The Pyruvate-Tricarboxylic Acid Cycle Node

A FOCAL POINT OF VIRULENCE CONTROL IN THE ENTERIC PATHOGEN YERSINIA PSEUDOTUBERCULOSIS*

Received for publication, June 20, 2014, and in revised form, August 13, 2014 Published, JBC Papers in Press, August 27, 2014, DOI 10.1074/jbc.M114.581348

René Bücker‡§, Ann Kathrin Heroven‡, Judith Becker¶, Petra Dersch‡, and Christoph Wittmann‡†

From the ‡ Institute of Systems Biotechnology, Saarland University, 66123 Saarbrücken, the § Institute of Biochemical Engineering, Technische Universität, Braunschweig and the ¶ Department of Molecular Infection Biology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany

Background: Yersinia pseudotuberculosis is a human pathogen and the ancestor of Y. pestis.

Results: The pyruvate-tricarboxylic acid cycle node in the carbon core metabolism of Y. pseudotuberculosis is a focal point of its virulence control system.

Conclusion: Mutants genetically perturbed at this metabolic control point are less virulent in mouse infection studies.

Significance: Learning how pathogenic traits are controlled is crucial for finding novel drug targets against the pathogen.

Despite our increasing knowledge of the specific pathogenicity factors in bacteria, the contribution of metabolic processes to virulence is largely unknown. Here, we elucidate a tight connection between pathogenicity and core metabolism in the enteric pathogen Yersinia pseudotuberculosis by integrated transcriptome and [13C]metabolome analysis of the wild type and virulence-regulator mutants. During aerobic growth on glucose, Y. pseudotuberculosis reveals an unusual flux distribution with a high level of secreted pyruvate. The absence of the transcriptional and post-transcriptional regulators RovA, CsrA, and Crp strongly perturbs the fluxes of carbon core metabolism at the level of pyruvate metabolism and the tricarboxylic acid (TCA) cycle, and these perturbations are accompanied by transcriptional changes in the corresponding enzymes. Knock-outs of regulators of this metabolic branch point and of its central enzyme, pyruvate kinase (ΔpykF), result in mutants with significantly reduced virulence in an oral mouse infection model. In summary, our work identifies the pyruvate-TCA cycle node as a focal point for controlling the host colonization and virulence of Yersinia.

In mammals, including humans, Yersinia pseudotuberculosis causes gut-associated diseases, such as diarrhea, enteritis, and colitis, including several recent outbreaks of these diseases (1, 2). In addition to its clinical relevance, the pathogen is important as an evolutionary ancestor of Yersinia pestis, the agent of plague (3). Y. pseudotuberculosis is found in different external habitats and warm-blooded hosts (4). The typical infection route occurs via the oral uptake of Y. pseudotuberculosis and its binding to the intestinal epithelium (5). Subsequently, the bacterial cells spread into the lymphatic system, where they rapidly multiply and colonize deeper tissues, such as the mesenteric lymph nodes, liver, and spleen (6). The infection process is complex and is accompanied by frequent environmental changes in which the bacteria experience constant variations in nutrient availability. The ability to efficiently compete for nutrients is crucial for successful infection, i.e. the bacteria must outcompete the gut microbiota and persist long term within the intestinal tract (7, 8). Y. pseudotuberculosis has developed global regulatory cascades that coordinate physiological processes and virulence factors to initiate the infection. Several of the regulators suggest a link between the virulence and core metabolism of the pathogen. Over the last few years, it has become evident that the catabolite repression protein (Crp) and the carbon storage regulator A (CsrA) are key players in the virulence management system in addition to their well known function as metabolic regulators (Fig. 1) (9, 10). CsrA is a global RNA-binding regulator protein, which itself is controlled by the small noncoding RNAs CsrB and CsrC. This global regulator is also involved in controlling the major transcriptional regulator of virulence, RovA, and its transcriptional repressor, RovM (Fig. 1). In the absence of CsrA, the expression of RovM is reduced, and lower levels of the repressor then lead to higher amounts of RovA (11) and the subsequent expression of invasin, thus mediating attachment to and entry into the intestinal epithelium (12). The Crp protein influences the expression of rova through counter-regulation of the Csr RNAs either directly or through the UvrY response regulator (Fig. 1). Crp and CsrA are pivotal for a successful Yersinia infection (10). Crp also affects the virulence and metabolism of Y. pestis (13).

