsR system[11], we modified our reporter strain by deleting *yhjX*, which is sufficient to interrupt the feedback loop[1]. The resulting strain was then cultivated in 10-fold diluted (0.1x) LB medium for 1 h. At this time point, cells do not induce expression of *yjiY*, but experience soon carbon limitation (Fig. 4A, Fig. S1). However, expression of *yjiY* was rapidly triggered upon addition of pyruvate, and the induction level increased linearly with increasing pyruvate concentration (Fig. 4A). Addition of L-serine also induced *yjiY* expression, but only after a 20-min delay (Fig. 4B). Under these conditions the growth of *E. coli* did not differ significantly by addition of pyruvate or serine (Fig. S3). Only supplementation of 10 mM serine prolonged the exponential growth phase. Moreover, higher serine concentrations delayed *yjiY* expression for even longer, and decreased the level of induction attained (Fig. 4B). The threshold concentration of pyruvate required for detectable *yjiY* expression was 10 μM, and that for L-serine 50 μM. None of the other tested compounds (each of the other 19 amino acids, phosphoenolpyruvate, lactate, oxaloacetate, α-ketoglutarate, valeriate, propionate, acetate, malate) were able to induce *yjiY* in this context. These results suggest that extracellular pyruvate acts as a direct stimulus for BtsS/BtsR-mediated *yjiY* expression, whereas delayed *yjiY* induction in response to L-serine
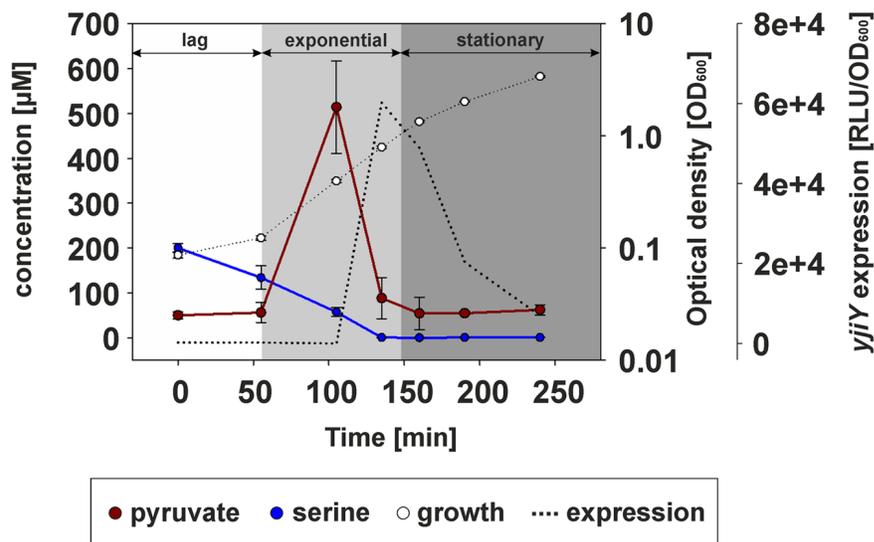
Figure 3. Determination of changes in extracellular concentrations of serine and pyruvate during growth of *E. coli*. *E. coli* MG1655/pBBR *yjiY-lux* was cultivated in LB medium, and growth (OD_{600}) and luminescence were monitored. At the times indicated, cells were harvested, and serine and pyruvate levels were quantified by hydrophilic interaction liquid chromatography. All experiments were performed in triplicate, and the error bars indicate the standard deviation of the means. The growth phases of *E. coli* are marked as following: lag phase (white), exponential growth (light grey) and stationary phase (dark grey).
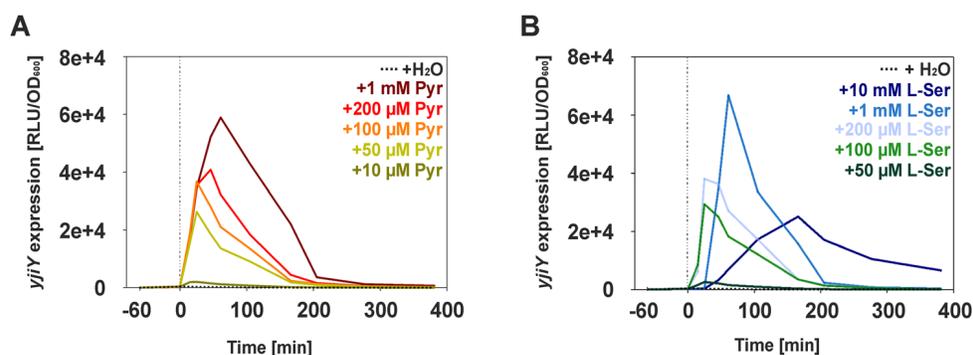
Figure 4. Stimulus-dependent *yjiY* expression under nutrient-limiting conditions. *E. coli* MG1655 mutant $\Delta yhjX$ harboring pBBR *yjiY-lux* was cultivated in 0.1x LB medium to establish low nutrient conditions. After 1 h (time point 0), the indicated concentration of pyruvate (**A**), or L-serine (**B**), or the equivalent volume of water was added. Experiments were performed at least three times (standard deviation <10%), and results of a representative experiment are shown.

may depend on uptake of the amino acid, its conversion to pyruvate and excretion of the pyruvate into the culture medium.

The sensor histidine kinase BtsS binds pyruvate with high affinity. BtsS cannot be autophosphorylated, possibly owing to a defective ATP-binding site within the G1 box[3] (data not shown). Therefore, we used the method of differential radial capillary action of ligand assays (DRaCALA)[12] to determine whether BtsS physically interacts with pyruvate as ligand. The technique is based on the ability of proteins that have been immobilized on a nitrocellulose membrane to bind a radiolabeled ligand, whereas unbound ligands undergo radial diffusion. DRaCALA allows rapid detection of both the total ligand and the ligand sequestered by proteins. The fraction of ligand bound to the protein, defined as F_B, is calculated from the signal intensity of the area with protein (inner circle) and the total signal intensity of the area (outer circle)[12]. For the DRaCALA we used unsealed membrane vesicles prepared from *E. coli* cells overproducing BtsS (MV BtsS) and calculated an F_B value of >0.15 (Fig. 5A). Control membrane vesicles (MV, lacking overproduced BtsS) were used and the low value of F_B (0.05) reflected only minor, non-specific binding. Therefore this assay was judged to be suitable for membrane vesicles, and it clearly indicated binding of radiolabeled pyruvate to BtsS. We also tested ³H-serine binding to BtsS in membrane vesicles using the DRaCALA technique. However, we only observed unspecific binding (data not shown).
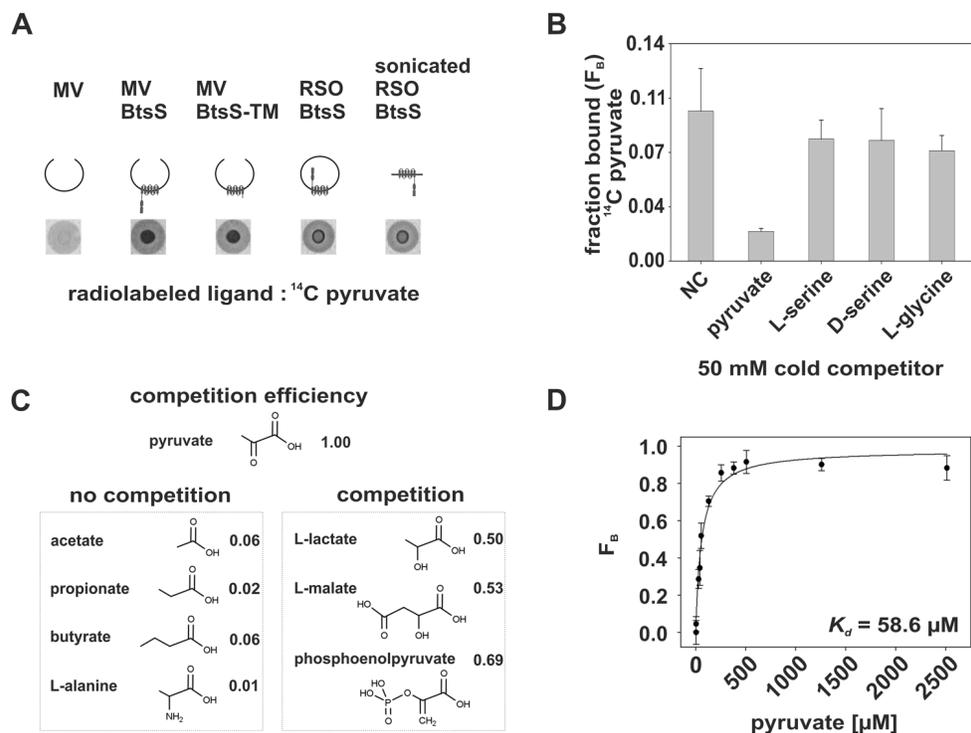
Figure 5. Analysis of the interaction of BtsS with selected ligands by DRaCALA. (**A**) A mixture of membrane vesicles (MV) or right-side-out vesicles (RSO) enriched with the corresponding proteins (indicated by graphical representations) and radiolabeled ^{14}C pyruvate (5 μM) is dropped onto a nitrocellulose membrane, and ligand migration via capillary action is analyzed. (**B**) Competition assays. Binding of radiolabeled pyruvate (5 μM) to BtsS in MVs was analyzed in the presence of various unlabeled competitors (each 50 mM). NC, no competitor. (**C**) Relative efficiency of competition by various carboxylic acids. Binding of radiolabeled pyruvate (5 μM) to BtsS in MVs was analyzed in the presence of various carboxylic acids (each 50 mM). The efficiency of competition by cold pyruvate was set to 1.00, and the effect of the indicated compounds was calculated accordingly. (**D**) Determination of the dissociation constant (K_d) for pyruvate to BtsS using DRaCALA. For each reaction radiolabeled pyruvate was used at 5 μM. Normalized F_B values [$F_B = (F_{B(NC)} - F_{B(pyr)})/F_{B(NC)}$, see Methods for details] were plotted as function of the pyruvate concentration. The best-fit line was determined by nonlinear regression using the equation $y = B_{max} * x/(K_d + x)$.

In addition, we tested MVs harboring a membrane-integrated truncated variant of BtsS (MV BtsS-TM) lacking its soluble domains, and found that this truncated sensor also binds pyruvate ($F_B > 0.15$). Furthermore, we prepared sealed right-side-out vesicles (RSO BtsS), in which membrane proteins retain their native orientation[13]. BtsS in these vesicles also showed pyruvate binding, and no significant change in binding was observed when these vesicles were fragmented by sonication and had a random orientation (sonicated RSO BtsS) (F_B values of each 0.18) (Fig. 5A). These data reveal that pyruvate binds to the external side of the membrane-spanning domain of BtsS.

The specificity of pyruvate binding to BtsS was then addressed by using a competition assay (Fig. 5B), in which several competitors were added in excess to the reaction mixture. Only unlabeled ("cold") pyruvate was able to prevent binding of radiolabeled pyruvate. L-serine, D-serine or glycine did not interfere with pyruvate binding to BtsS, suggesting that pyruvate binds specifically to BtsS.

Next we investigated the effects of varying the length and side-chain charge on ligand binding by BtsS. Using competition assays, we found that acetate, propionate and butyrate all failed to compete with pyruvate for binding, indicating the importance of the C-α keto group of pyruvate (Fig. 5C). The influence of polarity at the C-α position was then tested by addition of lactate and malate, which contain C-α hydroxyl groups. These molecules were able to decrease pyruvate binding by about 50% suggesting that a negative charge seems to be recognized. It should be noted that these compounds were tested in a 10^4-fold excess over pyruvate. Phosphoenolpyruvate, which harbors a phosphoryl group at the C-α position, also competed with pyruvate, reducing binding by approximately 50%. In contrast, a positive charge at the C-α position in the form of the amino group in alanine had no effect, reducing pyruvate binding by 1% (Fig. 5C). Notably, none of these compounds were able to induce *yjiY* expression *in vivo*, which emphasizes the specificity of BtsS for pyruvate. The dissociation constant (K_d) for pyruvate binding to the histidine kinase was found to be 58.6 ± 8.8 μM (Fig. 5D).

To our knowledge this is the first application of DRaCALA to measure the interaction between a ligand and a membrane-embedded protein. Moreover, these assays unambiguously demonstrated that pyruvate binds specifically and with high affinity to BtsS.
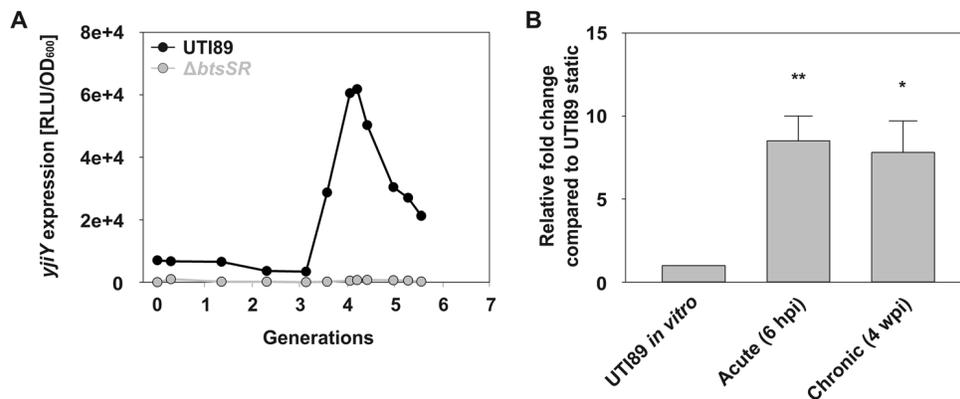
Figure 6. BtsS/BtsR importance during urinary tract infection. (**A**) The plot indicates the activity of the pBBR *yjiY-lux* reporter fusion in WT UTI89 and the isogenic *btsS/btsR* mutant during growth in LB, and depicts a representative of at least 6 biological replicates. (**B**) Change in the level of the *yjiY* transcript during acute and chronic phases of UTI relative to that measured in samples incubated *in vitro* without agitation. Data shown are the mean values of samples extracted from three different mice per time point. RNA was extracted from dissected bladders for each time point and enriched for bacterial RNA by depleting ribosomal and mammalian RNA as described in Materials and Methods. Profiling was performed using an *yjiY*-specific probe. Expression was normalized to that of the housekeeping gene *gyrB* and compared to expression in cDNA samples corresponding to the starting inoculum (static *in vitro* culture, prepared as described in Materials and methods). Relative fold change was measured using the method described by Pfaffl *et al.*[54] (hpi, hours post infection; wpi, weeks post infection).

BtsS/BtsR importance during urinary tract infection.   To understand the potential implications of BtsS/BtsR for pathogenesis, we therefore turned our attention to uropathogenic *E. coli* (UPEC) that colonize a nutritionally demanding environment – the mammalian bladder. However, responses to nutritional stress are of the utmost importance for the survival of both commensal and pathogenic bacteria within a given host.

UPEC strains are the primary causative agent of urinary-tract infections (UTIs) worldwide, accounting for over 85% of all reported episodes[14]. In the bladder, bacteria adhere to the apical surface of the epithelium and are internalized, before replicating to form biofilm-like pods within host bladder cells[15, 16]. Subsequent to this transient intracellular cascade, adverse outcomes such as chronic colonization can ensue as a result of an aberrant host immune response[17]. Previous studies have demonstrated that successful UTI requires aerobic respiration and amino acid utilization[18–20]. To investigate the significance of BtsS/BtsR in bladder infection, we first created a *btsS/btsR* mutant in the UPEC cystitis isolate UTI89. Using the same reporter fusion, we demonstrated that this strain, unlike the wild-type parental UTI strain, failed to induce transient expression of *yjiY* (Fig. 6A). Both pyruvate and L-serine are present in human and murine urine, and levels are elevated in diabetic populations[21–23]. Given that BtsS/BtsR responds directly to extracellular pyruvate levels and indirectly to L-serine, we asked whether BtsS/BtsR is active in the bladder lumen during acute and chronic UTI.

Mice were infected with the cystitis isolate UTI89 and RNA samples were extracted from bladder homogenates at 6 h post infection (coinciding with the acute stage of UTI), and at 2 weeks post infection from mice that went on to develop chronic cystitis. At this stage, the majority of bacteria are found on the bladder epithelial surface in the form of an extracellular biofilm[17]. Subsequent TaqMan-based qPCR analysis compared the expression of *yjiY* in the corresponding cDNA samples to the expression of *yjiY* in the starting bacterial inoculum. Our results demonstrated robust expression of *yjiY* in both the acute and chronic stages of infection from at least three separate mice per time-point (Fig. 6B). Taken together, these data suggest that, in the murine UTI model BtsS/BtsR responds to serine/pyruvate fluctuations and could play a role in promoting the infection process.

Discussion

Although numerous studies continue to demonstrate the importance of histidine kinase-mediated signal transduction in bacterial physiology, the natural ligands have been identified for very few histidine kinases[24–26]. This study presents compelling evidence demonstrating that the histidine kinase BtsS is a high-affinity receptor for extracellular pyruvate. Induction of the BtsS/BtsR target gene *yjiY*, which codes for a "carbon starvation" CstA-like transporter, correlates with the depletion of serine in complex medium, explaining the observation that BtsS/BtsR senses low serine. At the same time, while serine is being depleted from LB medium, *E. coli* excretes a substantial amount of pyruvate from overflow metabolism into the medium. Shortly afterwards, the concentration of extracellular pyruvate returns to its basal level (Fig. 3 and ref. 11). In previous studies, we demonstrated activation of a different system, YpdA/YpdB in response to high levels of extracellular pyruvate[11] and showed that induction of YpdA/YpdB enhances BtsS/BtsR activation[1]. The current work provides a comprehensive characterization of how BtsS/BtsR directly responds to extracellular pyruvate levels and fine-tunes the metabolic fitness of *E. coli* under low-nutrient conditions. Sequence comparison between YpdA and YehU did not identify putative amino acids involved in pyruvate binding. In non-pathogenic *E. coli*, each of these signaling systems induces the expression of exactly one gene, which codes for a transporter. One (YhjX) belongs to the major facilitator

superfamily, and is assumed to be a low-affinity carboxylate antiporter[27]. The other one (YjiY) belongs to the CstA-like transporters, which are characterized by an unusually large number of transmembrane helices (17 in the case of YjiY). Neither transporter has been characterized thus far. It is hypothesized that both are involved in nutrient uptake, but with different affinities. The interconnectivity between the high-affinity pyruvate signaling system BtsS/BtsR with the putative low-affinity pyruvate signaling system YpdA/YpdB by a positive and a negative feedback loop[1] would provide *E. coli* with a network that could tailor pyruvate uptake in each individual cell according to its availability. Switching between low- and high-affinity phosphate transporters based on the needs of the individual cell was recently demonstrated for *S. cerevisiae*[28], and seems to be a widely distributed strategy for the maintenance of nutrient homeostasis as stocks of essential nutrients decline[29].

Although nutrient sensing is crucial for host-microbe and microbe-microbe interactions, the majority of studies in non-pathogenic *E. coli* strains have focused on metabolic engineering, aiming to understand how processes such as elevated intracellular pyruvate levels affect metabolite distribution[30, 31] or how central mutations trigger/alter metabolic fluxes[32–34]. In recent years, technological advances have permitted detailed analyses of *in vivo* metabolism[35], revealing its complexity and its influence on virulence and pathogenesis[36]. These studies have shown pyruvate to be one of the major factors connecting cellular metabolism to cell division[37]. Moreover, pyruvate levels are thought to reflect the quantitative relationship between carbon and nitrogen availability in the cell, and affect amino acid biosynthesis[38].

Investigations of how metabolic decisions determine pathogen fitness within host niches are increasingly uncovering opportunities for the development of robust and pathogen-specific drugs. Different *E. coli* pathotypes cause various clinical syndromes, depending on their genetic makeup and expression patterns. They obviously differ extensively from each other and from commensal *E. coli*. For example, uropathogenic and enterohemorragic *E. coli* differ significantly in genome content, and employ different strategies to infect their niches - the bladder and the gut, respectively. Very recent work has pointed to the need for aerobic respiration and amino acid utilization for optimal colonization of the urinary tract by UPEC[18–20]. Pyruvate is a crucial molecule required for fueling the aerobic arm of the tricarboxylic acid cycle, which is used by UPEC during infection[18, 19, 39]. Thus, it is reasonable to postulate that UPEC has the capacity to sense and respond to pyruvate fluctuations within the urinary tract. Here we demonstrate that BtsS/BtsR mediates *yjiY* expression during infection by a clinically relevant UPEC isolate, which strongly suggests that BtsS/BtsR constitutes part of the metabolic circuitry engaged during UTI. Thus understanding how UPEC can sense the metabolic signature of the bladder lumen, the kidney and the urothelial cell cytosol (in which they form intracellular bacterial communities) will be vital for a complete understanding of pathogen strategies and the design of more effective therapeutics. However, the study in *Salmonella enterica* Serovar Typhi and Typhimurium showed no detectable effect on the ability of *yehUT* mutant strain to invade cultured epithelial cells or induce colitis in a murine model[40].

In summary, this study has uncovered a signal transduction network that responds with exquisite sensitivity to extracellular pyruvate levels and fine-tunes carbon utilization in *E. coli* strains. Furthermore we provide direct evidence for receptor-ligand interactions between BtsS and pyruvate, and demonstrate that BtsS/BtsR mediates *yjiY* expression during UTI. Future studies will focus on understanding the contribution of this system to pathogenic *E. coli* intra-host fitness and dissecting molecular determinants that drive the fine-tuning of bacterial fitness in response to external metabolic cues.

Methods

Strains, plasmids, and oligonucleotides.
In this study we used the *E. coli* strains MG1655[41], *E. coli* MG1655 Δ*yhjX*[1], *E. coli* MG1655 Δ*yjiY*[1], BL21(DE3)[42], *E. coli* KX1468[43] and the cystitis isolate UTI89[44]. Plasmids used in this study include the transcriptional promoter-luciferase fusion construct for P$_{yjiY}$ activity (pBBR *yjiY-lux*)[3]. For protein production we used the arabinose-inducible expression vectors pBAD24[45], pBAD24-*yehU*[3] and pBAD24-*yehU*-TM (YehU (BtsS) amino acids 1–205). DNA fragments for plasmid construction were amplified from genomic DNA by PCR. Scarless deletion of *btsS*/*btsR* genes in UTI89 was performed using the method published by Murphy and Campellone[46], and verified via PCR with oligonucleotides flanking the deleted loci. All oligonucleotide sequences are available on request.

Molecular biological techniques.
Plasmid and genomic DNAs were isolated using a HiYield plasmid minikit (Suedlaborbedarf) and a DNeasy blood and tissue kit (Qiagen), respectively. DNA fragments were purified from agarose gels using a HiYield PCR cleanup and gel extraction kit (Suedlaborbedarf). Q5 DNA polymerase (New England BioLabs) was used according to the supplier's instructions. Restriction enzymes and other DNA-modifying enzymes were also purchased from New England BioLabs and used according to the manufacturer's directions.

Growth conditions.
All strains were grown overnight in LB medium. After inoculation, bacteria were routinely grown in LB, LB supplemented with the indicated amino acids or diluted LB medium [containing 1% (w/v) NaCl] under agitation (200 rpm) at 37 °C. For solid medium, 1.5% (w/v) agar was added. Where appropriate, media were supplemented with antibiotics (ampicillin sodium salt, 100 μg/ml; kanamycin sulfate, 50 μg/ml; gentamicin sulfate, 50 μg/ml). For infection studies in mice, UTI89 was inoculated into 10 ml of LB medium and grown without shaking at 37 °C for 24 h. This culture was used to seed a fresh flask with 10 ml of a 1:1000 dilution, which was incubated for another 24 h to maximize expression of type 1 pili, as previously described[47]. The growth phases of *E. coli* marked in graphs were according to the definition in ref. 48.

In vivo expression studies.
In vivo expression of *yjiY* was quantified by means of luciferase-based reporter-gene assays, using bacteria that had been transformed with plasmid pBBR *yjiY-lux*. Cells from an overnight culture were transferred to fresh medium to give a starting optical density at 600 nm (OD$_{600}$) of 0.05, and

incubated under aerobic growth conditions at 37 °C while OD_{600} and luminescence were continuously monitored. The optical density was determined in a microplate reader (Tecan Sunrise) at 600 nm. Luminescence levels were determined in a luminescence reader (Centro LB960; Berthold Technology) for 0.1 s and are reported as relative light units (RLU; counts s^{-1}).

Identification of quorum sensing-like molecule. The cells of *E. coli* MG1655 Δ*yjiY*/pBBR *yjiY-lux* were cultivated in M9-minimal medium supplemented with 20 mM gluconic acid and OD_{600} and luminescence were constantly monitored. Cells were harvested shortly before, at and after the induction of *yjiY*. The supernatant was sterile filtrated (Stericup®, Filter Unit Millipore Express® PLUS(PES), 0.22 μm and separated by high pressure liquid chromatography (HPLC) using column C18-Hypersil Gold, with the gradient 1–100% (v/v) acetonitrile in 40 min. Fractions were collected, concentrated and used in reporter strain *E. coli* KX1468[43]/pBBr *yjiY-lux* grown in M9-minimal medium supplemented with 20 mM succinate. The fraction with the highest induction potential was then analyzed via UPLC-UHR-ToF-MS using a Waters XBridge Amide column.

Production of BtsS-6His in membrane vesicles. *E. coli* BL21(DE3) cells were transformed with the vector pBAD24-*yehU*[3], which codes for BtsS-6His, and grown in LB medium at 37 °C to an OD_{600} of 0.5. Recombinant gene expression was induced by addition of 0.2% (w/v) arabinose. After 3 h of further incubation, cells were harvested by centrifugation, disrupted and fractionated. At each step, the pellet was resuspended in buffer consisting of MES/NaOH (pH 6), 10% (v/v) glycerol and 10 mM $MgCl_2$. To produce BtsS-6His in right-side-out vesicles, cells were cultivated as described before. After harvesting the cells by centrifugation, lysozyme (50 μg/ml) and EDTA (10 mM) were added for 45 min at room temperature to remove the cell wall. After low-speed centrifugation, the spheroplasts were resuspended in buffer consisting of 100 mM MES/NaOH (pH 6), 30% (w/v) sucrose, 20 mM $MgSO_4$, DNase and RNase, and then diluted 100-fold into pre-warmed buffer (100 mM MES/NaOH (pH 6)) to trigger osmotic lysis. After centrifugation, the pellet was resuspended in 100 mM MES/NaOH (pH 6), 10% (v/v) glycerol and 10 mM $MgCl_2$. Formation of spheroplasts and right-side-out vesicles was monitored under the microscope. His-tagged BtsS was detected by Western blot analysis with an anti-His antibody (Qiagen) and an alkaline phosphatase-conjugated anti-mouse antibody as the secondary antibody.

Protein-ligand interactions. Protein-ligand interactions were analyzed via DRaCALA, a method established by Roelofs *et al.*[12]. Membrane vesicles enriched for overproduced BtsS-6His were mixed with 5 μM radiolabeled (3-^{14}C) pyruvic acid sodium salt (55 mCi mmol^{-1}, Biotrend) or radiolabeled (^{3}H) L-serine (11.0 Ci mmol^{-1}, Hartmann Analytics) and incubated for 5 min at room temperature. Triplicate 5-μl aliquots were transferred to a nitrocellulose membrane, dried and visualized by a PhosphorImager. Quantification of radioactive signal was done with the software ImageQuant. The fraction bound to protein was calculated according to the signal intensities using an equation defined earlier: $F_B = (I_{inner} - I_{background})/I_{total}$[12]. For the evaluation the corresponding cold ligand (50 mM) was added to the reaction mixture and incubated for further 3 min. To estimate the K_d value, increasing concentrations of cold pyruvate (0 μM to 2.5 mM) were used. In this case the F_B value was normalized as follows: $F_B = (F_{B(NC)} - F_{B(pyr)})/F_{B(NC)}$. Here, $F_{B(NC)}$ is calculated from the sample with pure ^{14}C-labeled pyruvate, and $F_{B(pyr)}$ are all values for mixtures containing ^{14}C-labeled pyruvate together with increasing concentrations of unlabeled pyruvate. The amount of ^{14}C-pyruvate was kept constant in all assays.

Extraction and determination of extra- and intracellular metabolites. The reporter strain *E. coli* MG1655/pBBR *yjiY-lux* was cultivated in LB medium, and OD_{600} and luminescence were constantly monitored. At selected time points, cells were harvested and the supernatants were saved. Cells were washed with 10% (v/v) glycerol, and subsequently cell pellets and supernatants were analyzed via hydrophilic interaction liquid chromatography (HILIC). Acetonitrile (ACN), methanol (MeOH), ammonium acetate and ammonium hydroxide (all of LC-MS grade) were obtained from Sigma-Aldrich (Sigma-Aldrich GmbH). Water was purified using a Merck Millipore Integral water purification system to a resistance of 18 MΩ and TOC < 5 ppb. Sodium pyruvate and serine were also obtained from Sigma-Aldrich and dissolved in water at a concentration of 100 mM and further diluted with ACN. Cell pellets were extracted with 200 μl ice-cold water/MeOH (50/50, v/v). Samples were vortexed vigorously and lysed in an ice-cold sonic bath for 15 min. After centrifugation at 20,000 × *g* and 4 °C for 10 min, supernatants were transferred to autosampler vials.

Pyruvate and serine were quantified using a modified version of the method published by Yuan *et al.*[49]. Separation was achieved using a Waters XBridge Amide column (3.5 μm, 100 mm × 4.6 mm ID) and an ACN/water gradient using a Waters Acquity UPLC system coupled to a Bruker maXis UHR-ToF-MS (Bruker Daltonics). Eluent A consisted of 5% (v/v) ACN, 95% (v/v) water, 20 mM ammonium hydroxide, 20 mM ammonium acetate, pH 9.0 and eluent B was pure ACN.

Metabolites were eluted with the following gradient: After 3 min of 85% B, a linear decrease to 2% B over 9 min, isocratic hold for 3 min and return to initial conditions for 1 min and a 7-min re-equilibration time. Sample (5 μl) was injected via partial loop injection.

For quantification a calibration curve was generated from different concentrations of standard compounds (0 μM, 0.5 μM, 1.0 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM, 250 μM). If the concentration of a sample was beyond the range of the calibration curve, it was appropriately diluted with water/MeOH. Quantification was performed with Bruker Quant Analysis.

Murine infections. A cohort (15) of 7- to 8-week old female C3H/HeN mice was infected with strain UTI89 via transurethral catheterization as described in Hung *et al.*[50]. Five mice were sacrificed at 6 h after infection, marking the acute stage of UTI in this murine model[51]. Bladders were excised for CFU enumeration and RNA extraction. The remaining 10 mice were monitored for chronic infection using longitudinal urinalysis, as previously described[52]. Mice that consistently shed more than 100,000 CFUs/ml of urine were separated from the

rest of the cohort, and flagged as chronically infected. These mice were sacrificed at 4 weeks post infection and bladders were obtained for CFU enumeration and RNA extraction.

To ensure the proper and humane treatment of animals, all animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the Vanderbilt University Medical Center's Institutional Animal Care and Use Committee (IACUC), who approved all protocols. Statistical analyses were performed using two-tailed Mann-Whitney t-test.

RNA extraction, cDNA synthesis and qPCR. RNA extraction and bacterial RNA enrichment were performed as described by Conover *et al*.[47]. DNase treatment, reverse transcription, and real-time quantitative PCR were done as described by Guckes *et al*.[53]. qPCR analysis was carried out with three concentrations of cDNA (50 ng, 25 ng, 12.5 ng), each in triplicate for each sample, and levels of *gyrB* (DNA gyrase) were used for normalization. The following primers (Integrated DNA Technologies) were used for amplification: *yjiY*_Fwd (5′-GGCACGACGCCGAAACT-3′), *yjiY*_Rev (5′-GCCGTAGCCGATGAAACG-3′), *gyrB*_L (5′-GATGCGCGTGAAGGCCTGAATG-3′), *gyrB*_R (5′-CACGGGCACGGGCAGCATC-3′). The following probes (Applied Biosystems) were used for quantification; *yjiY* (5′FAM-TGGCTAATGAAACCGACGC-MGBNFQ-3′); *gyrB* (5′VIC-ACGAACTGCTG-GCGGA-MGBNFQ-3′).

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Author Contributions

S.B., M.W., P.S.-K., M.H. and K.J. designed the experiments. S.B., I.K., M.W., E.B., A.E., C.S. performed the experiments. S.B., I.K., M.H., and K.J. wrote the manuscript.

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Chapter 3

BtsT - a novel and specific pyruvate/H$^+$ symporter in *Escherichia coli*

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1   BtsT - a novel and specific pyruvate/H^{+} symporter in *Escherichia coli*

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7   Running head: Pyruvate transporter BtsT in *Escherichia coli*

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15 **ABSTRACT**

16 The CstA (the peptide transporter carbon starvation CstA family; TC# 2.A.114) family of

17 peptide transporters belongs to the second largest superfamily of secondary transporters, the

18 <u>a</u>mino acid/<u>p</u>olyamine/organo<u>c</u>ation (APC) superfamily. No representative of the CstA family

19 has previously been characterized either biochemically or structurally, but we have now

20 identified the function of one of its members, the transport protein YjiY of *Escherichia coli*.

21 Expression of the *yjiY* gene is regulated by the LytS-like histidine kinase BtsS, a sensor of

22 extracellular pyruvate, together with the LytTR-like response regulator BtsR. YjiY consists of

23 716 amino acids, which form 18 putative transmembrane helices. Transport studies with intact

24 cells provided evidence that YjiY is a specific and high-affinity transporter for pyruvate (K_m

25 16 μM). Furthermore, reconstitution of the purified YjiY into proteoliposomes revealed that

26 YjiY is a pyruvate/H$^+$ symporter. It has long been assumed that *E. coli* possesses

27 transporter(s) for pyruvate, but the present study is the first to definitively identify such a

28 protein. Based on its function, we propose to rename the uncharacterized gene *yjiY* as *btsT*

29 (for "<u>B</u>renz<u>t</u>rauben<u>s</u>äure" <u>t</u>ransporter, from the German word for pyruvate).

30

31 **IMPORTANCE**

32 BtsT (formerly known as YjiY) is found in many commensal and pathogenic representatives

33 of the *Enterobacteriaceae*. This study for the first time characterizes a pyruvate transporter in

34 *Escherichia coli*, BtsT, as a specific pyruvate/H$^+$ symporter. When nutrients are limiting, BtsT

35 takes up pyruvate from the medium, thus enabling it to be used as a carbon source for the

36 growth and survival of *E. coli*.

37

38

INTRODUCTION

40   YjiY belongs to a small family of transporters, named for the peptide transporter CstA

41 (the peptide transporter carbon starvation CstA family; TC# 2.A.114) (1). The CstA family is

42 part of the amino acid/polyamine/organocation (APC) superfamily of secondary transporters

43 (2). The latter is the second largest superfamily of secondary transporters, and its

44 representatives cover a broad substrate spectrum, ranging from amino acids to metal ions and

45 peptides (2). Within the APC transporters, the CstA family displays the highest diversity in

46 sequence and topology. Some members of the CstA family are already characterized: CstA of

47 *E. coli* (3), CstA of *Campylobacter jejuni* (4), CstA and YjiY of *Salmonella enterica* serovar

48 Typhimurium (5). In both species, *E. coli* and *C. jejuni*, the gene *cstA* is upregulated under

49 carbon starvation, and knockout mutants have a lower growth rate in the presence of peptides

50 as nitrogen source (3, 6). Furthermore, a *cstA* or a *yjiY* mutant of *S.* Typhimurium is impaired

51 in the utilization of several dipeptides (5). However, no representative of CstA family has yet

52 been biochemically analyzed to ascertain the identity of its substrate(s).

53   Our studies focus on YjiY, which displays high sequence similarity (75.4%) and

54 identity (61.1%) to CstA from *E. coli* (7). The corresponding gene *yjiY* is the sole target gene

55 of the histidine kinase/response regulator system BtsS/BtsR, and its expression leads to the

56 synthesis of a 716-amino acid protein (7). In the *E. coli* genome *yjiY* is disconnected from the

57 *yehUT* operon. However, in other bacterial genomes these genes are adjacent to each other (7,

58 8), which suggests that they might be functionally related (7). Expression of *yjiY* is

59 additionally regulated by cAMP-CRP and post-transcriptionally modulated by CsrA (7).

60   It has been hypothesized that YjiY is involved in nutrient uptake, most probably the

61 transport of pyruvate (9). This assumption is supported by proteome studies of *E. coli* under

62 different growth conditions, which demonstrated that levels of YjiY are increased by up to 74-

63 fold when cells are grown in minimal medium with pyruvate as a carbon source compared to

64 cells grown in amino-acid-rich LB medium (10). Additionally, a quantitative fitness screen of

65 bar-coded *E. coli* BW25113 transposon mutants revealed that the *yjiY* mutant displays

66 reduced fitness when grown in minimal media containing pyruvate as sole carbon source (11).

67 Furthermore, pyruvate is vital to most living cells, both as a source of energy and carbon,

68 since it forms the central node of carbon metabolism (together with phosphoenolpyruvate) in

69 *E. coli* (12). Under conditions that require increased consumption of organic compounds

70 during exponential growth, *Escherichia coli* excretes acetate as well as pyruvate via overflow

71 metabolism (13). In later phases of growth, the extracellular pyruvate is then scavenged by the

72 cells (14). It was shown that BtsS/BtsR responds to such external pyruvate by activating the

73 expression of *yjiY* (9). The histidine kinase BtsS is a high-affinity receptor for extracellular

74 pyruvate (9). It is therefore tempting to speculate that YjiY is a pyruvate transporter.

75   To date, little is known about pyruvate transport in *E. coli*. It has been shown that a

76 separate transport system for pyruvate exists in *E. coli* (15), and that cells possess an active

77 uptake system for pyruvate (16). However, pyruvate transport has not been assigned to any

78 specific gene product. A gene located at around 15 centisomes on the *E. coli* gene map has

79 been suggested to encode an inducible pyruvate transport system (17), but has not been further

80 characterized. Uptake of pyruvate has also been shown in *Rhodopseudomonas spheroides*

81 (18) and *Lactobacillus plantarum* (19), but the transport systems responsible have not been

82 defined. MctC in *Corynebacterium glutamicum* (20), MctP in *Rhizobium leguminosarum* (21)

83 and TRAP-T in *Anabaena* sp (22) have all been characterized to function as pyruvate

84 transporters. However, the specificity of these transporters is not restricted to pyruvate. MctC,

85 a transporter that requires the electrochemical proton gradient, prefers acetate and propionate,

86 and has a low affinity for pyruvate ($K_{0.5}$ 250 µM). MctP, a sodium/solute transporter, has a

87 high affinity for pyruvate (K_m 3.8 µM), but shows a rather broad substrate specificity (alanine,

88 lactate, pyruvate and probably also propionate, acetate, butyrate and α-hydroxybutyrate). The

89   Trap-T transport system takes up several monocarboxylate 2-oxoacids including pyruvate (K_m

90   67 μM).

91   This study represents the first to be devoted to defining the function of YjiY. Analyses

92   with intact cells demonstrated that YjiY is able to transport pyruvate with a high affinity and

93   specificity. In addition, the reconstitution of purified YjiY in proteoliposomes proved that

94   pyruvate transport requires the proton motive force for energization. To indicate its role as a

95   pyruvate transporter in *E. coli* and highlight its functional relationship with the pyruvate-

96   responsive BtsS/BtsR histidine kinase/response regulator system (9), YjiY has been renamed

97   BtsT (for "<u>B</u>renz<u>t</u>rauben<u>s</u>äure" <u>t</u>ransporter), and is so referred to in what follows.

98

99   **RESULTS**

100   **CstA-like proteins in *Enterobacteriaceae*.** BtsT belongs to the CstA family, which

101   consists of 10 members in the Transporter Classification Database (www.tcdb.org), all which

102   share a high degree of sequence conservation (1). We were interested in elucidating the

103   relationship between the BtsT homologs found among the *Enterobacteriaceae* by comparative

104   genomics. For this purpose, we performed a local alignment search based on the full-length

105   amino acid sequences using Protein BLAST (23). Based on the alignment of 1,617

106   individually identified sequences, we generated a phylogenetic tree and grouped the

107   corresponding proteins **(Dataset S1)**. The tree possesses three distinct branches, which were

108   named BtsT-like, CstA1-like and CstA2-like **(Fig. 1)**, taking the two known BtsT and CstA

109   proteins in *E. coli* as branch markers. Within the *Enterobacteriaceae*, 43.5% of all sequences

110   were assigned to the BtsT-like branch of the tree, and 40.8%, including *E. coli* CstA, to the

111   CstA1-like branch. Surprisingly, the remaining 15.8% of the identified sequences formed an

112   additional, distinct branch, named CstA2-like. The genus *Escherichia* contained examples of

113   both BtsT and CstA1, while other genera like *Dickeya* and *Serratia* possess representatives of

114   both the CstA1 and CstA2 branches. No single genus contained representatives of all three

115   branches. Although the amino acid sequences show an extraordinarily high level of identity

116   (e.g. 61.1% between BtsT and CstA in *E. coli*), CstA sequences differ sufficiently to be

117   attributed to separate branches. Furthermore, the frequent presence of more than one CstA

118   representative within the same species suggests that members of different branches might

119   have different functions within the *Enterobacteriaceae*.

120   Since BtsT of *S. enterica* was proposed to function as peptide transporter (5), we

121   compared the sequences of BtsT of *E. coli* and *S. enterica* using a consensus-based approach.

122   A consensus derived from 148 BtsT sequences of *E. coli* was aligned to a consensus of 122

123   BtsT sequences of *S. enterica* **(Fig. S1)**. The sequence identity was determined to be 97%.

124   Most of the deviating amino acids are located in the loops of BtsT. Although the difference in

125   sequence identity is rather small, a single amino acid substitution might be sufficient to alter

126   the substrate specificity. It is worth mentioning, that there are differences in the metabolic

127   behavior of these two species. While *E. coli* excretes pyruvate during growth in an amino

128   acid-rich medium as part of an overflow metabolism, *S. enterica* did not excrete pyruvate to

129   the same extent (24). In addition, in *E. coli* the histidine kinase/response regulator system

130   BtsS/BtsR (its target gene is *btsT*) forms together with the histidine kinase/response regulator

131   system YpdA/YpdB a regulatory network (25). The YpdA/YpdB system is not encoded in *S.*

132   *enterica*. Here, we elucidate the function of BtsT, whose expression is controlled by the

133   BtsS/BtsR histidine kinase/response regulator system.

134

135   **Secondary structure model of BtsT.** CstA family members contain 13 to 18

136   transmembrane domains (TMs), which are organized into two central 5-TM repeat units with

137   1 to 4 extra TMs on each side. The first helix of the second repeat unit (helix 10 in **Fig. 2**)

138   contains a conserved motif CG-x(2)-SG of unknown function (2). The structural similarity

139   between BtsT and secondary transporters of the APC superfamily (TMs 4-17) was confirmed

140   by Phyre2, a protein homology/analogy recognition engine (26). Furthermore, hydropathy

141   analyses of BtsT with UniProt predicted that the membrane-integrated portion contains 17

142   putative TMs, while CstA displays 18 TMs **(Fig. S2)** (27).

143   Since the C-terminus of BtsT is located on the cytoplasmic side of the membrane (28),

144   the odd number of domains implied that the N-terminus of BtsT should extend into the

145   periplasm. Because a periplasmically located N-terminus is uncommon among integral

146   membrane proteins, we investigated its location in BtsT by using the MalE fusion strategy

147   (29). Native MalE is a periplasmic maltose-binding protein (MBP), and it contains a leader

148   sequence that enables it to be translocated through the cytoplasmic membrane. MalE without

8

149   the leader sequence is produced as cytoplasmic protein (30). We fused *malE* with or without a

150   leader sequence to the 5' end of *btsT* to code either for a periplasmically located MBP

151   (MBP$_P$-BtsT) or a cytoplasmically located MBP (MBP$_C$-BtsT). We predicted that only a

152   correctly folded hybrid protein would be inserted into the cytoplasmic membrane. We

153   individually overproduced both hybrid proteins in *E. coli* and determined the level and

154   localization of each by Western blot. The MBP$_C$-BtsT was predominantly found in the

155   membrane fraction **(Fig. S3A).** The level of MBP$_P$-BtsT was low, and no hybrid protein was

156   detectable in any cellular subfraction **(Fig. S3B).** In addition, we performed complementation

157   studies using the MBP-deficient *E. coli* mutant MM39, which only grows with maltose as a

158   sole carbon source, when it produces a periplasmically located MBP **(Fig. S4)**. *E. coli* MM39

159   producing either MBP$_P$-BtsT or MBP$_C$-BtsT was unable to grow, corroborating previous

160   results **(Fig. S3)**. These results revealed that only BtsT with a cytoplasmically fused MBP is

161   correctly integrated into the cytoplasmic membrane, and therefore suggest that the N-terminus

162   of BtsT is located on the cytoplasmic side of the membrane.