One of the key questions that arises from our current knowledge of the cell’s virulence revolves around the metabolic core machinery of the cell (14). Despite its obvious importance, the complex interplay between nutritional status, metabolism, and virulence is still not understood, and the metabolic requirements of Yersinia for adapting to and surviving in different host niches is largely unknown.

Here, we have utilized a systems biology approach to address this question with a focus on central carbon metabolism. In particular, the link between virulence and metabolism was

* This work was supported by the Deutsche Forschungsgemeinschaft (Host-Adapted Metabolism of Bacterial Pathogens) Grant SPP 1316.

‡ This article contains supplemental Tables S1 and S2.

§ To whom correspondence should be addressed: Institute of Systems Biotechnology, Saarland University, Campus A1.5, 66123 Saarbrücken, Germany. Tel.: 49-681-30271971; Fax: 49-681-30271972; E-mail: christoph.wittmann@uni-saarland.de.
investigated in the wild type and in specific mutants lacking key regulators that coordinate virulence and metabolism. To this end, we performed transcriptomic and \([^{13}C]\)fluxomic analysis. The latter involved \(^{13}C\) tracer studies, mass spectrometric labeling analysis, and comprehensive computer models to assess the pathway flux (15, 16). This work provided quantitative insight into the molecular fluxes of \(Y.\) pseudotuberculosis. We integrated the derived data sets into a carefully curated concept, which identified the tricarboxylic acid (TCA)\(^2\) cycle as the focal point of virulence control, and we verified its role with an \textit{in vivo} mouse model.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Mutant Constructions**—DNA manipulations and transformations were performed using standard genetic and molecular techniques as described previously (17, 18). The strains and plasmids used in this study are listed in Table 1. For construction of the mutagenesis plasmids pAKH175, pAKH177, and pAKH187, a PCR fragment harboring a kanamycin resistance gene inserted into the \(ptsN\), \(pykF\), or \(pdhR\) gene (\(ptsN\):\(Kan^R\), \(pykF\):\(Kan^R\), or \(pdhR\):\(Kan^R\)) was generated. The kanamycin gene was amplified using primer pair \(655/656\) and \(657/658\), respectively (Table 2). Each fragment contained 20 nucleotides homologous to the end of the kanamycin cassette (\(ptsN_{\text{down}}\), primer IV971/IV972; \(pykF_{\text{up}}\), primer IV977/IV978; \(pdhR_{\text{up}}\), primer V567/V548). The downstream fragment was amplified with a primer pair of which the forward primer contained an additional 20 nucleotides at the 3’-end, which were homologous to the start of the kanamycin cassette (\(ptsN_{\text{down}}\), primer IV971/IV972; \(pykF_{\text{down}}\), primer IV979/IV980; \(pdhR_{\text{down}}\), primer V549/V550). In the next step, PCR was performed with the forward and the reverse primer using the upstream and downstream PCR products of the respective target gene and the \textit{kan} gene fragment as templates. The resulting fragment was digested with ScaI and ligated into the ScaI site of pAKH3. The \(Y.\) pseudotuberculosis mutant strains used in this study are derived from wild type strain YPIII. Construction of the \(Y.\) pseudotuberculosis YPIII \(arcA::Kan^R\) mutant YP49 was performed by allelic exchange as described previously (11, 19). The kanamycin gene was amplified from pACYC177 using primer pair 360/361. The upstream and downstream fragment flanking the \(arcA\) gene were amplified with primer pair 655/656 and 657/658, respectively (Table 2). Each fragment contained 20 nucleotides homologous to the \textit{kan} gene. In the next step, PCR was performed with the forward primer of the upstream fragment and the reverse primer of the downstream fragment using the upstream and downstream PCR products of the respective target gene and the \textit{kan} PCR fragment as template. The PCR fragment was transformed into \(Y.\) pseudotuberculosis YPIII pKOBEG-sacB. Chromosomal integration of the fragment was selected by plating on LB supplemented with kanamycin. Mutants were subsequently grown on LB agar plates without NaCl plus 10% sucrose, and faster growing colonies without pKOBEG-sacB were selected and proven by PCR and DNA sequencing. The \textit{Yersinia} mutants YP252 (YPIII \(ptsN::Kan^R\)), YP253 (YPIII \(pykF::Kan^R\)), and YP274 (YPIII \(pdhR::Kan^R\)) were constructed as described previously (10) using the suicide plasmids pAKH175, pAKH177, and pAKH187.

**Cultivation**—Cells were cultivated at 25 °C under aeration on a rotary shaker. The first pre-cultures were grown in a 1:1 mixture of \(F\)’s F-12 Nutrient Mixture (Invitrogen) and liquid DMEM (Biochrom, Berlin, Germany). The second pre-cultures

---

\(^2\) The abbreviations used are: TCA, tricarboxylic acid; EMP, Emden-Meyerhof-Parnas; PEP, phosphoenolpyruvate; PP, Peyer’s patch; PPP, pentose phosphate pathway; MLN, mesenteric lymph node; PTS, phosphotransferase system.

---

**TABLE 1**

Bacterial strains and plasmids

| Strains, plasmids | Description | Source and ref. |
|-------------------|-------------|-----------------|
| **E. coli** | \(recA1\) thi pro hsdS\(^R\) \(RP4-2Tc::Mu\) \(Kan::Tn7\) Apir | 84 |
| **Y. pseudotuberculosis** | \(YP101\) plb1, wild type | 85 |
| \(YP3\) | plb1, \(rovA::Tn10(60)^{R}\) \(Cml^R\) | 12 |
| \(YP49\) | plb1, \(arcA\), \(Kan^R\) | This study |
| \(YP53\) | plb1, \(arcA\), \(Kan^R\) | 11 |
| \(YP49\) | plb1, \(arcA\), \(Kan^R\) | 10 |
| \(YP252\) | plb1, \(ptsN\), \(Kan^R\) | This study |
| \(YP253\) | plb1, \(pykF\), \(Kan^R\) | This study |
| \(YP274\) | plb1, \(pdhR\), \(Kan^R\) | This study |
| Plasmids | pACYC177 Cloning vector, Ap\(^R\), Kan\(^R\) | 86 |
| pAKH3 | pG7704, sacB\(^{R}\), Ap\(^R\) | 10 |
| pAKH175 | pAKH3, ptsC::Kan\(^R\) | This study |
| pAKH177 | pAKH3, pykF::Kan\(^R\) | This study |
| pAKH187 | pAKH3, pdhR::Kan\(^R\) | This study |
| pKD4 | Kanamycin cassette template, Ap\(^R\), Kan\(^R\) | 87 |
| pKOBEG-sacB | Recombination vector, sacB\(^R\), Cm\(^R\) | 19 |
and main cultures were grown in a Yersinia minimal medium (YMM) that was developed in this work. YMM contained the following per liter: 8 g of glucose, 6.62 g of KH$_2$PO$_4$, 13.26 g of K$_2$HPO$_4$, 0.31 g of NaCl, 5 g of (NH$_4$)$_2$SO$_4$, 0.20 g of MgSO$_4$·7H$_2$O, 30 mg of 3,4-dihydroxybenzoic acid, 0.5 mg of FeSO$_4$·7H$_2$O, 2 mg of FeCl$_3$·6H$_2$O, 2 mg of MnSO$_4$·H$_2$O, 1.3 mg of ZnSO$_4$·7H$_2$O, 0.2 mg of CuCl$_2$·2H$_2$O, 0.2 mg of Na$_2$B$_4$O$_7$·10H$_2$O, and 0.1 mg of (NH$_4$)$_2$Mo$_7$O$_24$·4H$_2$O. In tracer experiments for metabolic flux analysis, the natural glucose was replaced by 99% [1-13C]glucose (Euriso-top, Saint-Aubin Cedex, France), and the inoculum level was below 1% of the sampled cell concentration, so that the influence of nonlabeled biomass on the subsequent labeling analysis could be neglected (20). The dissolved oxygen concentration was quantitated online by immobilized sensor spots (21).