163   Then we compared the predicted secondary structures for BtsT and CstA, and found a

164   long loop between TM11 and TM12 in BtsT **(Fig. S2A)**. According to PSIPRED secondary

165   structure prediction (31), amino acids 417-437 in this loop should have a high propensity to

166   form a helix and therefore a TM. Based on the experimental evidence for the location of the

167   N-terminus and the structure prediction for CstA, we propose a secondary structure model of

168   BtsT with an intracellular N-terminus and 18 TMs **(Fig. 2)**.

169

170   **BtsT activity in intact cells.** Since pyruvate is an important metabolite of central

171   carbon metabolism, and other pyruvate transport systems in *E. coli* are yet unknown, we

172   started to work with a mutant strain deficient in *btsT* only to analyze BtsT activity in intact

173   cells. It is important to note that there were no growth defects for mutant MG1655 Δ*btsT*

174 neither in LB medium **(Fig. S5A)** nor in M9 minimal medium supplemented with pyruvate

175 **(Fig. S5B).** Uptake of ^{14}C-pyruvate into intact cells of *E. coli* MG1655 Δ*btsT* (25)

176 transformed with either pBAD24-*btsT* (7) or pBAD24 (32) was analyzed by a rapid filtration

177 assay. An initial experiment showed that measurements of ^{14}C-pyruvate are difficult due to

178 extremely fast uptake and/or metabolization (data not shown). Therefore, all subsequent

179 assays were performed at 15ºC. At this lower temperature, the rate of uptake of ^{14}C-pyruvate

180 by *E. coli* MG1655 Δ*btsT* containing the control plasmid pBAD24 was approximately linear

181 for 60 seconds **(Fig. 3)**. Cells transformed with pBAD24-*btsT* displayed a 6-fold higher linear

182 uptake rate for 30 s and reached 4-fold higher steady state (0.5 nmol/mg of protein) at about

183 40 s. Transport rates in intact cells of *E. coli* MG1655 Δ*btsT* transformed with pBAD24-*btsT*

184 were also measured at various external pH values **(Fig. S6)**. The highest pyruvate uptake rate

185 was found at pH 7.5, the pH-value of the buffer used in all the subsequent experiments. The

186 pyruvate uptake rate decreased at pH 6, no transport was detected at pH of 4.5 and 3.0.

187   In addition, we tested pyruvate uptake by BtsT in intact cells, which have a decreased

188 pyruvate metabolism. For this purpose, we used *E. coli* strain YYC202, which harbors

189 deletions in the pyruvate metabolizing pathways *aceEF*, *pflB*, *poxB* and *pps*, and therefore

190 cannot convert pyruvate into either acetyl-CoA, phosphoenolpyruvate (PEP) or acetate (33).

191 Uptake of ^{14}C-pyruvate into intact cells of *E. coli* YYC202 transformed with either pBAD24-

192 *btsT* or pBAD24 was measured, and we observed a pattern of pyruvate uptake very similar to

193 that in *E. coli* MG1655 Δ*btsT* transformed with these plasmids (**Fig. S7**). Therefore,

194 accumulation of pyruvate could not be increased in this mutant, since it is impossible to

195 completely block its metabolization. It is important to state here that expression of

196 chromosomally encoded *btsT* is tightly controlled by the BtsS/BtsR system, and therefore

197 requires specific environmental conditions (9).

198

199   **Characterization of pyruvate transport by BtsT in intact cells.** Previous

200   observations based on the effects of electron transport chain inhibitors on pyruvate transport

201   in *E. coli* K-12 suggested that the process is driven by the proton motive force (16). To test

202   this hypothesis, we used the hydrophobic protonophores 2,4-dinitro-phenol (DNP) and

203   carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) **(Fig. 4A).** Both reagents abolished

204   substrate accumulation by BtsT, suggesting active transport of pyruvate by BtsT. To

205   determine whether pyruvate transport by BtsT is accompanied by the movement of other ion

206   species, several ionophores were tested. Valinomycin is a highly selective ionophore for K^+

207   (34), while nonactin forms complexes with K^+, Na^+, NH_4^+ and some other cations with lower

208   affinity (35). Nigericin acts mainly as a potassium-proton antiporter, but can also transport

209   Pb^+ and H^+ into the cytosol (36). Pyruvate uptake by BtsT was not affected by any of these

210   ionophores (**Fig. 4A**).

211   Additionally, the specificity of BtsT was tested by assaying several compounds with

212   shared structural similarity for the ability to act as competitive inhibitors for the pyruvate

213   binding site when provided in a 100-fold access **(Fig. 4B)**. Br-pyruvate is a synthetic analogue

214   of pyruvate, and was found to act as a competitive inhibitor of BtsT, which is in agreement

215   with earlier studies in *E. coli* K-12 (16). None of the other compounds used – L-malate and

216   lactate (both configurations) with a C-α hydroxyl group, phosphoenolpyruvate with a C-α

217   phosphoryl group, or L-alanine with a positively charged C-α amino group – reduced the rate

218   of pyruvate uptake. We also tested monocarboxylates (acetate and propionate), a

219   dicarboxylate (succinate) and amino acids (L-serine, L-glycine), but the uptake rate was

220   significantly reduced only in the presence of pyruvate or its synthetic analog Br-pyruvate,

221   indicating the narrow specificity of the transporter. In order to determine the K_m for pyruvate

222   uptake, we quantified the initial rate of pyruvate uptake by BtsT at different concentrations of

223   pyruvate **(Fig. 4C).** Pyruvate can enter the cells by diffusion **(Fig. S8).** Therefore, the uptake

11

224   rates were corrected by the values determined for pure diffusion. The K_m of the transporter

225   BtsT for pyruvate measured in intact cells was 16.5 ± 6.4 μM.

226

227   **Effects of $\Delta\tilde{\mu}_{H+}$, $\Delta\Psi$ and ΔpH on BtsT-mediated pyruvate transport.** Energization of

228   pyruvate transport by BtsT was studied in detail in *E. coli* proteoliposomes by determining the

229   consequences for pyruvate transport of an imposed electrical potential ($\Delta\Psi$) across the

230   liposome membrane or a difference in proton concentration (ΔpH) between the interior of the

231   liposomes and the external medium, or a combination of both, thus generating a

232   transmembrane electrochemical proton gradient ($\Delta\tilde{\mu}_{H+}$). To this end, BtsT was first expressed

233   at high levels under the control of the pBAD promoter, affinity-purified and integrated into

234   proteoliposomes preloaded with potassium phosphate buffer, pH 7.6 (37) **(Fig. S9)**. The

235   proteoliposomes were then diluted 200-fold, in the presence of valinomycin, with either

236   potassium phosphate buffer pH 5.8 (ΔpH), potassium-free buffer pH 7.6 ($\Delta\Psi$), potassium-free

237   buffer pH 5.8 ($\Delta\tilde{\mu}_{H+}$) or sodium phosphate buffer pH 7.6 ($\Delta\tilde{\mu}_{Na+}$), and the rates of [14]C-

238   pyruvate transport were measured.

239   From the results, it is clear that the presence of an electrochemical H^{+} gradient ($\Delta\tilde{\mu}_{H+}$)

240   is associated with the accumulation of ^{14}C-pyruvate in proteoliposomes, and that uptake is

241   markedly reduced in the absence of such a gradient (dilution in potassium phosphate buffer

242   pH 7.6; **Fig. 5A**). Thus, the initial rate of uptake of pyruvate by BtsT in the presence of $\Delta\tilde{\mu}_{H+}$

243   is 27.7 nmol per mg of protein, and the steady state is achieved after about 1 min **(Fig. 5B)**.

244   Imposition of ΔpH decreased the initial rate by two-fold and a lower steady state (relative to

245      $\Delta\tilde{\mu}_{H+}$) was reached after 1 min. In the presence of a $\Delta\Psi$, we observed a similar initial rate of

246   pyruvate accumulation as in the presence of ΔpH, but it continued to rise for the (10-min)

247   duration of the experiment. ^{14}C-Pyruvate was also accumulated upon the application of $\Delta\tilde{\mu}_{Na+,}$

248   although the Na$^+$ ions did not stimulate uptake beyond the level seen with ΔpH alone (**Fig.**

249   **5A**).

250   Pyruvate could potentially cross the *E. coli* plasma membrane in its protonated form

251   by simple diffusion (38). In the experiments where a pH gradient is generated (ΔpH, interior

252   alkaline, exterior acidic), the ^{14}C-pyruvate is added to an extracellular environment with a low

253   pH. Therefore, ^{14}C-pyruvate might be protonated and transported by diffusion. To rule out

254   this possibility, liposomes without BtsT were prepared as a control (**Fig. S10**). Using these

255   liposomes, we observed only a slight pyruvate accumulation. Therefore, the protonation of

256   pyruvate at low pH does not significantly affect the outcome of these experiments (**Fig. S10**).

257   Lastly, we confirmed the functionality of the transporter BtsT in proteoliposomes in a

258   counterflow assay. In this case, we preloaded the proteoliposomes with a high internal

259   concentration (10 mM) of unlabeled ("cold") pyruvate and subsequently diluted them 200-

260   fold in the same buffer containing a low concentration of ^{14}C-pyruvate (4.7 μM). If pyruvate

261   was indeed transported, exchange of the internal (unlabeled) substrate and external (labeled)

262   pyruvate should lead to the accumulation of ^{14}C-pyruvate in the vesicles due to the

263   concentration gradient across the membrane. We observed such an accumulation of ^{14}C-

264   pyruvate via BtsT in proteoliposomes, which was low (0.28 nmol x mg^{-1} x min^{-1}), but

265   significant in light of the competitive inhibition by unlabeled pyruvate (**Fig. 5C**). In the

266   control experiment, proteoliposomes preloaded with 10 mM unlabeled lactate exhibited no

267   ^{14}C-pyruvate accumulation. These experiments thus demonstrate that BtsT-mediated uptake

268   of pyruvate can occur against a concentration gradient.

269   **DISCUSSION**

270   Pyruvate plays a central role in carbon metabolism. Nevertheless, little is known about

271   how intracellular levels of the compound are regulated in *E. coli*. It has been suggested that

272   pyruvate uptake is driven via specific active transporters (16, 17), but until now their

273   molecular identities have remained unknown.

274   However, interest in pyruvate transporters is growing, primarily as a result of increasing

275   evidence for their roles in biological fitness and virulence of *Enterobacteriaceae*. The

276   pyruvate-tricarboxylic acid cycle node was identified as a focal point for controlling the host

277   colonization and virulence of *Yersinia pseudotuberculosis* (39). *Y. pseudotuberculosis*

278   secretes high amounts of pyruvate when grown in minimal medium with glucose as carbon

279   source. However the pyruvate exporter or importer in *Y. pseudotuberculosis* has not yet been

280   characterized.

281   Metabolic engineering is also focusing its attention on pyruvate in efforts to achieve

282   optimized metabolite production in *E. coli*, *Corynebacterium glutamicum* or *Bacillus subtilis*

283   (40). However, to control the carbon flux between glycolysis and the TCA cycle, further

284   insights into the regulation and characterization of the enzymes and other proteins relevant to

285   the PEP-pyruvate-oxaloacetate node are still required.

286   Here, we were principally interested in elucidating the role of the pyruvate transporter

287   in *E. coli*. It is known that pyruvate overflow occurs under conditions of carbon excess during

288   exponential growth. At the end of the exponential phase, cells experience nutrient limitation

289   and initiate the rapid re-uptake of previously excreted metabolic intermediates, including

290   pyruvate (13). This extracellular pyruvate is sensed by the two-component system BtsS/BtsR,

291   which consequently activates the expression of *btsT* encoding the putative pyruvate

292   transporter BtsT (7, 9). BtsT belongs to the APC superfamily of secondary transporters (2,

293   41). Comparative genomic studies clearly separate BtsT from CstA, and it has been proposed

14

294 that the two have different functions (**Fig. 1**). Since the histidine kinase BtsS responds to

295 extracellular pyruvate (9), we hypothesized that BtsS/BtsR triggers the production of BtsT

296 transporter to enable cells to take up pyruvate as a scavenging strategy to cope with carbon

297 limitation. Here, we have shown that BtsT indeed serves as a pyruvate transporter in *E. coli*

298 and thus assigned a function to a representative of the CstA family for the first time.

299 First we studied the function of BtsT in intact cells and characterized the protein as a

300 highly specific pyruvate transporter with a K_m of 16 µM (**Fig. 4**). Furthermore, we showed

301 that the pronophores CCCP and DNP had a significant inhibitory effect on pyruvate transport

302 rates, indicating involvement of a $\Delta\Psi$ and/or ΔpH. In comparison, both valinomycin and

303 nigericin did not cause inhibition of pyruvate uptake by BtsT even though these compounds

304 dissipate either $\Delta\Psi$ or ΔpH, respectively. Likewise, valinomycin had no effect on pyruvate

305 transport by *L. plantarum* cells (19). The lack of an inhibitory effect of valinomycin suggests

306 that intact cells somehow circumvent inhibition by a thus far unknown mechanism.

307 Elimination of all the influences from intact cells is therefore required to obtain reliable

308 insights into the energization of pyruvate transport.

309 It is worth mentioning that *E. coli* probably harbors at least one additional pyruvate

310 transporter, based on the observed background pyruvate accumulation by intact cells of an *E.*

311 *coli* Δ*btsT* strain (**Fig. 3**), which cannot be solely attributed to diffusion (**Figs. S6 and S8).** It

312 was previously suggested that *E. coli* uses one export and two uptake transporters to modulate

313 the level of intracellular pyruvate (17). Unfortunately, we were unable to identify other

314 pyruvate transporters based on the BtsT sequence, and contrary to a previous suggestion (17),

315 we could find no BtsT-like coding sequence near the 15 centisome position in the *E. coli*

316 genome.

317 Further insights into the mode of action of BtsT were obtained by incorporating the

318 protein into *E. coli* proteoliposomes. In these experiments, a suspension of proteliposomes

15

319   harboring BtsT was assayed for ^{14}C-pyruvate transport under conditions known to generate

320   either $\Delta\tilde{\mu}_{H+}$, $\Delta\Psi$ or ΔpH **(Fig. 5)**. From the results, it is obvious that simultaneous imposition

321   of a membrane potential and a proton gradient ($\Delta\tilde{\mu}_{H+}$, interior negative and alkaline) is

322   required to drive pyruvate uptake into *E*. *coli* proteoliposomes. In the presence of either $\Delta\Psi$

323   (interior negative) or ΔpH (interior alkaline), the rate of transport was halved in comparison to

324   that observed in the presence of $\Delta\tilde{\mu}_{H+}$. The two gradients most likely contribute with their

325   actual extent to the energization of uptake **(Fig. 5)**. Therefore, it seems that the secondary

326   transport of pyruvate by BtsT is coupled to both the influx of protons and the movement of

327   electric charges. Hence, BtsT acts as a pyruvate/H^{+} symporter. Although the exact

328   pyruvate/H^{+} coupling stoichiometry is still unknown, the results suggest a ratio of more than

329   one proton per transported pyruvate.

330   In summary, BtsT is the first identified pyruvate transporter in *E*. *coli* and the first

331   characterized representative of the CstA family. This study opens the possibility to search for

332   the pyruvate-binding site in BtsT as well as to elucidate the 3D structure of this transporter. It

333   will be interesting to determine which of the 18 putative TMs in BtsT are essential for the

334   recognition and transport of pyruvate. Currently, we can only speculate that the two conserved

335   5-TM repeat units found in members of the CstA family (2) are crucial for this function. BtsT

336   contains 8 additional TMs (4 TMs each before and after the repeat unit) which might perhaps

337   interact with the histidine kinase BtsS (25) or have other regulatory or structural functions.

338

339

MATERIALS AND METHODS

340

341 **Bacterial strains and plasmids.** In this study we used the previously described *E. coli* strains

342 LMG194 [F⁻ Δ*lacX74 galE galK thi rpsL* Δ*phoA* Δ*ara714 leu*::Tn*10*] (32), MG16555 Δ*btsT*

343 (previously termed MG1655 Δ*yjiY)* (25), MM39 (*araD, lacI,* Δ*U1269* and *malE444*) (29) and

344 YYC202 (Δ*aceEF,* Δ*pflB,* Δ*poxB*, and Δ*pps*) (33), and constructed two new plasmids:

345 pMAL$_P$-*btsT* and pMAL$_c$-*btsT*. The corresponding *btsT*-6His gene was isolated from plasmid

346 pBAD24-*btsT* (previously known as pBAD24-*yjiY* (7)) by cleavage with EcoRI and XbaI, and

347 ligated into the cognate sites in vectors pMAL-p2X and pMAL-c2X (New England Biolabs).

348 For transport studies we used the arabinose-inducible vector pBAD24-*btsT* (7), which codes

349 for BtsT-6His, and pBAD24 (32) as control.

350

351 **Growth conditions.** All strains were grown overnight in LB medium (10 g/l tryptone, 5 g/l

352 yeast extract, 10 g/l NaCl) or M9 minimal medium containing 0.5% [wt/vol] maltose or 20

353 mM pyruvate. Cells from the overnight culture were then transferred to corresponding fresh

354 medium. When appropriate, media were supplemented with ampicillin (sodium salt, 100

355 μg/ml) and/or streptomycin (50 μg/ml). After inoculation, bacteria were grown under

356 agitation (200 rpm) at 37ºC and growth was monitored over time by measuring the optical

357 density at 600 nm (OD$_{600}$).

358

359 **Production of MBP hybrid proteins.** Cells were grown to an OD$_{600}$ of 0.5 as described

360 above. Recombinant gene expression was induced by addition of 0.2% (w/v) arabinose. After

361 3 h of further incubation, cells were harvested by centrifugation, disrupted [using a high

362 pressure cell disrupter (Constant Systems)] and fractionated. At each step, the pellet was

363 resuspended in a buffer consisting of 50 mM Tris/HCl buffer (pH 7.5), 10% (v/v) glycerol, 1

364 mM DTT, 1 mM PMSF and DNase. His-tagged BtsT was detected by Western blot analysis

17

365 with an anti-His antibody (Abcam) and an alkaline phosphatase-conjugated anti-rabbit
366 antibody (Rockland) as the secondary antibody.

367

368 **Comparative genomic studies.** BtsT-like proteins were identified by Protein BLAST search
369 within the *Enterobacteriaceae* (taxid: 543) using the full-length amino acid sequence of
370 *E. coli* BtsT to query the RefSeq protein database (23). In order to cover a huge amount of
371 highly diverse proteins an Expect (E) value was kept at < 10. An amino acid length tolerance
372 was set to 10% as default parameter resulting in 1,617 sequences. To elucidate the
373 relationship between the BtsT homologs, all sequences were aligned and a phylogenetic tree
374 was generated using CLC Main Workbench 7 (CLC Bio Qiagen). BtsT and CstA of *E. coli*
375 were used as branch markers. Comparison of BtsT conserved in different species, *E. coli* and
376 *S. enterica*, was done via a consensus alignment. For this purpose, a total of 148 sequences of
377 BtsT in *E. coli* (or 122 in *S. enterica*) was retrieved from the branch BtsT of the phylogenetic
378 tree. The consensus sequence was derived from their alignment using CLC Main Workbench
379 7 software.

380

381 **Transport measurements with intact cells.** *E. coli* strain MG1655 Δ*btsT* (25) was
382 transformed with pBAD24 (32) or pBAD24-*btsT* (7). Cells grown in LB medium in the
383 absence of arabinose (the leaky expression of *btsT* was suitable for complementation) were
384 harvested in the mid-log phase. Cells were washed twice and resuspended in 100 mM
385 Tris/MES buffer (pH 7.5) containing 5 mM $MgCl_2$, thereby adjusting the total protein
386 concentration to 0.35 mg/ml. In the experiments performed at different external pH values,
387 the cells were washed and resuspended in buffers: Tris/MES buffer (pH 6), 5 mM $MgCl_2$ or
388 100 mM citrate buffer (pH 3 / pH 4.5), 5 mM $MgCl_2$. Uptake of ^{14}C-pyruvate (50-60 mCi
389 mmol^{-1}, Biotrend) was measured at a total substrate concentration of 10 μM at 15ºC. At

390   various time intervals, transport was terminated by the addition of 100 mM potassium

391   phosphate buffer (pH 6.0) and 100 mM LiCl (stop buffer) followed by rapid filtration through

392   membrane filters (MN GF-5 0.4 μm, Macherey-Nagel). The filters were dissolved in 5 ml of

393   scintillation fluid (MP Biomedicals) and radioactivity was determined in a Liquid Scintillation

394   Analyzer (Perkin-Elmer). Effects of the cold substrates on pyruvate uptake by BtsT were

395   tested by simultaneous addition of cold compound (1 mM) and ^{14}C-pyruvate (10 μM). The

396   effects of protonophores and ionophores were tested after pre-incubation of cells in Tris/MES

397   buffer (pH 7.5) supplemented with 2 mM DNP or 20 μM CCCP, 6 μM nigericin, 10 μM

398   nonactin or DMSO (as control) at 25ºC for 30 min. An ionophore valinomycin was pre-

399   incubated in 100 mM potassium phosphate buffer, pH 7.5 at 25ºC for 30 min. In experiments

400   where the pyruvate concentration was varied, the amount of ^{14}C-pyruvate was kept constant

401   (9 nmol).

402

403   **Transport measurements with proteoliposomes.** *E. coli* LMG194 cells were transformed

404   with pBAD24-*btsT* (7), and BtsT-6His was produced as described above. At each step, the

405   pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.6) containing 5 mM

406    $MgCl_2$, 5% (v/v) glycerol, 1 mM DTT, 1 mM PMSF and DNase. For BtsT solubilization,

407   1.5% (w/v) *n*-dodecyl β-D-maltoside (Glycon Biochemicals) was added while stirring on ice

408   for 30 min, and the sample was centrifuged at 244,000 x *g* for 45 min at 4°C. Ni^{2+}-NTA resin,

409   which had been pre-incubated with buffer W [100 mM potassium phosphate buffer (pH 7.6),

410   5 mM $MgCl_2$, 5% (v/v) glycerol and 1 mM DTT], was then added to the supernatant and the

411   mixture was incubated for 1 h with gentle shaking at 4ºC. Unbound protein was removed by

412   washing with buffer W containing 30 mM imidazole. Subsequently, BtsT was eluted with

413   buffer W containing 300 mM imidazole. Purified BtsT was reconstituted into preformed

414   liposomes prepared from an acetone-ether-washed *E. coli* polar lipid extract as described

415   before (42). Prior to reconstitution, the liposomes were destabilized by the addition of 0.12%

416   (w/v) Triton X-100, and then mixed with purified protein at a ratio 100:1. Detergents were

417   removed by adding Bio-Beads SM-2 (Bio-Rad) at a bead/detergent ratio of 5:1. Fresh Bio-

418   Beads were added after 1 h and 2 h, and the mixture was gently stirred at 4ºC overnight. Bio-

419   Beads were removed by filtration through glass wool, and the proteoliposome suspension was

420   dialyzed twice against buffer A [100 mM potassium phosphate buffer (pH 7.6), 5 mM $MgCl_2$

421   and 5% (v/v) glycerol], and centrifuged at 160,000 x *g* for 1 h. The pellet was resuspended in

422   buffer A and stored in liquid N_2. Proteins were visualized by silver-staining and His-tagged

423   BtsT was detected by Western blot analysis as described before. After thawing, samples were

424   extruded through a 400-nm filter (Avestin) to obtain unilamellar proteoliposomes of

425   homogeneous size, and adjusted to 4 mg/ml of protein in buffer A. Aliquots of

426   proteoliposomes were diluted (1:200) into buffers A-E containing 80 µM valinomycin and 40

427   µM ^{14}C-pyruvate to initiate pyruvate transport, and at defined times (25ºC) the entire sample

428   was filtered through 0.2-µm Millipore filters [buffer B (ΔpH): 100 mM potassium phosphate

429   buffer (pH 5.8) and 5 mM $MgCl_2$, buffer C (ΔΨ): 100 mM Tris/MES buffer (pH 7.6) and 5

430   mM $MgCl_2$, buffer D ($\Delta\tilde{\mu}_{H+}$): 100 mM Tris/MES buffer (pH 5.8) and 5 mM $MgCl_2$, buffer E

431   ($\Delta\tilde{\mu}_{Na+}$): 100 mM Tris/MES (pH 5.8), 5 mM $MgCl_2$ and 50 mM NaCl]. For the counterflow

432   assay, proteoliposomes were preloaded with 10 mM pyruvate or lactate at 4ºC overnight. The

433   resulting suspension was diluted 200-fold into buffer A containing ^{14}C-pyruvate at a

434   concentration of 4.7 µM (final external pyruvate concentration of 54.7 µM). At defined times

435   (25ºC) the entire sample was filtered, and the radioactivity of the sample was analyzed as

436   described above.

437

438   **Statistical analysis.** All experiments were repeated at least three times with independently

439   prepared samples. Statistical analysis was carried out using GraphPad Prism (version 5.03 for

440　　Windows). Data was assessed for significance between tested groups by using one-way

441　　analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test. Error

442　　bars represent standard errors of the mean.

443

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566

FIGURE LEGENDS

568 **Figure 1. Phylogenetic tree of BtsT-like proteins in *Enterobacteriaceae*.** The tree was

569 constructed based on the alignment of 1,617 individual BtsT-like sequences, and visualized

570 with CLC Main Workbench 7. The lengths of the branches represent the relative amount of

571 evolutionary divergence between any two sequences in the tree.

572

573 **Figure 2. Schematic model of the secondary structure of *E. coli* BtsT.** The model is based

574 on the results of an analysis carried out by the Uniprot program (27), coupled with the

575 experimental evidence for the cytoplasmic location of the N-terminus and the alignment with

576 CstA. The secondary structure of BtsT was visualized using the Protter tool (43).

577 Transmembrane domains (TMs) are numbered. The conserved motif CG-x(2)-SG within CstA

578 homologs is marked in yellow (2). PP, periplasm; CM, cytoplasmic membrane; CP.

579 cytoplasm.

580

581 **Figure 3. Time course of pyruvate uptake by *E. coli* MG1655 Δ*btsT*.** Rates of ^{14}C-pyruvate

582 uptake by the BtsT-producing strain *E. coli* MG1655 Δ*btsT* pBAD24-*btsT* (green) and the

583 control strain *E. coli* MG1655 Δ*btsT* pBAD24 (gray) were measured at a final pyruvate

584 concentration of 10 µM at 15°C. Standard deviations are estimated from three biological

585 replicates.

586

587 **Figure 4. Characterization of BtsT-mediated pyruvate uptake by intact *E. coli* cells.** [14]C-

588 pyruvate uptake was analyzed in freshly grown cells expressing *btsT* from pBAD24-*btsT*.

589 Initial uptake rates were measured at a pyruvate concentration of 10 µM. (A) Effects of the

590 indicated protonophores and ionophores on pyruvate uptake by BtsT. Cells were pre-

591   incubated at room temperature with the inhibitors for 30 min. (B) Effects of the indicated

592   substrates on pyruvate uptake by BtsT. Each individual compound (1 mM) was added

593   simultaneously with ^{14}C-pyruvate. ANOVA was used to calculate the statistical significance

594   in panels A and B (*P* values are denoted as * $P \leq 0.05$, ** $P \leq 0.01$). Standard deviations (A

595   and B) are estimated from three biological replicates with no background correction. (C) The

596   K_m value was determined by quantification of the initial rate of pyruvate uptake by BtsT in the

597   presence of various concentrations of the substrate. The values were corrected by the

598   determined diffusion rates (Fig. S8). The best-fit line was determined by nonlinear regression

599   using the equation y = Bmax * x / (Kd + x). Error bars represent standard error of the mean.

600

601   **Figure 5. Influence of $\Delta\tilde{\mu}_{H+}$, $\Delta\Psi$, ΔpH and $\Delta\tilde{\mu}_{Na+}$ on BtsT-mediated pyruvate uptake into**

602   **proteoliposomes of *E. coli*.** Uptake of ^{14}C pyruvate was analyzed using proteoliposomes

603   reconstituted with purified BtsT. Uptake rates were measured at a pyruvate concentration of

604   40 μM. (A) Time course of pyruvate uptake in the presence of artificially imposed $\Delta\tilde{\mu}_{H+}$

605   (green), $\Delta\Psi$ (orange), ΔpH (red) or $\Delta\tilde{\mu}_{Na+}$ (brown), or in the absence of any gradient (gray).

606   (B) Initial rates of pyruvate uptake by BtsT calculated using linear regression during the linear

607   uptake phase. (C) Counterflow experiment. Uptake of ^{14}C-pyruvate into proteoliposomes

608   preloaded with pyruvate (green). In the control experiment proteoliposomes were preloaded

609   with lactate (gray). ANOVA was used to calculate the statistical significance (*P* values in B

610   are denoted as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). Standard deviations are estimated

611   from three technical replicates.

612

FIG1

FIG 2

FIG 3

FIG 4

A

B

C

K_m = 16.5 μM

FIG 5

Chapter 4

A single-cell view of the BtsSR/YpdAB pyruvate sensing network in *Escherichia coli* and its biological relevance

A Single-Cell View of the BtsSR/YpdAB Pyruvate Sensing Network in *Escherichia coli* and Its Biological Relevance

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ABSTRACT Fluctuating environments and individual physiological diversity force bacteria to constantly adapt and optimize the uptake of substrates. We focus here on two very similar two-component systems (TCSs) of *Escherichia coli* belonging to the LytS/LytTR family: BtsS/BtsR (formerly YehU/YehT) and YpdA/YpdB. Both TCSs respond to extracellular pyruvate, albeit with different affinities, typically during post-exponential growth, and each system regulates expression of a single transporter gene, *yjiY* and *yhjX*, respectively. To obtain insights into the biological significance of these TCSs, we analyzed the activation of the target promoters at the single-cell level. We found unimodal cell-to-cell variability; however, the degree of variance was strongly influenced by the available nutrients and differed between the two TCSs. We hypothesized that activation of either of the TCSs helps individual cells to replenish carbon resources. To test this hypothesis, we compared wild-type cells with the *btsSR ypdAB* mutant under two metabolically modulated conditions: protein overproduction and persister formation. Although all wild-type cells were able to overproduce green fluorescent protein (GFP), about half of the *btsSR ypdAB* population was unable to overexpress GFP. Moreover, the percentage of persister cells, which tolerate antibiotic stress, was significantly lower in the wild-type cells than in the *btsSR ypdAB* population. Hence, we suggest that the BtsS/BtsR and YpdA/YpdB network contributes to a balancing of the physiological state of all cells within a population.

IMPORTANCE Histidine kinase/response regulator (HK/RR) systems enable bacteria to respond to environmental and physiological fluctuations. *Escherichia coli* and other members of the *Enterobacteriaceae* possess two similar LytS/LytTR-type HK/RRs, BtsS/BtsR (formerly YehU/YehT) and YpdA/YpdB, which form a functional network. Both systems are activated in response to external pyruvate, typically when cells face overflow metabolism during post-exponential growth. Single-cell analysis of the activation of their respective target genes *yjiY* and *yhjX* revealed cell-to-cell variability, and the range of variation was strongly influenced by externally available nutrients. Based on the phenotypic characterization of a *btsSR ypdAB* mutant compared to the parental strain, we suggest that this TCS network supports an optimization of the physiological state of the individuals within the population.

KEYWORDS histidine kinase, nutrient limitation, overflow metabolism, persister cells

Typical two-component systems (TCSs) consist of a membrane-bound histidine kinase (HK), which perceives a stimulus, and a cytoplasmic response regulator (RR), which triggers an appropriate response (1, 2). *Escherichia coli* contains 30 TCSs in all.

FIG 1 Model of the nutrient-sensing BtsS/BtsR and YpdA/YpdB network in *E. coli*. The scheme summarizes the signal transduction cascades triggered by the BtsS/BtsR and YpdA/YpdB systems and the influence of other regulatory elements. Activating (→) and inhibitory (⊢) effects are indicated. PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm. See the text for details.

Members of the LytS/LytTR family make up one prominent class of TCSs, representatives of which are found in many microorganisms. Examples include AgrC/AgrA from *Staphylococcus aureus*, which is involved in the transition from the persistent, avirulent state to the virulent phenotype (3), while FsrC/FsrA from *Enterococcus faecalis* is responsible for the production of virulence-related proteases (4), and VirS/VirR from *Clostridium perfringens* induces the synthesis of exotoxins and collagenase (5, 6). In our laboratory, we are studying the only two known members of the LytS/LytTR family in *E. coli*: BtsS/BtsR (previously YehU/YehT) and YpdA/YpdB (7–10). These two TCSs not only share the same domain structure, they also display over 30% identity at the amino acid sequence level (9). BtsS/BtsR activation leads to the expression of *yjiY*, YpdA/YpdB activation results in *yhjX* expression (Fig. 1). Both target genes code for transporters, which belong to different transporter families: YjiY is a member of the CstA family, and YhjX has been assigned to the oxalate/formate antiporter (OFA) family (7, 8, 11). In addition, the cyclic AMP (cAMP) receptor protein (CRP) complex (CRP-cAMP) upregulates *yjiY* at the transcriptional level (7), whereas the carbon storage regulator A (CsrA) upregulates *yhjX* and downregulates *yjiY* at the posttranscriptional level.

In previous studies we found functional interconnectivity of the two TCSs (9). Deletion of either component of the TCS or its target gene influences the level of expression of the target gene regulated by the other TCS and vice versa (9). In addition, *in vivo* protein-protein interaction assays suggested that the two systems form a single, large signaling unit (Fig. 1). Moreover, when *E. coli* was grown in tryptone-based (LB) medium, both systems are activated at the onset of the post-exponential growth phase (9). A more refined study revealed that the BtsS/BtsR system is activated in the presence of extracellular pyruvate (at a threshold concentration of 50 μM) under nutrient-depleted conditions (10). Biochemical studies confirmed that BtsS is a high-affinity pyruvate receptor ($K_d = 58.6$ μM) (10). Recently, the corresponding YjiY transporter was characterized as a high-affinity pyruvate/H$^+$ symporter (12). The YpdA/YpdB system also responds to extracellular pyruvate, albeit at a higher threshold concentration of 600 μM (8).

The biological significance of the BtsS/BtsR and YpdA/YpdB network is still unclear. To explore this issue, we determined the activation states of the two systems at the single-cell level in *E. coli* populations. Using separate fluorescence reporter strains for each system, we found a correlation between the available nutrient resources and the degree of heterogeneity in the transcriptional responses of the target gene promoters in individual cells. Based on this finding and further phenotypic analyses, we suggest that the BtsS/BtsR and YdpA/YpdB systems play a role in optimization of the physiological status of the individual cells within the population.
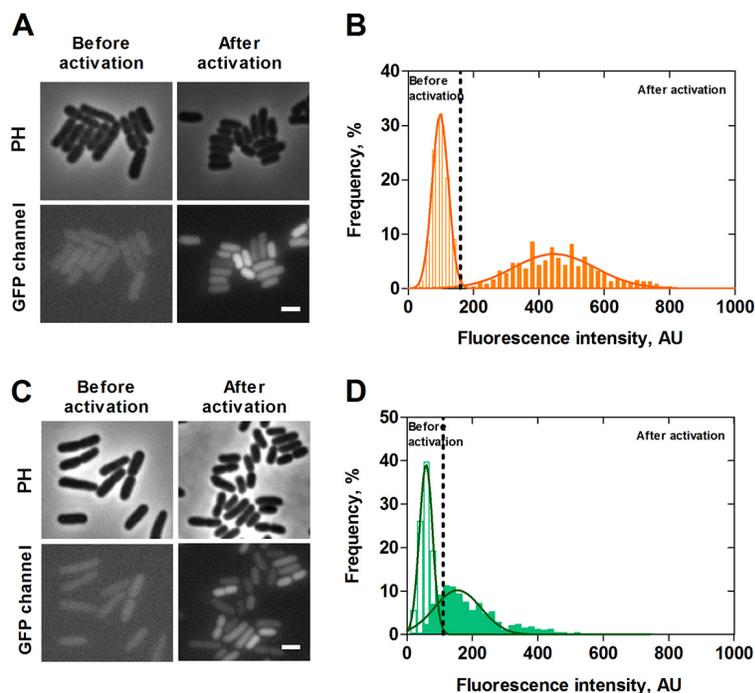
FIG 2 Single-cell analysis of P_{yhjX} and P_{yjiY} activation during growth in LB medium. *E. coli* cells expressing *gfp* under the control of the *yhjX* or *yjiY* promoter, respectively, were grown in LB medium, and fluorescence micrographs were taken before (exponential growth phase) and after activation (post-exponential growth phase) of the two TCSs. Representative fluorescence and phase-contrast images of P_{yhjX}-*gfp* and P_{yjiY}-*gfp* reporter strains are shown in panels A and C, respectively. The corresponding distributions of the fluorescence intensity of the P_{yhjX}-*gfp* and P_{yjiY}-*gfp* reporter strains are depicted in panels B and D. Unfilled bars refer to values prior to activation, and filled bars refer to values observed after activation. Dashed lines represent the threshold of activation for each of the reporter strains. A total of 200 cells were analyzed in each experiment, and frequency refers to the percentage of cells with the indicated intensity (see Materials and Methods for details). The continuous curves represent Gaussian fits based on the histograms of the fluorescence intensity. PH, phase contrast; AU, arbitrary units. Scale bar, 2 μm. Experiments were performed independently three times.

RESULTS

Heterogeneous activation of P_{yhjX}-*gfp* and P_{yjiY}-*gfp*. For the BtsS/BtsR and YpdA/YpdB systems, population-based studies have shown that the promoters of their respective target genes, *yjiY* and *yhjX*, are activated in cells which face nutrient limitation and sense the presence of external pyruvate (9, 10). Since both systems are linked to form a network, we analyzed the activation of these two promoters at the single-cell level. We constructed fluorescent reporter strains by fusing the promoter regions of *yhjX* and *yjiY* to *gfp* and introduced each fusion separately into the genome of *E. coli* MG1655 via single homologous recombination at the native locus. Using this strategy, the regulatory inputs to the native promoters of *yjiY* and *yhjX* were maintained (9), as the promoter fused to *gfp* is inserted upstream of the original one (13). The fluorescence intensity of green fluorescent protein (GFP) was used to quantify the activity of the two promoters, thus allowing us to study the transcriptional activation of *yjiY* and *yhjX* in single cells. The growth rates in LB medium of strains containing a chromosomal copy of either promoter fusion (from now on referred to as P_{yhjX}-*gfp* and P_{yjiY}-*gfp*) were similar to that of the MG1655 wild-type strain (see Fig. S1 in the supplemental material).

From population studies it is known that in cells grown in LB medium, which is rich in amino acids and leads to the overflow of pyruvate, both promoters are activated at the onset of the post-exponential growth phase (9). Hence, as expected, at the single-cell level neither P_{yhjX}-*gfp* nor P_{yjiY}-*gfp* showed any activity during the exponential growth phase (Fig. 2A and C, before activation) in LB medium. However, when cells reached the end of the exponential growth phase, we observed activation of the *yhjX*
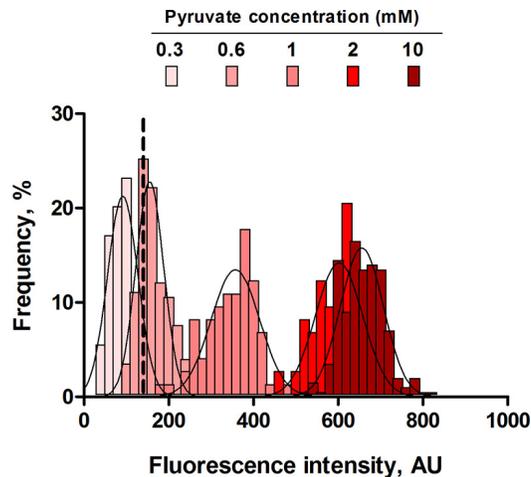
FIG 3 Effects of different external pyruvate concentrations on P_{yhjX}-*gfp* activation at the single-cell level. *E. coli* cells expressing *gfp* under the control of the P_{yhjX} promoter were grown in M9 minimal medium containing increasing concentrations of pyruvate (supplemented with succinate; final carbon concentration, 20 mM) and analyzed by fluorescence microscopy. A total of 200 cells was analyzed in each experiment at the time point of maximal expression, and frequency refers to the percentage of cells with the indicated intensity (see Materials and Methods). Histograms of the fluorescence intensities of cells were fitted using a Gaussian distribution (solid line). The dashed line represents the threshold of activation for the reporter strain. AU, arbitrary units. Experiments were performed independently three times.

promoter, as indicated by a shift of the distribution of fluorescence intensities to higher levels (Fig. 2A and B, after activation) in the majority of the population, albeit with a high degree of cell-to-cell variability as seen in the width of the Gaussian distribution (noise value [standard deviation divided by the mean] = 0.27). Less than 4% of the population was found to be nonfluorescent and therefore did not respond (the threshold of activation is marked by the dashed line in Fig. 2B). To differentiate these cells from dead cells, we stained cells with propidium iodide and found that dead cells made up only 0.4% of the population (data not shown).

Cells of the P_{yjiY}-*gfp* strain cultivated in LB medium also showed heterogeneous activation upon entry into the post-exponential growth phase. These strains exhibited an even higher noise value of 0.52 and a higher percentage of nonresponding cells (9%) (Fig. 2D) (the percentage of dead cells was determined to be 0.6%).

To determine the basal noise level of a promoter in cells at this growth phase, we performed a control experiment, in which *gfp* expression is controlled by a synthetic vegetative promoter (pXGSF). Cells harboring the vector pXGSF activate this promoter at the post-exponential growth phase (R. Hengge, unpublished data). In this experiment the promoter was activated in all the cells, and the variability was lower (i.e., 0.13) than that observed for either P_{yjiY}-*gfp* or P_{yhjX}-*gfp*. Taken together, these results indicate a heterogeneous, almost unimodal pattern of transcriptional activation for each of the two target genes of the BtsS/BtsR and YpdA/YpdB systems at the end of the exponential phase, when cells are grown in LB medium.

The degree of heterogeneity of P_{yhjX}-*gfp* activation depends on the external pyruvate concentration. Although the exact nature of the primary stimulus for the YpdA/YpdB system remains elusive, we know from previous studies that P_{yhjX} is activated in cells which are exposed to extracellular pyruvate concentrations greater than 0.6 mM (9). Aiming to further explore the single-cell behavior of this promoter activity, we analyzed the pyruvate dependence of the activation of YpdA/YpdB by determining the fluorescence intensities of P_{yhjX}-*gfp* reporter cells cultivated in M9 minimal medium supplemented with increasing concentrations of pyruvate (succinate was added to keep the total carbon concentration constant at 20 mM) (Fig. 3). As expected, a pyruvate concentration below the threshold (0.3 mM) failed to activate the YpdA/YpdB system in single cells. At pyruvate concentrations above the threshold, all
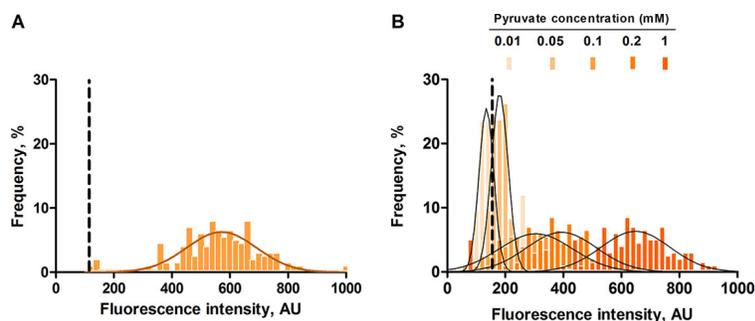
FIG 4 Effects of different external pyruvate concentrations on P$_{yjiY}$-*gfp* activation at the single-cell level. *E. coli* cells expressing *gfp* under the control of the P$_{yjiY}$ promoter were grown in a nutrient-poor environment (0.1× LB medium) for 1 h. The medium was then supplemented with 20 mM pyruvate (A) or with increasing pyruvate concentrations (B), and the cells were subsequently analyzed by fluorescence microscopy. A total of 200 cells were analyzed for each experiment, and frequency is represented as a percentage of the cells (refer to Materials and Methods for a detailed explanation). Histograms of the fluorescence intensities of cells were fitted using a Gaussian distribution (solid line). Dashed lines represent the threshold of activation for the reporter strain. AU, arbitrary units. Experiments were performed three independent times. For further details, see the legends to Fig. 2 and 3.

cells in the population homogenously activate the *yhjX* promoter. The presence of 0.6 mM pyruvate in the medium generated a low, but detectable P$_{yhjX}$-*gfp* signal in the cells and the presence of 1 mM pyruvate shifted the expression level toward higher values. Interestingly, the response was markedly less heterogeneous (noise value of 0.18) in cells grown under these conditions than in the cells grown in LB medium (noise value of 0.27). Further increases in the external pyruvate concentration (2 and 10 mM) boosted the signal intensities, while the variability further decreased (to 0.09 and 0.07, respectively) (Fig. 3). These results reveal a correlation between external pyruvate availability and P$_{yhjX}$-*gfp* activation.

The degree of heterogeneity of P$_{yjiY}$-*gfp* activation is influenced both by the external pyruvate level and the metabolic state of the cells. As previously described, growth of cells in M9 minimal medium with pyruvate as sole carbon source (20 mM) is not sufficient to activate the P$_{yjiY}$ promoter, because both extracellular pyruvate and nutrient limitation are needed to trigger BtsS/BtsR activation (10). Therefore, our reporter strain had first to be exposed to nutrient limitation (growth in 0.1× LB medium for 1 h) before pyruvate was added. Under these conditions, no activation of P$_{yjiY}$-*gfp* was detected (data not shown), in accordance with our previous studies (10). Pyruvate was then added to the cell culture at a final concentration of 20 mM, and cells were analyzed by fluorescence microscopy at various time points. Cells responded within 70 min and exhibited a higher average *gfp* intensity than cells grown in LB medium, which confirmed the strong response of the BtsS/BtsR system to pyruvate after exposure of cells to nutrient limitation (Fig. 4A). Remarkably, in this case activation of P$_{yjiY}$-*gfp* remained heterogeneous (noise value of 0.27) in spite of the abundance of pyruvate. Subsequently, we tested five different pyruvate concentrations to assess the pyruvate concentration dependence of BtsS/BtsR activation (Fig. 4B). Below the threshold of 50 μM (0.01 mM) to which BtsS/BtsR responds, there was no detectable P$_{yjiY}$-*gfp* signal. As expected, only a few cells produced a weak GFP signal in an environment containing 0.05 mM pyruvate. Starting at a concentration of 0.1 mM pyruvate, P$_{yjiY}$ activation was found in all cells, but with a high degree of cell-to-cell variability (noise value of 0.27). At higher pyruvate concentrations, the signal intensity increased, but the noise values were unchanged. The broad Gaussian distribution found at 1 mM pyruvate resembled the profile found for cells at 20 mM pyruvate (Fig. 4A). A *t* test was performed on the mean values of the two distributions, and the *P* value was determined to be 0.88. This value revealed that there is no significant difference between the cellular responses at 1 and 20 mM pyruvate. These results confirmed at the single-cell level that BtsS/BtsR-mediated activation of P$_{yjiY}$-*gfp* is not only dependent on the pyruvate concentration but is also influenced by internal nutrient limitation.
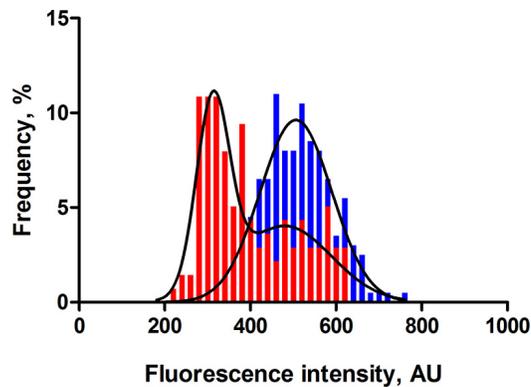
FIG 5 In the absence of the BtsSR/YpdAB network, *rrnB* P1 promoter activity is low and bistable. Wild-type *E. coli* MG1655 (blue) or mutant *btsSR ypdAB* (red) cells harboring a chromosomally encoded *rrnB* P1-*gfp* fusion were grown in LB medium and examined by fluorescence microscopy. For further details, see the legends to Fig. 2 and 3. A total of 200 cells were analyzed for each experiment at the post-exponential growth phase, and frequency is represented as a percentage of the cells (refer to Materials and Methods for detailed explanation). Histograms of the fluorescence intensities of cells were fitted using a Gaussian distribution (solid line). AU, arbitrary units. Experiments were performed three independent times.