Substrate and Product Analysis—Glucose concentrations were measured by enzymatic analysis (YSI 2700 Select, YSI Inc., Yellow Springs, OH). Organic acids and alcohols were quantified by HPLC (22). Amino acid quantification was conducted as described previously (23). The cell concentration ($A_600$) was measured as the absorbance at 600 nm. In addition, the cell dry weight (CDW) was determined by gravimetric analysis. The correlation factor from both measurements was 0.325 (g$_{CDW}$ liter$^{-1}$) = 1 $A_{600}$ unit.

Gene Expression Profiling—The global gene expression was analyzed by microarray (Agilent, Waldbronn, Germany, 8 × 15 K format). The analysis included three biological replicates for each strain. In addition, samples were taken at three different time points during the exponential growth phase to validate constant expression during the cultivation. The array design, RNA extraction, and hybridization were performed as described previously (10). Microarray data processing was conducted using the Limma package (24) from the R/Bioconductor framework (25). Unprocessed array intensity values were read-in using the function read.maimages, and the background was corrected with the improved saddle-point approximation to the maximum likelihood method (26) using an intensity offset of 50. The array intensities were normalized within each array using loess normalization (27) and between arrays using quantile normalization (28, 29) to obtain similar distributions of expression intensities. To obtain reliable gene expression values, we averaged the normalized intensities of at least three probes targeting the same gene. Differentially expressed genes were determined using the lmFit function for linear modeling and by computing moderated $t$-statistics and log-odds with the function eBayes (30). $p$ values from the moderated $t$-tests were corrected for multiple testing using the Benjamini-Hochberg procedure for controlling the false discovery rate. Finally, the set of differentially expressed genes was filtered by the fold change ([log2FC] $>$ 0.8) (supplemental Table S1). All array data generated in this study were deposited in the Gene Expression Omnibus (GEO) database and are available under accession number GSE54547.

Western Blot Analysis—Wild type Y. pseudotuberculosis was grown to the stationary phase, and whole cell extracts were prepared (18). For DNA digestion, 1% by volume of benzonase (Merck) was added to the whole cell extracts and incubated for 1 h at 37 °C. Separation of the cell extracts and Western blotting were performed as described previously (31).

Metabolic Network and Biomass Requirements—The metabolic network (supplemental Table S2) of Y. pseudotuberculosis, used for flux estimation, was constructed from its genome-encoded pathway repertoire (NCBI Reference Sequence, NC_010465.1). This network included all relevant central metabolic pathways of carbon core metabolism, i.e., the Emden-Meyerhof-Parnas (EMP) pathway, the pentose phosphate pathway, the Entner-Doudoroff pathway, and the TCA cycle, as well as the anaerobic glyoxylate shunt, phosphoenolpyruvate (PEP) carboxylase, PEP carboxykinase, and the malic enzyme. Furthermore, the anabolic routes from the intermediary precursors into biomass and the pathways to the excreted by-products (pyruvate, lactate, formate, ethanol, acetate, fumarate, succinate, and 2-oxoglutarate) were implemented. The requirement of Y. pseudotuberculosis for anabolic precursors was estimated on the basis of its cellular composition, which was experimentally determined.
Interplay between Metabolism and Virulence in Yersinia

Analysis of Cellular Composition of Y. pseudotuberculosis—The protein content was determined after enzymatic treatment of frozen cell pellets. The pellet was resuspended in lysis buffer with 25 units ml⁻¹ benzonase and 10 KU ml⁻¹ lysozyme (Bug Buster, Merck, Germany) and incubated for 1 h at 170 rpm and 25 °C on a rotary shaker (Lab Shaker, B. Braun Melsungen, Melsungen, Germany) with a shaking diameter of 5.0 cm. To ensure efficient digest, the concentration of the biomass was adjusted in a range between 3 and 15 mg liter⁻¹. The protein content was then quantified by UV absorption measurement at 280 and 260 nm (Nanodrop ND-1000, Thermo Fisher Scientific, Waltham, MA) in the supernatant, obtained by centrifugation. The protein concentration was calculated as described previously (32). The amino acid composition of the protein was quantified after acidic hydrolysis of biomass and subsequent quantification by HPLC as described above. The values for cysteine, methionine, and tryptophan, respectively, were taken from the closely related bacterium Escherichia coli (33), because these amino acids are degraded during the hydrolysis. For quantification of the RNA content, cells were washed twice with an ice-cold killing buffer (20 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 20 mM NaN₃) to prevent RNA degradation. RNA preparation was performed as described previously (34), followed by quantification of the RNA content using a Nanodrop ND-1000 at 260 nm. The nucleotide composition of RNA was calculated from the sequence of the 5 S, 16 S, and 23 S rRNA genes, based on the assumption that the total RNA pool is mainly composed of rRNA (35). The DNA content was determined after phenol/chloroform extraction. Cells were first harvested by centrifugation at 16,100 × g and 4 °C for 5 min (Centrifuge 5415R, Eppendorf, Hamburg, Germany). An appropriate amount (0.2–0.5 mg) was then resuspended in 560 µl of lysis buffer, pH 8.0, containing 2.88 liter⁻¹ Tris, 6.96 liter⁻¹ EDTA, 97.80 g liter⁻¹ sucrose, and 0.48 mg liter⁻¹ lysozyme and then disrupted by incubation at 800 rpm and 30 °C for 30 min (Thermomixer Comfort, Eppendorf, Hamburg, Germany). A second disruption was performed with FastPrep-24 (MP Biomedicals, Santa Ana, CA) involving two cycles of 40 s each at 6.0 m s⁻¹ and 4 °C. Subsequently, 140 µl RES buffer with RNase A (Nucleo Bond Xtra, Macherey-Nagel, Germany) was added to degrade RNA residues, otherwise interfering with subsequent analysis. The aqueous DNA solution was then extracted twice with 700 µl of Roti-P/C/1 (Carl-Roth, Karlsruhe, Germany) and 700 µl of chloroform. Precipitation of the DNA was performed by adding 65 µl of 3 M sodium acetate and 1.3 ml of 100% ethanol, respectively. The DNA pellet was then washed with 70% ethanol, dried, and suspended in deionized water. All centrifugation steps were performed at 16,100 × g and 4 °C for 10 min. Quantification was carried out by measuring the absorbance at 260 nm using a Nanodrop ND-1000. The nucleotide composition of the DNA was calculated from the genomic GC content of 47.5% (NCBI reference sequence of the genome, NC_010465). Data for glycogen, lipids, and minor shares of further biomass constituents were taken from E. coli (33).

Metabolic Flux Analysis and Statistical Evaluation—For determination of metabolic fluxes, cells were grown on 99% [1-¹³C]glucose. At different time points during exponential growth, mass isotopomer distributions of amino acids from hydrolyzed and lyophilized cell protein were quantified by GC-MS after derivatization with N-methyl-N-tert-butyldimethylsilyl-trifuoroacetamide (36). GC-MS analysis was performed as described previously using a GC system (7890A gas chromatograph) and a quadrupole MS detector (inert MSD 5979C) from Agilent Technologies (Waldborn, Germany) (37, 38). To check for potential isobaric interference, all samples were first measured in scan mode. The labeling analysis of the amino acids was then conducted in duplicate by selective ion monitoring of representative ion clusters for each analyte. Similar to previous flux studies with other bacteria (39), the amino acids alanine, valine, glycine, serine, phenylalanine, tyrosine, aspartate, threonine, and glutamate yielded high quality data and were thus considered for flux calculation. Following general guidelines for CO₂ flux analysis (20), other amino acids were not considered because of the isobaric signal overlay (proline), unambiguous fragments (leucine and isoleucine), background interference due to weak signal intensity (arginine, histidine, and lysine), or were not available due to oxidative breakdown (cysteine, methionine, and tryptophan) and deamination (glutamine and asparagine) during the hydrolysis process. For flux calculation, the reconstructed metabolic network (supplemental Table S2) was implemented into the open source software OpenFlux 2.1, which uses Matlab 7.11 (MathWorks Inc., Natick, MA) as a numerical computing environment (40), involves isotopomer and metabolite balancing, and simulates the propagation of the ¹³C label through the network by the elementary metabolite unit framework (41), whereby ¹³C transition by bidirectional reactions, ¹³C scrambling in symmetric molecules, ¹³C incorporation from CO₂, and naturally occurring isotopes in the tracer substrate were fully considered (40). Shortly, metabolic fluxes were estimated from measured extracellular fluxes of substrate uptake and product secretion, of anabolic fluxes determined from cell growth and cell composition, and from the set of ¹³C labeling data, using a least square algorithm applying a weighted sum of least squares as error criterion (20). Statistical evaluation of metabolic fluxes was conducted by a Monte-Carlo approach (40).