Cellular physiology in the post-exponential growth phase. We have shown thus far that transcriptional activation of both target genes of the BtsS/BtsR and YpdA/YpdB network occurs heterogeneously. Furthermore, their activation is influenced by the availability of external pyruvate, albeit with different thresholds.

Since the two systems are activated in the post-exponential growth phase in LB medium, we decided to explore the impact of the BstS/BtsR and YpdA/YpdB systems on the overall physiological state of *E. coli* during this growth phase. In order to do so, we investigated individual cells of both *E. coli* MG1655 (the wild-type [WT] strain) compared to a strain lacking both systems: MG1655 Δ*btsSR* Δ*ypdAB* (abbreviated as the *btsSR ypdAB* mutant).

Fast-growing cells express high levels of 16S RNA from the *rrnB* P1 promoter (14). Cells with an inactive *rrnB* P1 promoter are likely to be dormant, antibiotic-tolerant persisters (14, 15). Recently, the strength of *rrnB* P1 promoter activation was shown to correlate with intracellular ATP levels (16). We therefore fused the ribosomal *rrnB* P1 promoter to *gfp* as previously described (14) and integrated this construct into the genomes of the two strains as a marker for their physiological states.

As expected, *E. coli* MG1655 WT *rrnB* P1-*gfp* showed a Gaussian distribution of GFP signal intensities, with a mean fluorescence value of 510 arbitrary units (AU) and noise level of 0.16 (Fig. 5). In contrast, the *btsSR ypdAB rrnB* P1-*gfp* mutant had a lower overall *rrnB* P1-*gfp* activity (average fluorescence intensity of 398 AU), which indicates a lower rate of ribosome synthesis within the population. Most strikingly, a bistable distribution of the signal was observed. These results suggest that, in the absence of both systems, the population differentiates into two subpopulations, one with a normal and another with a reduced ribosome synthesis rate.

The BtsSR/YpdAB network promotes protein overproduction. To test the idea that BtsS/BtsR and YpdA/YpdB systems together act to optimize the physiological state of cells within the population, we set out to metabolically challenge the *btsSR ypdAB* mutant and compare its response to that of the parental *E. coli* MG1655 WT strain. Interestingly, *E. coli* C41 (DE3), also known as the Walker strain, has been optimized for maximal overproduction of membrane and globular proteins (17). Subsequently, the genome of this strain was sequenced and, among other mutations, a point mutation in *btsS* was found that led to constitutive expression of *yjiY* (18). Based on these data, we hypothesized that the BtsS/BtsR and YpdA/YpdB systems might help cells to cope with the metabolic burden of protein overproduction.

In order to test this hypothesis, both strains (WT and the *btsSR ypdAB* mutant) were transformed with the overexpression vector pBAD24-*gfp*, which carries *gfp* under the
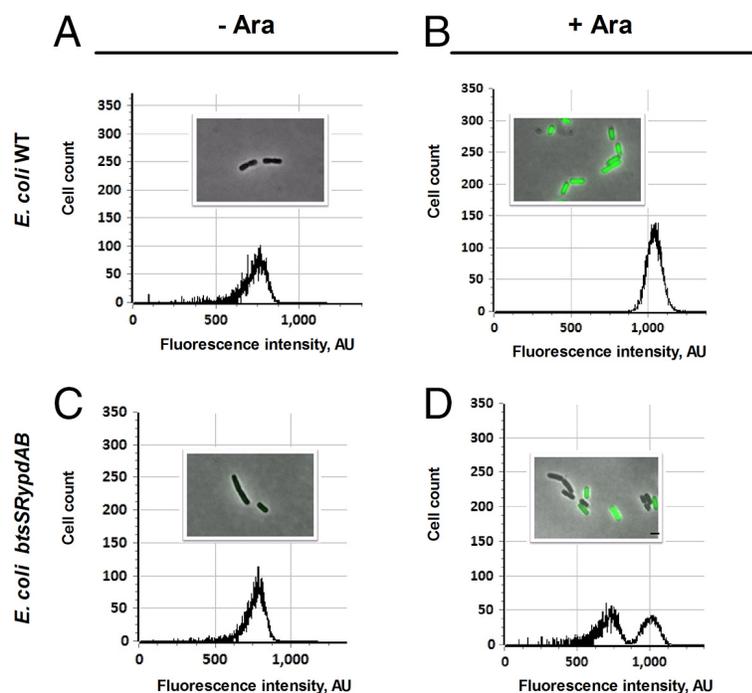
FIG 6 The BtsSR/YpdAB network promotes homogeneous protein overproduction in all cells. Wild-type (WT) or *btsSR ypdAB* mutant cells harboring the overproduction vector pBAD24-*gfp* were grown in LB medium. Samples were taken before and after the addition of the inducer arabinose (Ara) (0.2% [wt/vol]). The cells were analyzed by fluorescence microscopy and flow cytometry. Distributions of fluorescent cell counts and representative views of WT cells before and after addition of arabinose are shown in panels A and B, while the corresponding data for the *btsSR ypdAB* mutant are depicted in panels C and D. About 2,000 events were recorded for each plot. Cell counts represent the numbers of cells, and fluorescence intensity is expressed in arbitrary units (AU). Scale bar, 2 μm. Experiments were performed independently three times.

control of an arabinose-inducible promoter. Before induction with arabinose, fluorescence microscopy of single WT and *btsSR ypdAB* mutant cells showed no apparent GFP signals, and flow cytometry confirmed that the maximal fluorescence intensity of green cells (~750 AU) was low (Fig. 6A and C), indicating little or no GFP expression. One hour after induction with 0.2% (wt/vol) arabinose, cells of the WT population were producing GFP, which was clearly detected as an increase in the maximum fluorescence observed by flow cytometry (to ~1,100 AU). This result was corroborated by the detection of labeled single cells with fluorescence microscopy (Fig. 6B). In contrast, flow cytometric analysis of the mutant under inducing conditions detected two peaks: one at 1,100 AU, as in the WT, and a second at 750 AU. The accompanying micrographs revealed the presence of fluorescent and nonfluorescent cells (Fig. 6D). Therefore, the low-intensity peak in the flow cytometer represents cells that are unable to produce GFP in large amounts. The C41 (DE3) strain was also tested and was found to be capable of a homogeneously high protein overproduction, as expected (data not shown).

We also tested the overproduction of (i) GFP under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lac* promoter (pCOLA-P*lac*-*gfp*), (ii) the periplasmic protein DppA fused to the Tat translocation sequence (19) and under the control of the arabinose promoter (pBAD24-RR-*gfp-dppA*), and (iii) the membrane protein LysP fused to a different fluorophore and under the control of an arabinose-inducible promoter (pBAD33-*lysP-mcherry*) (see Table S1 in the supplemental material). The results obtained for the IPTG-inducible GFP reporter were similar to those for the arabinose-controlled system. The *btsSR ypdAB* mutant was hardly able to overproduce the periplasmic DppA or the membrane protein LysP. In summary, the BtsS/BtsR and YpdA/YpdB sensing network helps *E. coli* to cope with the additional metabolic burden imposed by protein overproduction.
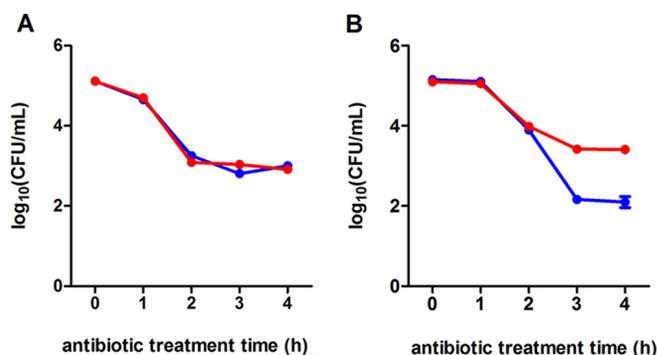
FIG 7 The BtsSR/YpdAB network reduces the proportion of persister cells in populations. Either WT (blue lines) or mutant *btsSR ypdAB* (red lines) cells were grown in LB medium. Before (exponential growth phase) (A) and after (B) activation (post-exponential growth phase) of the systems, the cells were exposed to ampicillin (200 μg/ml). Samples were taken and analyzed for CFU. Three independent experiments were performed, and error bars indicate the standard deviations of the means.

The BtsSR/YpdAB network limits the proportion of persister cells in WT populations. We hypothesized that the heterogeneous distribution of the capacity for protein overproduction among the *btsSR ypdAB* mutant population might be related to the presence of a subpopulation of cells that are unable to sense nutrient limitation and consequently fail to activate transporters to acquire needed resources. Persister cells survive exposure to antibiotics owing to their altered metabolic activity and low growth rate, but they can subsequently resume growth to form an antibiotic-sensitive population (20). We were interested to know whether the BtsS/BtsR and YpdA/YpdB network has an influence on persister cell formation. To address this question, we performed population-based studies by exposing growing WT or *btsSR ypdAB* mutant cells to ampicillin and determining the number of CFU. Only cells able to recover from the stress will form CFU. We subdivided a growing culture and exposed cells to ampicillin before and after the natural activation of the signaling systems, namely, at an optical density at 600 nm (OD_{600}) of 0.4 (exponential growth phase) and 1.2 (post-exponential growth phase) (9). After treatment with ampicillin a biphasic time-dependent killing curve was observed, which is typical for persister formation (Fig. 7) (20). When cells were exposed to ampicillin prior to activation of the signaling systems, the two strains exhibited almost identical patterns of response, characterized by a steep initial decrease in CFU, followed by a slower killing rate, revealing persister cells (Fig. 7A). Ampicillin treatment of cells after activation of the signaling systems resulted in a considerably higher level of persister cells in the mutant (2.15%) than the WT (0.14%) population (Fig. 7B).

In parallel, the minimum time taken to kill 99% of the population (MDK_{99}) (21) was determined for both strains after exposure to ofloxacin. The value for the WT was determined to be 0.49 h, and for the *btsSR ypdAB* mutant it was 1.98 h, which is compatible with the higher fraction of persisters in the mutant population (see Fig. S2 in the supplemental material).

These results reveal a novel role for the BtsS/BtsR and YpdA/YpdB signaling network in reducing the percentage of persister cells in a growing population. They are also in accordance with the idea of a contribution of both systems to help individual cells to replenish nutrient resources.

DISCUSSION

BtsS/BtsR (formerly YehU/YehT) is one of the most widespread TCSs in bacteria and is found in many human and plant pathogens. Although most gammaproteobacteria contain this system, some, including *Escherichia*, *Citrobacter*, and *Serratia*, have a second homologous system, YpdA/YpdB (22). Both belong to the LytS/LytTR family. Previous systematic studies failed to identify a function for these TCSs (23, 24). We have now identified the HK BtsS as a high-affinity pyruvate receptor (K_d = 58.6 μM) and

YpdA/YpdB as a system that responds to higher levels (>0.6 mM) of the same compound (8, 10). The target genes regulated by the two systems code for the high-affinity pyruvate/H$^+$ symporter YjiY (recently renamed BtsT [12]) and a transporter of unknown function, YhjX. However, the biological significance of the BtsS/BtsR and YpdA/YpdB systems has remained unclear.

Therefore, we first investigated the activation of the target genes of each system at the single-cell level using promoter fusions. We found that in clonal populations the chromosomally integrated copies of either P$_{yjiY}$-*gfp* or P$_{yhjX}$-*gfp* were heterogeneously activated when grown in LB medium, which is rich in amino acids (Fig. 2), and that induction of P$_{yjiY}$-*gfp* was slightly more variable than that of P$_{yhjX}$-*gfp*. In both cases, a predominantly unimodal Gaussian distribution of activation levels was observed, and only a very small percentage of cells remained in the OFF state. This pattern of activation differs markedly from the "all-or-nothing," switch-like gene expression described for the *lac* or *ara* promoter (25, 26). However, the heterogeneous, but unimodal activation of *yhjX* and *yjiY* can, in principle, be explained by the multiple factors known to affect their expression: (i) binding of the respective transcriptional activators BtsR and YdpB (27), (ii) the influence of the cAMP/CRP protein (P$_{yjiY}$), (iii) fine-tuning by the carbon starvation regulator CsrA (Fig. 1), and (iv) variations in the physiological state between cells (see below).

YpdA/YpdB-mediated activation of P$_{yhjX}$ was found to be dependent on the concentration of pyruvate in the medium and became homogenous when cells were grown in minimal medium containing pyruvate (20 mM) as the sole carbon source (Fig. 3). In contrast, under all tested conditions BtsS/BtsR-mediated activation of P$_{yjiY}$ was characterized by high cell-to-cell variability, which was virtually unaffected by the amount of pyruvate in the medium (Fig. 4). It is important to note that the BtsS/BtsR systems, whose target gene codes for a high-affinity pyruvate transporter, is only activated by external pyruvate when cells concurrently face nutrient limitation (Fig. 4) (10). The high degree of heterogeneity might reflect variations in the nutritional state of individual cells and differing needs for the high-affinity pyruvate transporter YjiY. Therefore, the BtsS/BtsR system responds only when cells are in need of a high-affinity uptake transporter to scavenge traces of available nutrients, e.g., pyruvate.

It has been proposed that cellular metabolism is both inherently stochastic and a generic source of phenotypic heterogeneity (28). In this general context, the results of our single-cell studies can be accommodated by the following model for the role of the two LytS/LytTR-type systems in *E. coli*. Under certain conditions, e.g., during growth in LB medium, cells excrete pyruvate due to overflow metabolism. Subsequently, other nutrients are depleted, and cells sense the availability of pyruvate. Depending on the external pyruvate concentration and their particular nutritional needs, individual *E. coli* cells activate either the high-affinity BtsS/BtsR and/or the low-affinity YpdA/YpdB system upon entry into the post-exponential growth phase. The interplay between transporters with different affinities for the same substrate has already been described, and this seems to be a successful strategy under nutrient limitation (29).

By using a reporter for the rate of ribosome synthesis, we found that only populations of reporter cells harboring the nutrient-sensing network exhibited unimodal activation of the *rrnB* P1 promoter, whereas the *btsSR ypdAB* mutant was characterized by a bimodal expression pattern (Fig. 5). The heterogeneous activation of either P$_{yjiY}$ or P$_{yhjX}$ in individual WT cells allows uptake of nutrients, e.g., pyruvate, according to the individual requirement of the cells. This results in a unimodal distribution of the activation level of the *rrnB* P1 promoter characteristic of growing cells. It should be noted that previous physiological studies revealed that *E. coli* has more than one pyruvate transporter (30), although only YjiY has thus far been characterized as high-affinity pyruvate transporter (12). Therefore, we assume that individuals within the population of the *btsSR ypdAB* mutant can cope with the lack of the sensing/transport of pyruvate and have normal ribosome synthesis. In addition, we imposed a metabolic burden by forcing cells to overproduce particular proteins. This is a natural scenario, since many pathogens have to produce virulence factors, exoenzymes, siderophores,

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description[a]	Source or reference
E. coli strains		
MG1655	F⁻ λ⁻ *ilvG rfb50 rph-1*	35
ST18	S17lpir Δ*hemA*	36
DH5α	*fhuA2 lacΔU169 phoA glnV44 φ80′ lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	37
MG 35	MG1655 Δ*btsSR* Δ*ypdAB*	This study
MG 2	MG1655 Δ*yehUT* = Δ*btsSR*	7
MG 20	MG1655 Δ*ypdAB*	8
MG1655 P*yhjX*-*gfp*	Integration of P*yhjX*-*gfp* at the native locus in *E. coli* MG1655	This study
MG1655 P*yjiY*-*gfp*	Integration of P*yjiY*-*gfp* at the native locus in *E. coli* MG1655	This study
MG1655 P*rrnB* P1-*gfp*	Integration of P*rrnB* P1-*gfp* at the native locus in *E. coli* MG1655	This study
MG 35 P*rrnB* P1-*gfp*	Integration of P*rrnB* P1-*gfp* at the native locus in *E. coli* MG 35	This study
Plasmids		
pRed/ET	λ-RED recombinase in pBAD24; Amp^r	Gene Bridges
pCP20	FLP-recombinase, λcI 857⁺, λpR Rep^ts; Amp^r Cm^r	38
pNPTS138-R6KT	*mobRP4⁺ ori*-R6K *sacB*, suicide plasmid; Kan^r	13
pNPTS138-R6KT-P*yhjX*-*gfp*	300 bp of P*yhjX* fused to *gfp* and cloned into EcoRI/PspOMI sites of pNPTS138-R6KT; Kan^r	This study
pNPTS138-R6KT-P*yjiY*-*gfp*	300 bp of P*yjiY* fused to *gfp* and cloned into EcoRI/PspOMI sites of pNPTS138-R6KT; Kan^r	This study
pNPTS138-R6KT-P*rrnB* P1-*gfp*	300 bp of P*rrnB* P1 fused to *gfp* and cloned into EcoRI/PspOMI sites of pNPTS138-R6KT; Kan^r	This study
pXGSF	*gfp* under the control of a vegetative synthetic promoter	G. Klauck and R. Hengge, unpublished data
pBAD24	Arabinose-inducible P_BAD promoter, pBR322 ori; Amp^r	39
pBAD24-*gfp*	*gfp* cloned in the EcoRI and NcoI sites of pBAD24	26
pBAD24-RR-*gfp*	*gfp-mut2* cloned in the NheI and HindIII sites of pBAD24 (p8754 derivative)	19
pBAD24-RR-*gfpmut-dppA*	*dppA* cloned in the HindIII site of pBAD24-RR-*gfpmut2*	This study
pCOLA Duet-1	Expression vector, ColA ori; Kan^r	Merck
pCOLA-P*lac*-*gfp*	*gfp* under the control of the IPTG-inducible *lac* promoter cloned in the BamHI and HindIII sites of pCOLA-Duet-1	This study
pBAD33-*lysP*	*lysP* in pBAD33; Cm^r	40
pBAD33-*lysP-mcherry*	*mcherry* cloned in the XbaI and SalI sites of pBAD33-*lysP*; Cm^r	This study

[a]Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance.

etc., in large amounts. Although all WT cells managed to cope with this burden, about 50% of the mutant cells failed to overproduce the test protein, GFP, a pattern which we also observed for the activation of the *rrnB* P1 promoter (Fig. 6). It should be noted that the evolved *E. coli* C41(DE3) strain, which has been optimized for protein overproduction, has a point mutation in *btsS* that leads to stimulus-independent expression of *yjiY* (18). In light with the results presented here, the constitutive expression of the high-affinity pyruvate transporter YjiY in strain C41 guarantees a sufficient uptake of pyruvate in all cells independent from external or internal factors. Finally, a population-based persister assay revealed that *btsSR ypdAB* populations contain a higher percentage of antibiotic-tolerant persister cells (dormant cells) than do WT populations (Fig. 7).

Taking these results into account, the model described above can be further extended. Sensing of external pyruvate by the BtsS/BtsR and YpdA/YpdB systems and the tight regulation of expression of the two transporters YjiY and YhjX depending on the needs of the individual cell ensures an optimization of the physiological state within the whole population to withstand upcoming metabolic stress. These findings are important not only in light of the host colonization of pathogenic species and their persistence but also for metabolic engineering.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains, including their genotypes, and the plasmids used in this study are listed in Table 1. Mutants were constructed using an *E. coli* Quick-and-Easy gene deletion kit (Gene Bridges) and a BAC modification kit (Gene Bridges), as previously reported (31). Both kits rely on the Red/ET recombination technique (31). The oligonucleotide sequences are available on request.

E. coli MG1655 strains (Table 1) were grown overnight in lysogeny broth (LB; 10 g/liter NaCl, 10 g/liter tryptone, 5 g/liter yeast extract). After inoculation, bacteria were routinely grown in LB medium under agitation (200 rpm) at 37°C. For solid medium, 1.5% (wt/vol) agar was added. Where appropriate, media were supplemented with antibiotics (kanamycin sulfate, 50 μg/ml; ampicillin sodium salt, 100 μg/ml). For the "low-nutrient environment" experiments, cells from an overnight culture in LB were inoculated into 0.1× diluted LB at a starting OD_{600} of 0.05 and grown for 1 h. Pyruvate was then added to the cultures to a final concentration of 0.01, 0.05, 0.1, 0.2, 1, or 20 mM.

E. coli MG1655 strains were also grown overnight in M9 minimal medium with 0.5% (wt/vol) glucose as sole carbon source. Bacteria were then inoculated into M9 minimal medium supplemented with increasing concentrations of pyruvate (0.3, 0.6, 1, 2, and 10 mM), and the total carbon source concentration was adjusted to 20 mM using succinate. The conjugation strain *E. coli* ST18 was grown in the presence of 50 μg/ml 5-aminolevulinic acid.

Construction of fluorescence reporters. Molecular manipulations were carried out according to standard protocols (32). Plasmid DNA and genomic DNA were isolated using a HiYield plasmid minikit (Sued-Laborbedarf) and a DNeasy blood and tissue kit (Qiagen), respectively. DNA fragments were purified from agarose gels using a HiYield PCR cleanup and gel extraction kit (Sued-Laborbedarf). Q5 DNA polymerase (New England BioLabs) was used according to the supplier's instructions. Restriction enzymes and other DNA-modifying enzymes were also purchased from New England BioLabs and used according to the manufacturer's directions. Replicative plasmids were transferred into *E. coli* strains using competent cells prepared as described previously (33).