Hierarchical Clustering Analysis—The hierarchical clustering of transcriptome data were conducted with the heatmap2 function of the R package gplots.

Ethics Statement—All animal work was performed in strict accordance with the German Recommendations of the Society for Laboratory Animal Science and the European Health Recommendations of the Federation of Laboratory Animal Science Associations. The animal protocol was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (animal licensing committee permission no. 33.9-225212-1/1010). Animals were handled with appropriate care and welfare, and all efforts were made to minimize suffering.

Mouse Infections—The bacteria used for oral infection were grown overnight at 25 °C in LB medium and resuspended in PBS. To assess the effect of arcA, ptsN, pykF, and pdhR on Y. pseudotuberculosis virulence, groups (n = 20) of 7-week-old female BALB/c mice (Janvier, Saint Berthevin, France) were orally infected with an equal mixture of 10⁷ bacteria of the wild type strain (YP111) and an isogenic mutant strain, i.e. YP49
Interplay between Metabolism and Virulence in Yersinia

Iron Availability Triggers the Expression of the Early Stage Virulence Regulator RovA in Y. pseudotuberculosis—A thorough comparison of growth media revealed a significant influence of monovalent and bivalent ions on the expression level of RovA in Y. pseudotuberculosis. Increased levels of bivalent iron and of the iron chelator 3,4-dihydroxybenzoic acid stimulated RovA expression, whereas calcium and sodium ions reduced the level of the virulence regulator (Table 3). These findings were integrated to formulate a Yersinia minimal medium (YMM, see under “Materials and Methods”) that enabled efficient expression of RovA under defined growth conditions, an important pre-requisite for the subsequent fluxome analysis.

Glucose-grown Y. pseudotuberculosis Secretes High Amounts of Pyruvate—When grown on minimal medium, Y. pseudotuberculosis completely consumed the glucose carbon source within 8.8 h, indicating high activity of the cultured bacteria (Fig. 2). Indeed, the specific rates of growth (0.32 h⁻¹) and substrate uptake (7.1 mmol g⁻¹ h⁻¹) were consistently maintained at high levels throughout the cultivation (Table 4). Glucose, however, was only partially converted into biomass, as indicated by a low biomass yield. A range of metabolites was secreted into the medium. Surprisingly, high amounts of pyruvate accumulated (22.6 mmol liter⁻¹). Lactate and acetate were also observed (Fig. 2). Additional by-products (formate, succinate, ethanol, α-ketoglutarate, and fumarate) were found at lower levels (Table 4). The level of dissolved oxygen, which was monitored on-line, remained above 30% of the saturation value throughout the entire cultivation period. This finding indicates that the observed overflow metabolism was active in the fully aerobic cells. The triplicate cultures showed high consistency. Similarly, the developed set-up enabled highly reproducible growth for all deletion mutants investigated in this study (Fig. 3).