For construction of the promoter-*gfp* fusions, 300-bp segments of the region immediately upstream of the coding sequence were amplified using oligonucleotide pairs containing EcoRI/PspOMI restriction sites. The resulting promoter fragments were ligated into the γ-origin-dependent vector pNPTS138-R6KT-*gfp* after restriction with EcoRI/PspOMI. Chromosomal insertions of promoter-*gfp* into the designated *E. coli* strains were achieved by integrating the resultant suicide vectors pNPTS138-R6KT-P$_{yhjX}$-*gfp* and pNPTS138-R6KT-P$_{yjiY}$-*gfp* via RecA-mediated single homologous recombination as described previously (13). The donor strain *E. coli* ST18, containing the required plasmids, was cultivated together with the recipient *E. coli* MG1655 strain in LB medium, supplemented with additives as described, to an OD_{600} of about 0.8. Recombination-positive clones were selected on kanamycin plates, and correct chromosomal integration was checked by PCR and sequencing. To prevent duplication instability, the reporter strains were always cultivated in the presence of kanamycin.

Single-cell fluorescence microscopy and analysis. To measure promoter activity in individual cells of the reporter strains, cells were cultivated as described above in a rotary shaker. Samples were taken (10 μl) and analyzed on an agarose pad (0.5% [wt/vol] agarose in phosphate-buffered saline [PBS; pH 7.4]), which was placed on a microscope slide and covered with a coverslip.

Images were taken on a Leica microscope (DMI 6000B) equipped with a Leica DFC 365 Fx camera (Andor, 12 bit). An excitation wavelength of 460 nm and a 512-nm emission filter with a 75-nm bandwidth were used for visualization of GFP fluorescence, and an excitation wavelength of 546 nm and a 605-nm emission filter with the same bandwidth were used for visualization of red fluorescence. At least 200 cells per condition were analyzed. The digital images were analyzed using Fiji (34), and statistical analysis was performed using Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA). The background fluorescence was subtracted from each field of view.

The noise was calculated by dividing the standard deviation by the mean. The higher the noise value the more heterogeneous the distribution. The percentage of dark cells was determined from the number of cells whose fluorescence levels overlapped with the negative control (before activation) and the total number of cells quantified. The frequency distributions depict the fraction of values which lie within the range of values that define the bin. The bin range was kept constant at 20 AU. Propidium iodide (Invitrogen, Eugene, OR) was added to the cell cultures at a final concentration of 5 μM to stain dead cells (red fluorescence).

Overproduction experiments. Overnight cultures of *E. coli* MG1655 transformed with the plasmid pBAD24-*gfp* were diluted 100-fold in 20 ml of fresh LB medium supplemented with 100 μg/ml of ampicillin sodium salt and incubated aerobically at 37°C until an OD_{600} was reached 0.6 (early exponential phase). The cells were induced with L-arabinose 0.2% (wt/vol) for 1 h. Before and after induction, 100-μl samples were taken, diluted 1:1,000 in PBS, and analyzed in a BD Accuri C6 flow cytometer equipped with a solid-state laser (488-nm emission; 20 mW). The green fluorescence emission from GFP was collected by the FL1 filter (BP 533/30 filter). Forward-angle light scatter (FSC) and side-angle light scatter (SSC) were collected in the FSC detector and SSC filter (BP 488/10 filter), respectively. The detection threshold was adjusted for FSC to eliminate noise, and the gate was set on the FSC-SSC dot plot to exclude debris. The sheath flow rate was 14 μl/min, and no more than 100 events/s were acquired. For each sample run, a maximum of 2,000 events were collected. Analysis of data was carried out using Cytospec software (http://www.cyto.purdue.edu/Purdue_software).

Persister cell assay. To determine the number of persister cells, the number of CFU per ml was measured after exposure of the culture to 200 μg/ml ampicillin. Overnight cultures were diluted 100-fold in 20 ml of fresh LB medium and incubated aerobically at 37°C until the OD_{600} reached 0.4 or 1.2. Aliquots were then transferred to a new 100-ml flask (final $OD_{600} = 1$), and the antibiotic was added. Every hour during antibiotic treatment, samples were taken, serially diluted in PBS, plated on LB agar, and incubated at 37°C for 16 h. CFU were counted as a measure of surviving persister cells. Persisters were calculated as the surviving fraction by dividing the number of CFU per milliliter in the culture after incubation with the antibiotic by the number of CFU per milliliter in the culture before addition of the antibiotic. Each experiment was repeated on three different days.

For calculation of the minimum duration of killing (MDK_{99}), the procedure described above was performed using ofloxacin (at a final concentration of 5 μg/ml) as the antibiotic. The MDK_{99} value corresponds to the time (in hours) needed to kill 99% of the initial population.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB .00536-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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Chapter 5

Concluding discussion

Bacteria are living in constantly fluctuating environments in which they need to keep balance of important nutrients. For this purpose they evolved TCSs to sense various stimuli and respond by rapid alteration of gene expression.

Under carbon limitation, *E. coli* monitors extracellular concentration of pyruvate by the TCS BtsS/BtsR (previously named YehU/YehT) (**Chapter 2**). It was biochemically shown, that the HK BtsS is as a specific receptor for extracellular pyruvate. The activation of BtsS/BtsR leads to upregulation of the transporter BtsT (previously named YjiY). Transport assays with intact cells and proteolisomes demonstrated that BtsT is a specific and high-affinity pyruvate/H$^+$ symporter (**Chapter 3**).

Overall the TCS BtsS/BtsR fine tunes pyruvate uptake by BtsT to match its demand. These results led us to formulate the biological role of the TCS BtsS/BtsR. Together with the TCS YpdA/YpdB, which also responds to extracellular pyruvate with different affinity, they contribute to a balanced physiological state of the individual cells within a population (**Chapter 4**).

5.1 Role of the TCS BtsS/BtsR upon carbon starvation

During exponential growth in a nutrient rich environment, *E. coli* can utilize many available carbon sources. To avoid the carbon accumulation in fast growing cells, several intermediates (mainly acetate and pyruvate) are released into the environment, by the process named as overflow metabolism or Crabtree effect (Paczia *et al.*, 2012). Acetate overflow especially occurs under conditions of high glucose concentrations in comparison to pyruvate overflow, which is mainly derived from amino acid metabolism. Extracellular accumulation of pyruvate or acetate is later on followed by a decline of an intracellular nutrient concentration in bacteria. To survive prolonged periods of starvation, *E. coli* coordinates nutrient scavenging and retrieves excreted by-products as alternative substrates (Peterson *et al.*, 2005). When *E. coli* depletes all available carbon sources, bacterial growth rate decelerates and soon enter a stationary phase.

    E. coli grown in mixtures of amino acids consumes serine as the first amino acid (Prub *et al.*, 1994). It was shown that the TCS BtsS/BtsR responds to such depletion (**Chapter 2**). Concomitantly, the HK BtsS senses the extracellular pyruvate (step 1) and activates BtsR (step 2) to induce expression of *btsT* (step 3), which codes for the pyruvate transporter BtsT (steps 4,5). BtsT is therefore very advantageous for circumventing the carbon limitation by taking up pyruvate (step 6). Collectively these findings establish a comprehensive model of pyruvate sensing and transport by the TCS BtsS/BtsR in *E. coli* (steps 1-6 in **Fig. 5.1**).

    The regulatory role at the transition from exponential to stationary phase has been also described for the LytS/LytTR-like TCS AgrC/AgrA in *Staphylococcus aureus*. To adapt to an environment that does not support anymore a fast growth, bacteria can autoactivate synthesis of signaling molecules (AIP). After a threshold concentration of AIP in the environment, bacteria will regulate expression of virulence genes by the TCS AgrC/AgrA (Novick and Geisinger, 2008).

    In addition, the growth of *E. coli* is limited not just by carbon, but also nitrogen and phosphorus. The ratio between all three elements is different in each environment. For all these types of starvation, *E. coli* developed scavenging strategies. However, if scavenging is insufficient, bacteria enter the stationary phase (Peterson *et al.*, 2005). The regulatory role
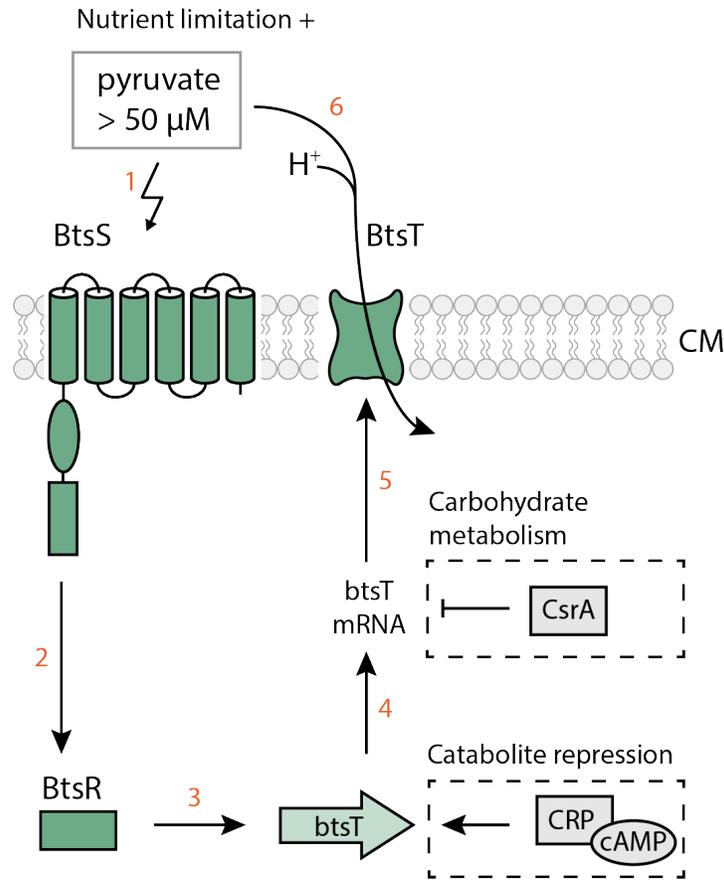
Figure 5.1: Fine-tuning of pyruvate levels in *E. coli* by BtsS/BtsR two-component system. The scheme summarizes the perception of pyruvate by BtsS under nutrient limitation ($\lightning$), signal transduction within the TCS BtsS/BtsR, an influence of global regulators CsrA and CRP, and a symport of pyruvate/H^+ by BtsT. CM, cytoplasmic membrane.

of TCSs during nutrient scavenging is well described. A TCS PhoR/PhoB surveys inorganic phosphate levels by a Pst transport system. Upon diminished activity of the Pst transporter, the TCS PhoR/PhoB activates sRNA transcription to stimulate translation of *rpoS* mRNA (Wanner, 1990). RpoS is a master regulator of general stress response, which regulates under direct or indirect control up to 10% of the *E. coli* genes (Weber *et al.*, 2005). On the other hand the TCS NtrB/NtrC analyzes the nutrient status of *E. coli* through the intracellular metabolite glutamine and thus monitors the levels of the nitrogen starvation. However, if and how the TCS NtrB/NtrC regulates RpoS is unclear (Peterson *et al.*, 2005).

It has been shown that the TCS BtsS/BtsR responds to carbon limitation (**Chapter 2**) however how it surveys the carbon levels is unknown. It was proposed that the TCS

BtsS/BtsR senses a low intracellular serine concentration since serine is able to delay response to activate promoter of *btsT* in growing *E. coli*. However, the effect was shown to be indirect by *in vivo btsT* expression studies under nutrient-limiting conditions (**Chapter 2**). Furthermore, it remains unclear if the TCS BtsS/BtsR serves just for pyruvate uptake or also initiate the stationary phase if carbon scavenging is insufficient for the cells. The possibility that BtsS/BtsR affects transcription of *rpoS* should be addressed in order to fully understand the role of the TCS BtsS/BtsR upon carbon starvation in *E. coli*.

5.2 Function of the histidine kinase BtsS

Based on biochemical studies, it has been revealed that BtsS is a high-affinity sensor for pyruvate (**Chapter 2**). Pyruvate binding has been observed to an external side of the transmembrane domain of BtsS. The pyruvate binding to BtsS is very specific with a dissociation constant of 58 μM.

These protein-ligand interaction results have been obtained by a rapid method differential radial capillary action of ligand assay (DRaCALA) (Roelofs *et al.*, 2011). Since BtsS is a membrane protein, DRaCALA is a very advantageous method enabling usage of membrane vesicles with overproduced BtsS, with no requirement of further protein solubilization.

The identification of pyruvate as a substrate sensed by BtsS may bring insights into stimuli perceived by the whole family of LytS-like HKs. Recently, another LytS/LytTR-like TCS, LytS/LytT in *B. subtilis*, has also been shown to be essential for growth on pyruvate (van den Esker *et al.*, 2017). DRaCALA can be as well used for proving the pyruvate binding to the HK LytS in *B. subtilis*. It is worth to mention that the TCS LytS/LytT is proposed to have similar function as the TCS BtsS/BtsR. In nutrient rich environments both *B. subtilis* and *E. coli* release pyruvate into the environment, in the process of overflow metabolism. It is possible that pyruvate receptors in bacteria monitor the external pyruvate levels and such bacteria reflect an available growth condition. Additionally, it is suggested that LytT regulates *ysbA* transcription to produce YsbA, a putative transporter for pyruvate (van den Esker *et al.*, 2017).

The putative binding site for pyruvate in BtsS was not yet elucidated (**Chapter 2**). Actually, pyruvate was once copurifed and crystallized with an extracytoplasmic domain

of KinD in *B. subtilis* however its relevance was not yet understood (Wu *et al.*, 2013). A comparison of the structures of KinD in complex with pyruvic acid to a predicted structure of BtsS did not detect any similarities (unpublished observation). Further research should concentrate on determining the pyruvate binding site of BtsS.

In addition, the understanding of how BtsS perceives external pyruvate might help to gain insights into how BtsS generates an intracellular response. Up to now *in vitro* phosphorylation of BtsS has not been observed (Kraxenberger *et al.*, 2012). The reason why is often attributed to an incomplete ATP-binding domain of BtsS lacking amino acids that are usually conserved in other LytS-like HKs in *E. coli* (BtsS lacks the first region of characteristic DxGxG and GxG motif for nucleotide binding) (Kraxenberger *et al.*, 2012). Therefore, it has been hypothesized that the signalling within the TCS BtsS/BtsR is mediated by protein-protein interactions. Indeed it was proved that BtsS interacts with the RR BtsR by *in vivo* protein-protein interaction assays (Behr *et al.*, 2014). For some TCSs, e.g. AmiC/AmiR in *P. aeruginosa*, the signal transduction is achieved via a ligand-induced release of the RR AmiR rather than usual phosphorylation (O'Hara *et al.*, 1999). Therefore, it could be speculated, that also pyruvate has an effect on the strength of protein-protein interactions between BtsS and BtsR, and thus we could get further insights into the signal transduction within the TCS BtsS/BtsR. A ligand influencing the regulatory interplay has already been reported in *E. coli*. Here, lysine is transferred from a co-sensor LysP to a pH sensor CadC and thus induces lysine-dependent adaptation under acidic stress (Rauschmeier *et al.*, 2014).

Last but not least, the HK BtsS might have another ligand binding site. It is possible that a ligand binds to an intracellular site of BtsS, which might answer the question of how the TCS BtsS/BtsR monitors levels of carbon starvation. Such dual sensor has already been observed in *E. coli* to balance extracellular and intracellular K^+ concentration (Schramke *et al.*, 2016). However, for this purpose the method DRaCALA is not suitable. A limitation of DRaCALA is the requirement of radioactive ligand, which does not enable rapid screening of unknown ligands related to the stationary phase or carbon metabolism. For this reason, e.g. thermofluor-based binding assay (Ericsson *et al.*, 2006) should be used to identify if the HK BtsS also contains the intracellular binding site for a specific ligand. It has been proposed that a GAF domain of BtsS might be involved in such binding, since in other proteins the GAF is capable to bind amino acids, ions and nucleotides (Zoraghi *et al.*, 2004).

5.3 Importance of the pyruvate transporter BtsT

The transporter BtsT has been extensively investigated for its transport activity (**Chapter 3**). Based on the transport studies in intact cells, BtsT is a specific pyruvate transporter with an apparent K_m of 16 μM. Reconstitution of a purified BtsT into *E. coli* proteoliposomes revealed that BtsT functions as a pyruvate/H$^+$ symporter.

BtsT represents the first pyruvate transporter to be identified in *E. coli*. The characterization of other pyruvate transporters in *E. coli* might be tricky since pyruvate is located in a central node of a carbon metabolism and its cellular concentration is tightly controlled (Vemuri *et al.*, 2006). Pyruvate plays an important role in amino acid catabolism (**Fig. 5.2**). During aerobic growth, pyruvate is converted to acetyl-CoA, which enters tricarboxylic acid (TCA) cycle. In addition, pyruvate is a precursor for amino acids (Ala, Ile, Leu and Val). Under anaerobic conditions, pyruvate is converted to lactate and ethanol.
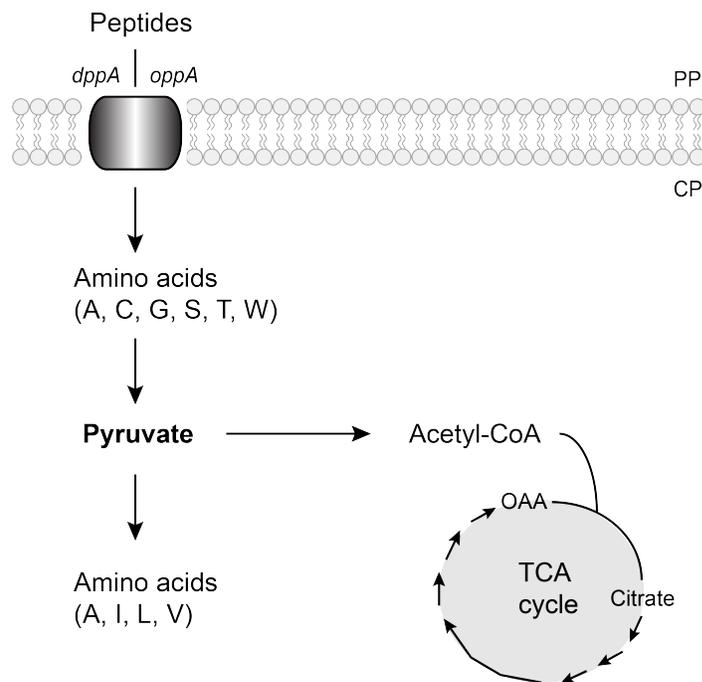
Figure 5.2: Central role of pyruvate in *E. coli* carbon metabolism. Figure summarizes import of peptides by *E. coli* into the cytoplasm, their degradation into amino acids. Amino acids alanine, cysteine, glycine, serine, threonine and tryptophan are directly converted to pyruvate. Under aerobic conditions, pyruvate is degraded to acetyl-CoA, which enters the tricarboxylic acid cycle. Pyruvate is also a precursor for synthesis of amino acids: alanine, isoleucine, leucine and valine. OAA, oxaloacetate, PP, periplasm, CP, cytoplasm.

Pyruvate transport systems have been predicted since it was shown that bacteria excrete and then retrieve pyruvate under several growth conditions (Kodaki *et al.*, 1981). Considering that pyruvate is an acid of pK_a of 2.5, under physiological conditions the deprotonated molecule cannot diffuse through a bacterial membrane and requires an active transporter. Transport assays in intact cells of *E. coli* claimed that there are active transporters however without their further characterization (Lang *et al.*, 1987). Later on, the knowledge about pyruvate transporters have been expanded by a research suggesting two importers and one exporter in *E. coli* yet also lacking their identification (Kreth *et al.*, 2013).

Since BtsT is the pyruvate transporter, it could help to identify other transporters in *E. coli*. A mutation of BtsT can decrease the pool of pyruvate inside *E. coli* and thus enable easier identification of other systems. Further studies of BtsT should also be focused on the elucidation of the pyruvate binding site, that might help to bioinformatically find possible candidates of other pyruvate transporters.

In addition, BtsT contains putative 18 TM domains and it is unknown which contribute for pyruvate uptake. Currently, we can only speculate that the two conserved 5-TM repeat units in members of the CstA family are essential for this function (Vastermark *et al.*, 2014) (**Fig. 5.3**). Furthermore, in the first TM of the second repeat has been identified a motif (CG-x(2)-SG) in the CstA family, which might be essential for function of these transporters (Vastermark *et al.*, 2014). BtsT contains 8 additional TMs, which can have other structural and/or regulatory function.

It is worth to mention that pyruvate transporters are of increasing physiological importance, mainly in biological fitness and virulence of *Enterobacteriaceae*. In *Yersinia pseudotuberculosis*, the high rate of glycolysis induces pyruvate overflow due to a metabolic bottleneck. It is suggested that high rates of nutrient uptake of easily metabolizable compounds, like pyruvate, provides a competitive advantage with other organisms in the gut environment (Bucker *et al.*, 2014). However, the pyruvate exporter or importer in *Y. pseudotuberculosis* has not yet been elucidated.

Even metabolic engineering is focusing its attention on pyruvate in order to achieve optimized metabolite production in *E. coli*, *C. glutamicum* or *B. subtilis* (Sauer and Eikmanns, 2005). The identification of carbon monocarboxylic acid transporters e.g. for pyruvate might gain further insights into metabolic adjustments within the PEP-pyruvate-oxaloacetate node.
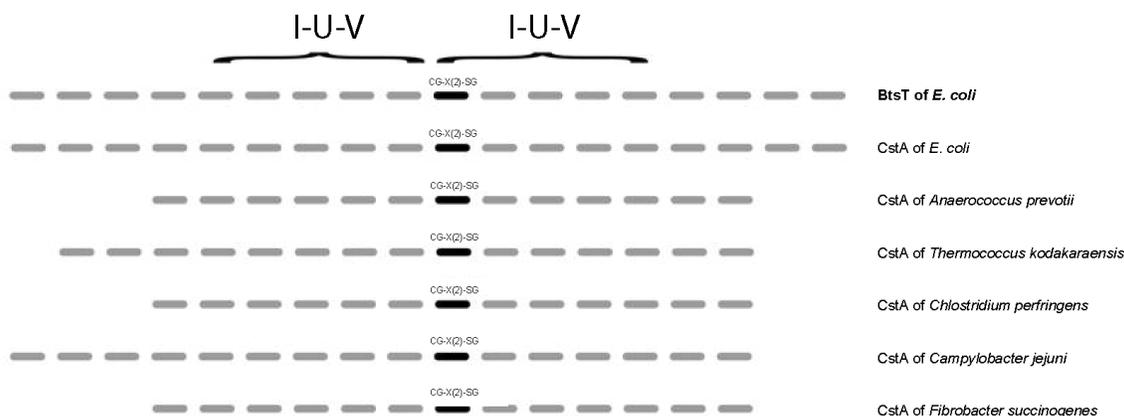
Figure 5.3: Topological prediction of BtsT in *E. coli* and CstA homologues in 6 different bacterial species. The CstA family exhibit 5 TM repeat unit and N- or C- terminal TMs. The I, U, V labels physically look like mentioned letters in 3D structures of APC superfamily proteins and refer to TMs 1, 2-3, 4-5, respectively, in each repeat unit. The location of the CG-x(2)-SG motif is shown. Adopted from (Vastermark *et al.*, 2014).

Indeed, the pyruvate transporter has been identified and characterized in *C. glutamicum*. Biochemical studies revealed that monocarboxylic acid transporter MctC actively take up acetate, propionate and with low affinity pyruvate ($K_{0.5}$ 250 μM) (Jolkver *et al.*, 2009). For *B. subtilis* nothing is known about pyruvate transport. The function of pyruvate transporter is suggested for YsbA, however, the transporter still awaits its characterization (van den Esker *et al.*, 2017). Nonetheless, to control the carbon flux between the glycolysis and the TCA cycle, further insights into the regulation and characterization of the enzymes and other relevant proteins of the mentioned node are still required.

5.4 Role of the TCS BtsS/BtsR

The TCS BtsS/BtsR plays a role in a nutrient sensing network of *E. coli* and in host colonization.

The TCS BtsS/BtsR forms a functional network with the TCS YpdA/YpdB, which also responds to pyruvate but with a lower affinity (Behr *et al.*, 2014). Single-cell analysis of the activation of their corresponding target genes, *btsT* and *yhjX*, respectively, showed cell-to-cell variability. Comparing the wild-type strain with the *btsSR ypdAB* mutant revealed differences under two metabolically modulated conditions. The *btsSR ypdAB* mutant was

impaired in protein production and had higher percentage of persister cells in a growing population (**Chapter 4**). It suggests, that the mentioned nutrient sensing network in *E. coli* maintain the optimal carbon supply for each individual cell. The similar mechanism is also proposed for other two TCSs. The RRs NarL and NarP regulate two operons depending on the nitrate concentration (Wang and Gunsalus, 2003). Here, a dual adjustment is used to fastly react to environmental changes in levels of nitrate and formate.

The importance of the TCS BtsS/BtsR for pathogenesis has been observed in uropathogenic *E. coli* (UPEC) that colonizes a mammalian bladder. This pathogen accounts for over 85% of urinary-tract infection worldwide (Foxman, 2010). In the UPEC cystitis isolate UTI89, a *btsS/btsR* mutant failed to induce expression of *btsT* however in the wild-type strain *btsT* was transiently expressed. In addition, it was elucidated that BtsS/BtsR is active during both the acute and chronic stages of urinary tract infections (**Chapter 2**).

Actually the bladder of mammals is a nutritionally demanding environment that also contains pyruvate (Foxman, 2010). Metabolic breakdown products, mainly amino acids are degraded to pyruvate or other TCA cycle intermediates (**Fig. 5.2**). Pyruvate is further degraded to acetyl-CoA, which enters the TCA cycle. Both the TCA cycle as well as gluconeogenesis have been shown to be required for UPEC *in vivo* fitness (Foxman, 2010). Therefore, pyruvate plays a central role in UPEC metabolism. It seems like that the TCS BtsS/BtsR responds to pyruvate fluctuations and plays a role in promoting the infections process.

Furthermore, *btsT* was expressed in avian pathogenic *E. coli* (APEC) during the infection in chicken liver and spleen (Tuntufye *et al.*, 2012). This pathogen leads to extraintestinal infections in poultry that cause several diseases known as colibacillosis. During infection, APEC must coordinate genes relevant in virulence, metabolism and regulation. Studies focusing on the influence of BtsS/BtsR in APEC should be carried out to elucidate its importance in pathogenesis.

Last but not least, pyruvate sensing might provide also an advantage in an enteric environment, in intestines. This environment contains high amounts of amino acids (proline, alanine and serine), but also pyruvate (Nagata *et al.*, 2003; Nagata *et al.*, 2007). Therefore, monitoring pyruvate levels can propagate a succesful host colonization of enteric bacteria, e.g. *E. coli*, *S. typhimurium* and *Shigella*, in intestines (Bearson *et al.*, 1997).

5.5 Conclusion and outlook

In this thesis, it was demonstrated how *E. coli* senses and transports pyruvate. Based on several *in vivo* and *in vitro* studies, it was discovered that pyruvate is recognized by the high-affinity HK BtsS of the TCS BtsS/BtsR. Furthermore, it was found out that the target gene of this TCS, BtsT, is a specific pyruvate/H^+ symporter. Under nutrient limitation, such pyruvate sensing and transport are crucial in carbon scavenging before entry into the stationary phase. Lastly, it was identified that the individual cells use the TCS BtsS/BtsR to optimize their carbon requirements within the population and thus withstand upcoming metabolic stress.

Nevertheless, there are still open questions for future research. Solved structures of BtsS and BtsT, e.g. by 3D-crystallization might bring insights into structural arrangements of pyruvate binding and elucidate the function of other HKs of the LytS family or other transporters of the CstA family, respectively. It is also required to address if the HK BtsS contains an additional binding site for carbon compound and thus monitors carbon limitation. Last but not least, the function of the TCS YpdA/YpdB components should be addressed to fully understand the nutrient sensing network of the TCS BtsS/BtsR and the TCS YpdA/YpdB.

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Supplemental Material - Chapter 2

Supplementary Material:

Identification of a High-Affinity Pyruvate Receptor in *Escherichia coli*

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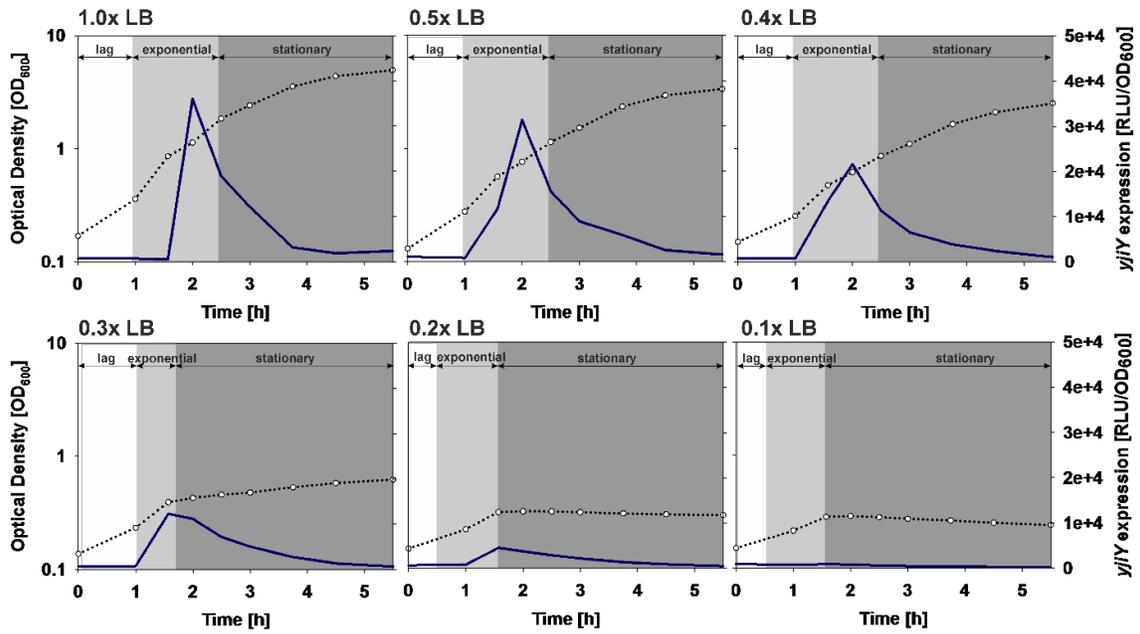
Fig. S1. Characterization of *yjiY* expression in different concentrations of LB medium. *Escherichia coli* MG1655 / pBBR *yjiY*-lux was cultivated under aerobic conditions and growth and luminescence were measured over time. Expression of *yjiY* over growth of *E. coli* cells in 1.0x, 0.5x, 0.4x, 0.3x, 0.2x and 0.1x diluted LB. The growth phases of *E. coli* are marked as following: lag phase (white), exponential growth (light grey) and stationary phase (dark grey).
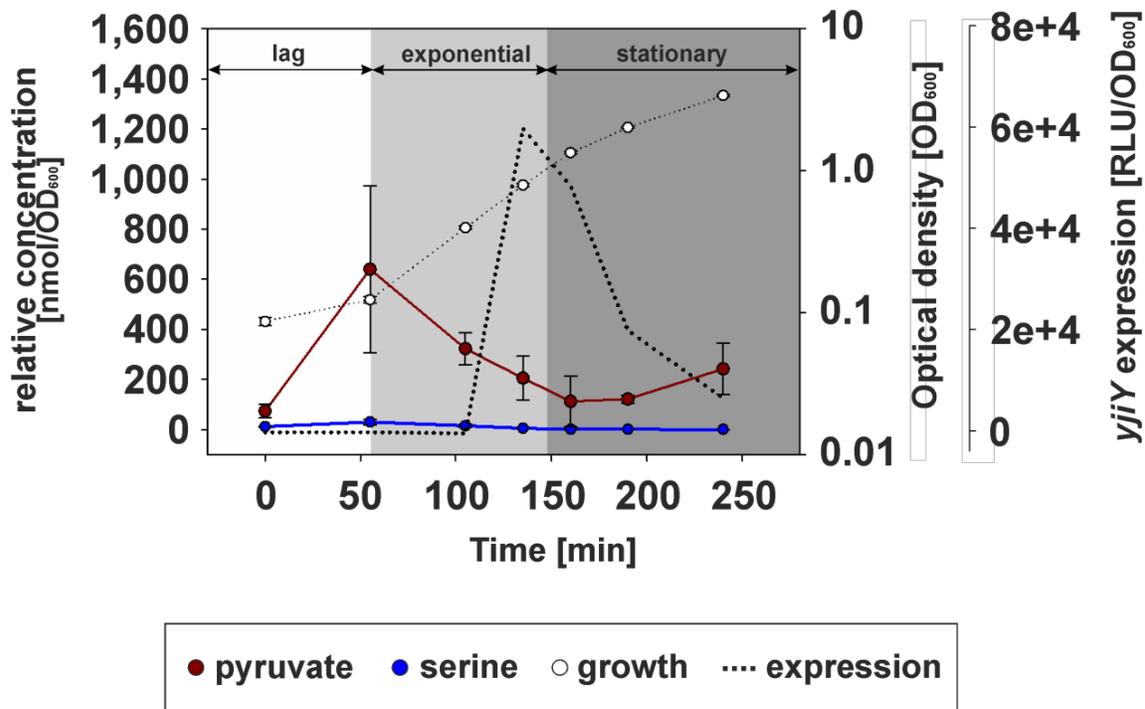
Fig. S2. Determination of changes in intracellular concentrations of serine and pyruvate during growth of *E. coli*. *E. coli* MG1655/pBBR *yjiY-lux* was cultivated in LB medium, and growth (OD600) and luminescence were monitored. At the times indicated, cells were harvested, and serine and pyruvate levels were quantified by hydrophilic interaction liquid chromatography. All experiments were performed in triplicate, and the error bars indicate the standard deviation of the means. The growth phases of *E. coli* are marked as following: lag phase (white), exponential growth (light grey) and stationary phase (dark grey).
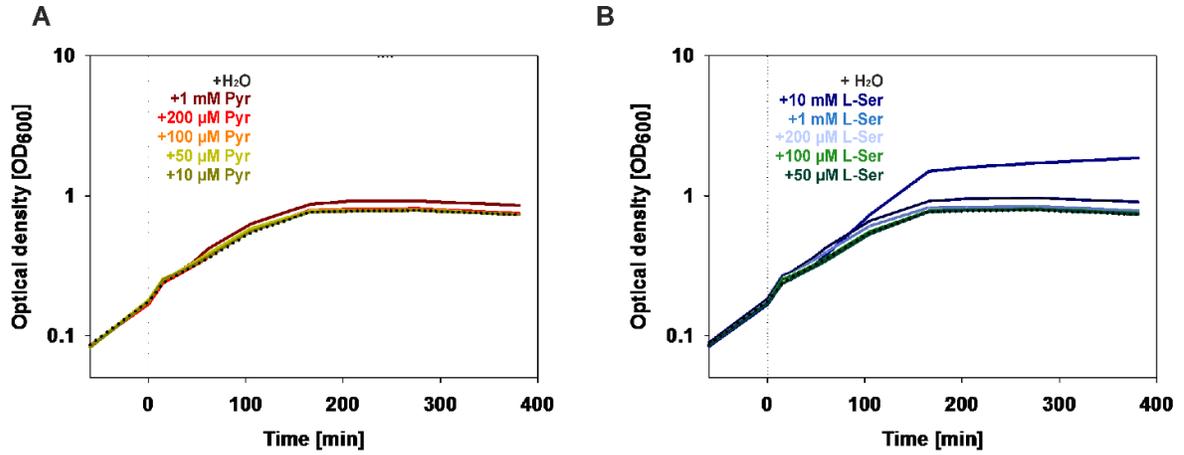
Fig. S3. Corresponding *E. coli* growth curves under nutrient-limiting conditions. *E. coli* MG1655 mutant Δ*yhjX* harboring pBBR *yjiY-lux* was cultivated in 0.1x LB medium. After 1 h (time point 0), the indicated concentration of pyruvate (A), or L-serine (B), or the equivalent volume of water was added. Growth was monitored over time.

Table S1. Summary of growth and *yjiY* expression data from *E. coli* cells grown in LB and diluted LB medium Growth rates for each time point (t(x)) were determined with μ = [ln (OD600 (x)) - ln (OD600 (x-1))] / [t(x) - t(x-1)].

Medium	Growth rate μ	max OD_{600}	OD_{600} at max. *yjiY* expression	max. *yjiY* expression [RLU/OD_{600}]
1.0x LB	1.27 h^{-1}	4.96	1.13	36,009
0.5x LB	1.18 h^{-1}	3.36	0.76	31,272
0.4x LB	0.95 h^{-1}	2.53	0.62	21,629
0.3x LB	1.05 h^{-1}	0.62	0.39	12,315
0.2x LB	0.75 h^{-1}	0.3	0.28	4,536
0.1x LB	0.63 h^{-1}	0.24	not detectable	no expression

Supplemental Material - Chapter 3

BtsT - a novel and specific pyruvate/H$^+$ symporter in *Escherichia coli*

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      E. coli   MDTKK IFKH I   PWVILGI IGA   FCLAVVALRR   GEHVSALWIV   VASVSVYLVA   50
   S. enterica  MDTKK IFKH I   PWVILGI IGA   FCLSVVALRR   GEHVSALWIV   VASVSVYLVA   50

      E. coli   YRYYSLYIAQ   KVMKLDPTRA   TPAVINNDGL   NYVPTNRYVL   FGHHFAAIAG   100
   S. enterica  YRYYSLYIAQ   KVMKLDPTRA   TPAVINNDGL   NYVPTNRYVL   FGHHFAAIAG   100

      E. coli   AGPLVGPVLA   AQMGYLPGTL   WLLAGVVLAG   AVQDFMVLFI   SSRRNGASLG   150
   S. enterica  AGPLVGPVLA   AQMGYLPGTL   WLLAGVVLAG   AVQDFMVLFI   SSRRNGASLG   150

      E. coli   EMIKEEMGPV   PGTIALFGCF   LIMIIILAVL   ALIVVKALAE   SPWGVFTVCS   200
   S. enterica  EMIKEEMGTV   PGTIALFGCF   LIMIIILAVL   ALIVVKALAE   SPWGVFTVCS   200

      E. coli   TVPIALFMGI   YMRFIRPGRV   GEVSVIGIVL   LVASIYFGGV   IAHDPYWGPA   250
   S. enterica  TVPIALFMGI   YMRFLRPGRV   GEVSVIGIVL   LVASIYFGGV   IAHDPYWGPA   250

      E. coli   LTFKDTTITF   ALIGYAFVSA   LLPWLILAP   RDYLATFLKI   GVIVGLALGI   300
   S. enterica  LTFKDTTITF   ALIGYAFVSA   LLPWLILAP   RDYLATFLKI   GVIVGLALGI   300

      E. coli   VVLNPELKMP   AMTQYIDGTG   PLWKGALFPF   LFITIACGAV   SGFHALISSG   350
   S. enterica  VILNPELKMP   ALTQYVDGTG   PLWKGALFPF   LFITIACGAV   SGFHALISSG   350

      E. coli   TTPKLLANET   DARFIGYGAM   LMESFVAIMA   LVAASIIEPG   LYFAMNTPPA   400
   S. enterica  TTPKLLACET   DARFIGYGAM   LMESFVAVMA   LVAASIIEPG   LYFAMNTPPA   400

      E. coli   GLGITMPNLH   EMGGENAPII   MAQLKDVTAH   AAATVSSWGF   VISPEQILQT   450
   S. enterica  GLGITMPNLH   EMGGENAPLI   MAQLKDVTAH   AAATVSSWGF   VISPEQILQT   450

      E. coli   AKDIGEPSVL   NRAGGAPTLA   VGIAHVFHKV   LPMADMGFWY   HFGILFEALF   500
   S. enterica  AKDIGEPSVL   NRAGGAPTLA   VGIAHVFHKV   LPMADMGFWY   HFGILFEALF   500

      E. coli   ILTALDAGTR   SGRFMLQDLL   GNFIPFLKKT   DSLVAGIIGT   AGCVGLWGYL   550
   S. enterica  ILTALDAGTR   SGRFMLQDLL   GNFVPFLKKT   DSLVAGIIGT   AGCVGLWGYL   550

      E. coli   LYQGVVDPLG   GVKSLWPLFG   ISNQMLAAVA   LVLGTVVLIK   MKRTQYIWVT   600
   S. enterica  LYQGVVDPLG   GVKSLWPLFG   ISNQMLAAVA   LVLSTVVLIK   MQRTKYIWVT   600

      E. coli   VVPAVWLLIC   TTWALGLKLF   STNPQMEGFF   YMASQYKEKI   ANGTDLTAQQ   650
   S. enterica  VIPAVWLLIC   TTWALGLKLF   SANPQMEGFF   YMANLYKEKI   ANGTNLTAQQ   650

      E. coli   IANMNHIVVN   NYTNAGLSIL   FLIVVYSIIF   YGFKTWLAVR   NSDKRTDKET   700
   S. enterica  IANMNHIVVN   NYTNAGLSIL   FLVVVYSIIF   YGFTTWMKVR   NSDKRTDKET   700

      E. coli   PYVPIPEGGV   KISSHH   716
   S. enterica  PYVPVPEGGV   KISSHH   716
```

Figure S1. Comparison of the BtsT consensus sequences of *E. coli* and *S. enterica*. A consensus-based approach sequence comparison was used. 148 sequences of BtsT of *E. coli* and 122 sequences of BtsT of *S. enterica* were aligned and consensus sequences for each bacterium were generated by using the CLC Main Workbench software. Subsequently, both consensus sequences were aligned. The amino acids were colored based on their polarity (red - acidic and polar; blue - basic and polar; green - neutral and polar; black - neutral and nonpolar). Red background color was used to highlight deviating amino acids.

A

B
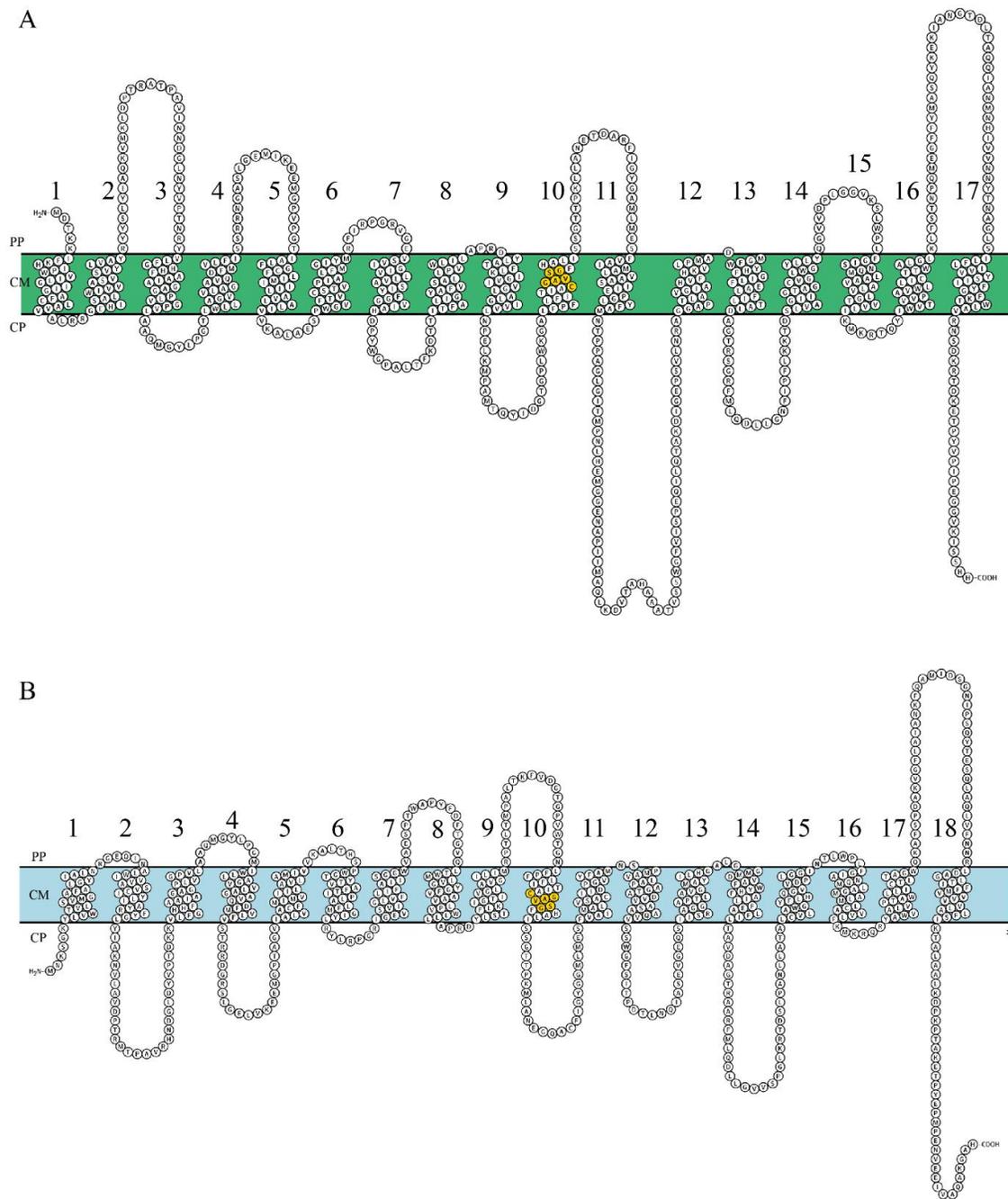
Figure S2. Schematic models of the secondary structure of (A) *E. coli* BstT and (B) *E. coli* CstA. Both models are based on the analysis of the secondary structure using the Uniprot program (1) and visualized with the Protter tool (2). Transmembrane domains (TMs) are numbered with numerals. The conserved motif CG-x(2)-SG with a high degree of sequence

conservation within CstA homologues is marked in yellow (3). PP periplasm, CM cytoplasmic

membrane, CP cytoplasm.
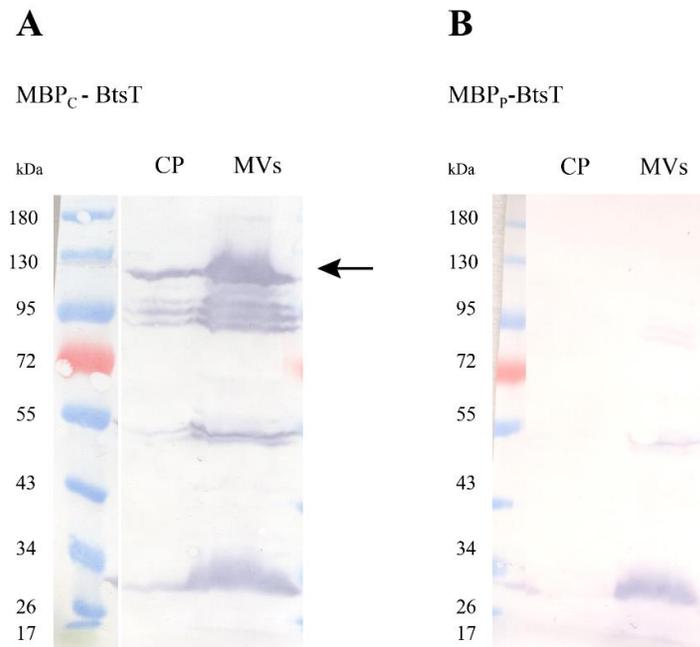
Figure S3. Localization of maltose-binding protein (MBP) hybrids. *malE* without or with leader sequence was fused to the 5' end of *btsT* encoding hybrid proteins with either a putative cytoplasmically located MPB (MBP$_C$-BtsT) (A) or a periplasmically located MBP (MBP$_P$-BtsT) (B), respectively. Cells with overproduced hybrids were fractionated to separate cytoplasm (CP) and membrane vesicles (MVs). Both fractions were adjusted to the same volume and separated by 12.5% (w/v) SDS-polyacrylamide gel electrophoresis and immunoblotted using a penta-His antibody for detection. The arrow indicates MBP$_C$-BtsT (about 120 kDa).
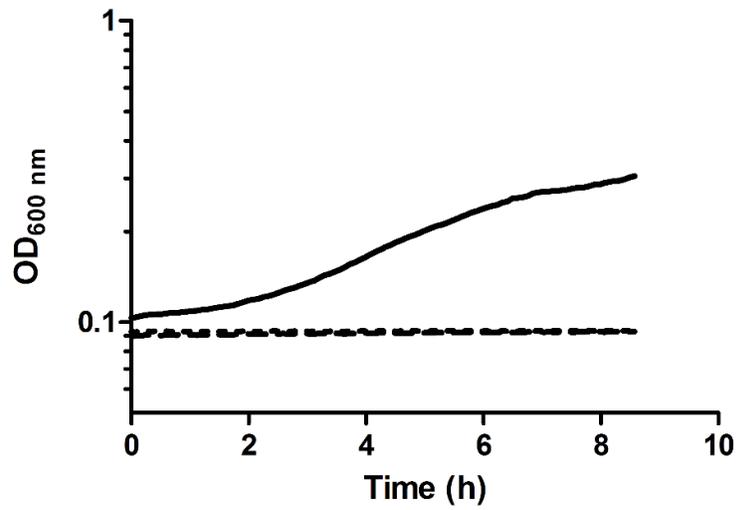
Figure S4. Complementation of *E. coli* MM39 with different MBP-BtsT hybrid proteins.

malE deficient *E. coli* MM39 cells were transformed with plasmids pMAL-p2x (solid line), pMAL$_P$-*btsT* (dotted line) and pMAL$_C$-*btsT* (dashed line), and their growth was monitored over time on maltose as the sole carbon source.
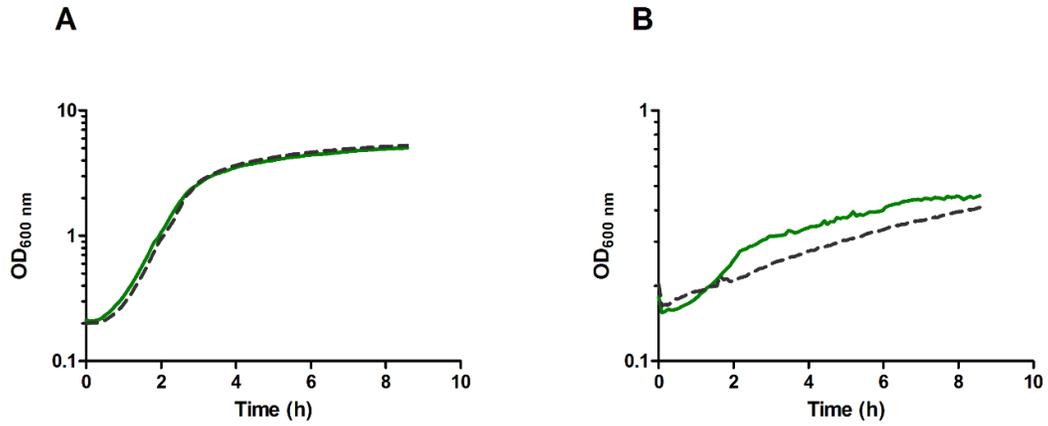
Figure S5. Growth of *E. coli* MG1655 and the *bstT* mutant in different media. *E. coli* MG1655 (green line) and *E. coli* MG1655 Δ*btsT* (dotted black line) were cultivated in LB medium (A) or in M9 minimal medium supplemented with pyruvate as carbon source (20 mM) (B). Samples were taken and analyzed every 5 min (A) or 2 hours (B).
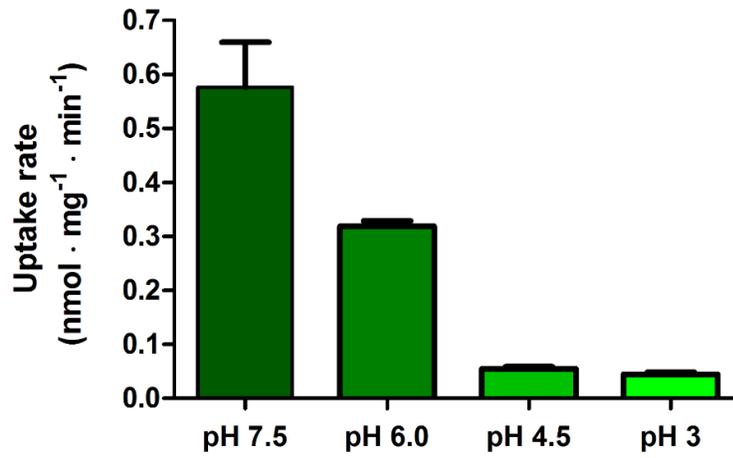
Figure S6. Rates of [14]C-pyruvate uptake by BtsT-producing strain *E. coli* MG1655 Δ*btsT* pBAD24-*btsT* at various external pH values. [14]C pyruvate was added at a final concentration of 10 μM.
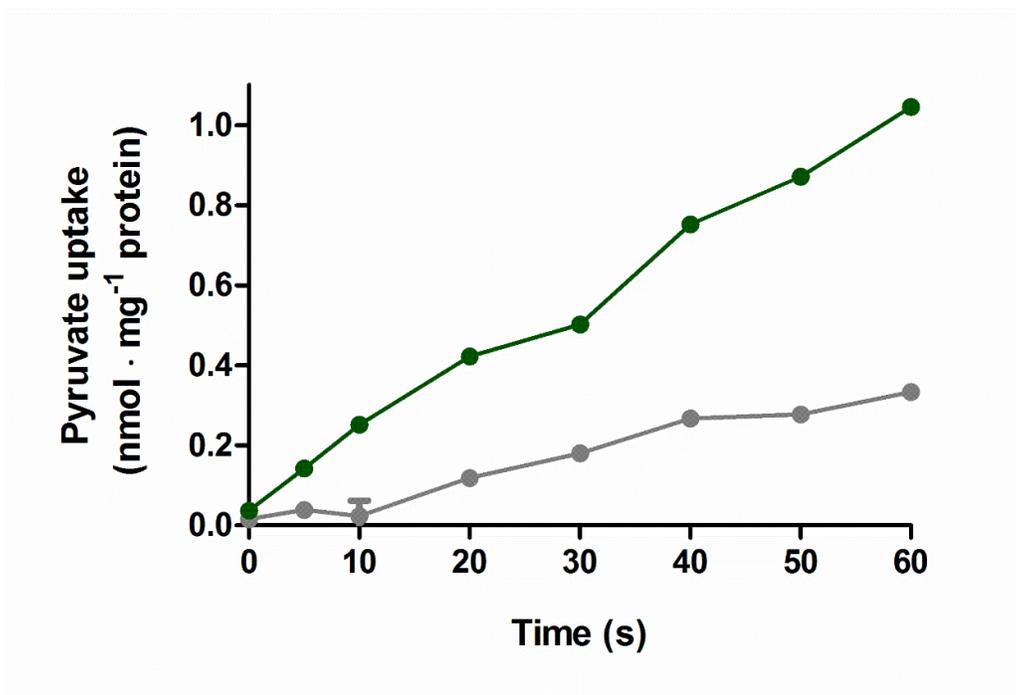
Figure S7. Time course of pyruvate uptake by *E. coli* YYC202. [14]C-Pyruvate uptake was determined at a final pyruvate concentration of 10 µM at 15°C. Rates of uptake accumulation: BtsT producing strain *E. coli* YYC202 pBAD24-*btsT* (green), control strain *E. coli* YYC202 pBAD24 (grey). Standard deviations are estimated from three biological replicates.
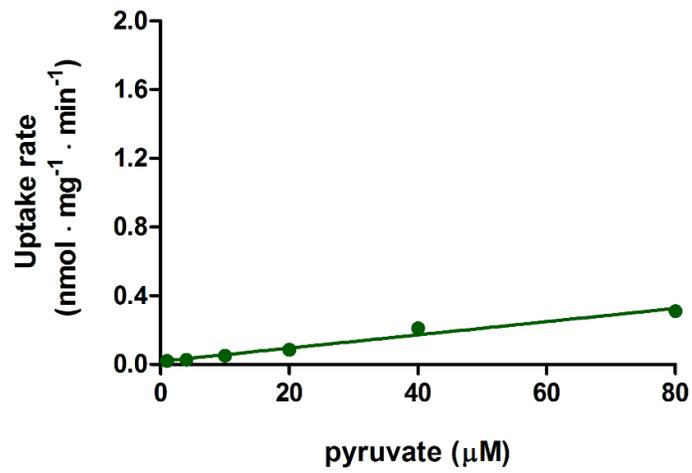
Figure S8. Pyruvate diffusion in intact *E. coli* cells. Uptake of ^{14}C-pyruvate by *E. coli* MG1655 Δ*btsT* transformed with pBAD24 was determined in the presence of increasing pyruvate concentrations. The best-fit line was determined by linear regression. Error bars represent standard error of the mean.
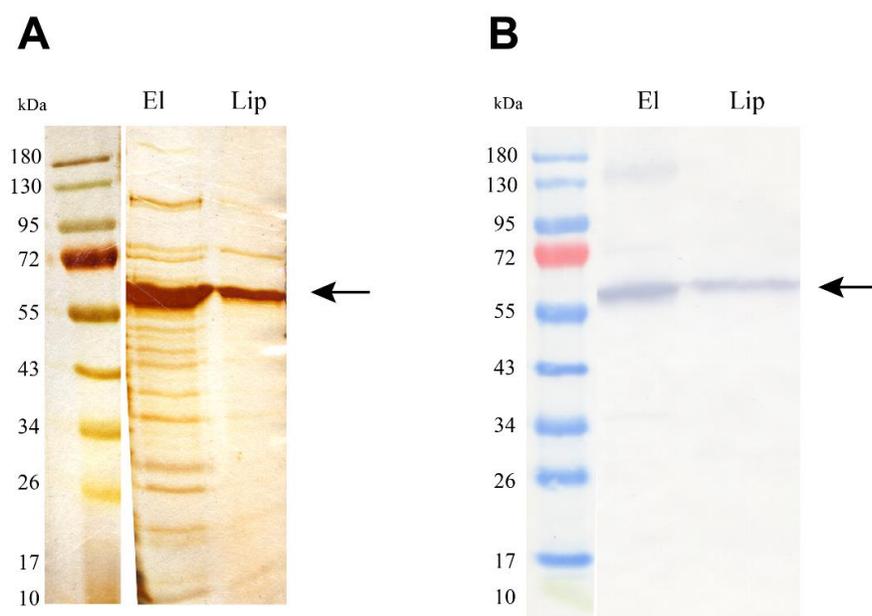
Figure S9. Purification of His-tagged BtsT. Membrane vesicles were prepared from *E. coli* cells after overproduction of BtsT-6His. Membrane proteins were then solubilized with 1.5% (w/v) n-dodecyl β-D-maltoside. The His-tagged BtsT was purified as described in Materials and Methods. El, BtsT eluted from the column with 300 mM imidazole (8.75 μg of protein). Lip, BtsT reconstituted into *E. coli* liposomes (10 μg of protein). Proteins were separated using 12.5% (w/v) SDS-polyacrylamide gel electrophoresis and stained with silver (A) or immunodetected by using a penta-His antibody (B). The arrows indicate BtsT-6His. In both images, non-relevant lanes were omitted for clarity.
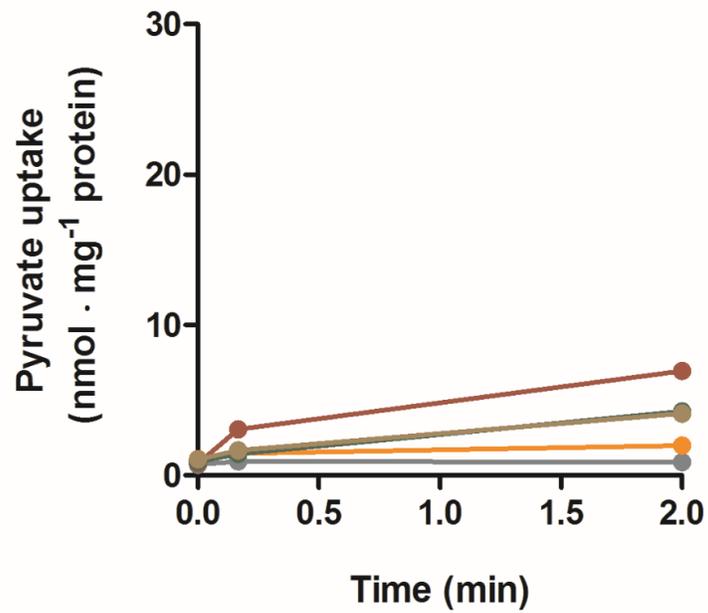
Figure S10. Pyruvate diffusion in *E. coli* liposomes. ^{14}C-pyruvate (40 μM) diffusion was analyzed in liposomes (in the absence of protein). Time course of pyruvate uptake in the presence of artificially imposed $\Delta\tilde{\mu}_{H+}$ (green), $\Delta\Psi$ (orange), ΔpH (red), $\Delta\tilde{\mu}_{Na+}$ (brown) or in the absence of any gradient (grey).

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2. Omasits U, Ahrens CH, Muller S, Wollscheid B. 2014. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics 30:884–886.

3. Vastermark A, Wollwage S, Houle ME, Rio R, Saier MH. 2014. Expansion of the APC superfamily of secondary carriers. Proteins 82:2797–2811.

Supplemental Material - Chapter 4

SUPPLEMENTARY MATERIAL

A single-cell view of the BtsSR/YpdAB pyruvate sensing network in *Escherichia coli* and its biological relevance

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Running Head: Phenotypic heterogeneity in *E. coli*

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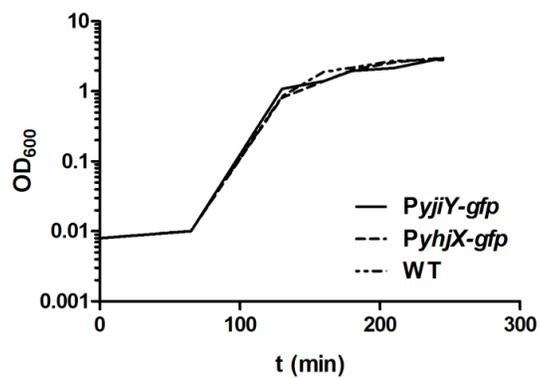
FIG S1 Growth of reporter strains. *E. coli* cells expressing *gfp* under the control of P_{yhjX} or P_{yjiY} and the MG1655 strain (WT, without promoter-*gfp* fusion) were grown in LB medium.
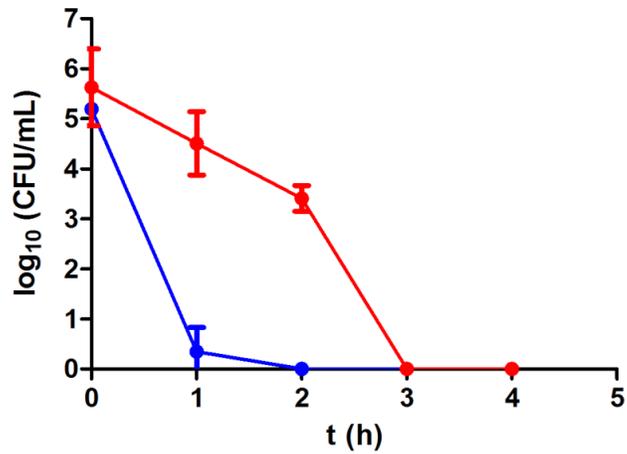
FIG S2 Determination of the minimum duration of killing (MDK) after ofloxacin treatment. *E. coli* cells of either WT (blue line) or mutant Δ*btsSRypdAB* (red line) were grown in LB-medium. At the post-exponential growth phase cells were challenged with ofloxacin (5 μg/ml). Samples were taken and analyzed for colony forming units (CFUs). The MDK_{99} value was taken as the time needed to kill 99% of the initial population. Experiments were performed three independent times and error bars indicate the standard deviations of the means.

STRAINS	- INDUCER		+ INDUCER	
	Cells (%)		**Cells (%)**	
	OFF	ON	OFF	ON
WT GFP (IPTG)	92.7	7.3	3.1	96.9
btsSRypdAB GFP (IPTG)	97.8	2.2	51.0	49.0
WT GFP-DppA (Arabinose)	94.9	5.1	24.4	75.6
btsSRypdAB GFP-DppA (Arabinose)	98.1	1.9	99.4	0.6
WT LysP-mCherry (Arabinose)	97.7	2.3	33.4	66.6
btsSRypdAB LysP-mCherry (Arabinose)	98.2	1.8	98.5	1.5

TABLE S1 The BtsSR/YpdAB network promotes overproduction of proteins. *E. coli* cells of either WT or the *btsSRypdAB* mutant harboring an overproduction vector with IPTG inducible promoter for the overproduction of GFP; an arabinose inducible promoter for the overproduction of DppA-GFP and an arabinose inducible promoter for the overproduction of LysP-mCherry were grown in LB medium. Samples were taken before (- inducer) and after (+ inducer) the addition of the inducer. Flow cytometry was used to count fluorescent cells (maximum of 2000

events), and the percentages of OFF (non-fluorescent cells) and ON cells (fluorescent cells) were calculated from the raw data. Experiments were performed three independent times and standard deviations were below 10%.

Acknowledgements

Looking back almost 4 years since I came to Munich, it was an amazing experience! I would like to thank all people who supported my research and made this thesis possible.

First, I would like to acknowledge the contribution of Prof. Kirsten Jung, who gave me the opportunity of doing scientific research in AG K Jung and for all the patient advice. Thanks goes to my TAC committee members, Prof. Marc Bramkamp and Prof. Jürgen Soll, for all their contribution and help to bring my PhD to its final form. Special thanks also to my thesis committee members, Prof. Wolfgang Enard, Prof. Jörg Nickelsen and Prof. Boshart for generously offering their time to examine my PhD thesis. I would also like to thank Prof. Heinrich Jung for all insightful conversations, and to Francisca Mende and all the members of LSM graduate school.

I am so glad for meeting so many interesting people over 4 years. Thanks to all YehUT team members, Stefan, Florian and all our students, my office mates Wolfram, Ralph and Felix and of course, my friends Ana, Bruno, Yang and Kim. It was an amazing time with you all. I would like to specially thank Cláudia, my YehUT team colleague, office mate, reviewer, secretary, business partner but most importantly, my best friend.

Last but not least, I consider myself very fortunate for all the support of my parents. And my last thanks goes to Stanislav, the one who was there for me all the time.

Ivica Krištofičová

Curriculum Vitae

▬▬ Education

2014–Present	**PhD in Microbiology**, *Ludwig Maximilians University*, Munich, Germany.

- Student of The Graduate School Life Science Munich (LSM)
- Supervisor:
  - Prof. Kirsten Jung
- PhD. Project:
  - Biochemical characterisation of a two-component system BtsS/BtsR by in vivo and in vitro studies in *Escherichia coli*.
- Publications:
  - Identification of a High-Affinity Pyruvate Receptor in *Escherichia coli* Sci Rep 2017

2011–2013	**Masters of Biochemistry**, *Charles University in Prague*, Prague, Czech Republic.

- Supervisors:
  - Marek Ingr PhD.
  - Doc. Jan Konvalinka
- Diploma thesis:
  - Study of the cleavage kinetics of Gag polyprotein from HIV-1 virus by the viral protease.

2008–2011	**Bachelor of Biochemistry**, *Comenius University*, Bratislava, Slovakia.

- Supervisor:
  - Prof. Jozef Nosek
- Bachelor thesis:
  - Quorum Sensing - communication of bacteria and its application in synthetic biology.

▬▬ Work

2011–2013	**Scientist**, *Institute of Organic Chemistry and Biochemistry*, Prague, Czech Republic.

I have joined there a research group under the supervision of Doc. Jan Konvalinka, that deals with the analysis, structure, activity and inhibition of proteases and other enzymes that are medically important.

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Awards

2016	Annual Conference 2016 of VAAM at Jena, Germany, Best Poster Presentation
2009–2010	Comenius University, Scholarship for excellent study results (top 10 % of students)
2008	Comenius University, Scholarship for excellent study results (top 5 % of students)

Skills

Certificates	Purification and Characterization of proteins, Malvern Instruments (2016), Hands-on training course NGS data analysis (2016), Workshop on "Sensory and regulatory RNAs in prokaryotes" (2016), MicroCal Course Advanced Isothermal Titration Calorimetry (2015)
Soft skills	Workshop "Presentation skills (Natural sciences and medical research)" (2015), Workshop "Writing and publishing of papers, reviews and theses in the life sciences" (2015), Workshop " Leadership Skills" (2015)
Other	LaTeX, Adobe Illustrator

Academic Meetings

2017	Attended SPP1617-progress report meeting in München, Germany
2017	Talk at the VAAM conference in Würzburg, Germany
2016	Attended SPP1617-progress report meeting in Düsseldorf, Germany
2016	Poster at the VAAM conference in Jena, Germany
2015	Poster at the 77th Harden Conference: Two Component Signalling in Bacteria: Integrating Approaches and Science in Warwick, UK
2015	Attended CAS Conference Synthetic Biology II in München, Germany
2015	Poster at the VAAM conference in Marburg, Germany
2015	Poster at the Blast XIII conference in Tucson, USA

Languages

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- Travelling
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