Influence of the Global Regulators RovA, CsrA, and Crp on Growth Behavior and Overflow Metabolism—Next, the manner in which RovA, CsrA, and Crp affect the overall growth behavior of Y. pseudotuberculosis was investigated. Deletion of each of the regulators affected the fitness and product formation. Y. pseudotuberculosis ΔrovA (YP3) showed a 21% enhanced secre-

(ΔarcA), YP252 (ΔptsN), YP253 (ΔpykF), or YP274 (ΔpdhR). At 5 days post-infection, the mice were euthanized by CO₂ asphyxiation. Peyer’s patches (PPs), mesenteric lymph nodes (MLNs), and the liver and spleen were isolated. The ileum was rinsed with sterile PBS and incubated with 100 μg ml⁻¹ gentamicin to kill the bacteria on the luminal surface. After 30 min, gentamicin was removed by extensive washing with PBS. Subsequently, all organs were weighed and homogenized in sterile PBS at 30,000 rpm for 20 s using a Polytron PT 2100 homogenizer (Kinematica, Lucerne, Switzerland). The bacterial organ burden was determined by plating three independent serial dilutions of the homogenates on LB plates with and without kanamycin. The colony-forming units (cfu) were counted and are given as cfu/g of organ/tissue. The levels of statistical significance for differences in the organ burden between test groups were determined by the Mann-Whitney test. The competitive index relative to the wild type strain YPIII was calculated as described previously (43).

### RESULTS

**Iron Availability Triggers the Expression of the Early Stage Virulence Regulator RovA in Y. pseudotuberculosis**

Table 3: Impact of nutrient status on the expression of the early stage virulence regulator RovA in Y. pseudotuberculosis

| Type of medium | Ca²⁺ [mmol L⁻¹] | Na⁺ [mmol L⁻¹] | Fe³⁺ [mmol L⁻¹] | 3,4-DHB [mmol L⁻¹] | RovA |
|----------------|----------------|----------------|----------------|-------------------|------|
| DMEM/F12       | 2.1            | 304            | 3              | -                 | -    |
| YMM            | 2.0            | 5              | 2              | -                 | -    |
| YMM            | 1.0            | 5              | 2              | -                 | -    |
| YMM            | -              | 93             | 2              | -                 | -    |
| YMM            | -              | 5              | 2              | -                 | 0.2  |
| YMM            | -              | 5              | 20             | 0.2               |      |

**FIGURE 2. Cultivation profile of wild type Y. pseudotuberculosis in YMM with glucose as the sole source of carbon and energy.** The data include the concentrations of glucose (gray circle), pyruvate (gray square), lactate (open circle), acetate (open triangle), and the absorbance (open diamond) and represent the mean of three biological replicates with the corresponding deviations.
Interplay between Metabolism and Virulence in Yersinia

growth characteristics of the Y. pseudotuberculosis wild type strain YPIII and the mutants YP3 (∆rovA), YP53 (∆csrA), YP89 (∆crp), YP49 (∆arcA), YP252 (∆ptsN), YP253 (∆pykF) and YP274 (∆dpdh)

The data include the specific growth rate (μ), the specific rate of glucose uptake (qS), and the yields on glucose for biomass (YX/s), pyruvate (YP/s), lactate (YL/s), acetate (YAc/s), formate (YF/s), succinate (Ysc/s), fumarate (Yfu/s), ethanol (Ye/s) and 2-oxoglutarate (Yyg/s). The values represent the mean of three biological replicates and the corresponding standard deviations. The specific growth rate was estimated from the semilogarithmic plot of the cell concentration. Yields were determined from the slope of the linear best fit between product formation and glucose consumption during the entire cultivation as described previously (42, 88). The specific glucose uptake rate was determined from the differentiated glucose profile and the corresponding cell concentration as the average value for each strain during the cultivation. Values below 0.1% for the by-product yields were below the detection limit.

| Strain | μ (h⁻¹) | qS (mmol g⁻¹ h⁻¹) | YX/s | YP/s | YL/s | YAc/s | YF/s | Ye/s | Yyg/s |
|--------|---------|-------------------|------|------|------|-------|------|------|-------|
| YPII (WT) | 0.32 ± 0.04 | 7.1 ± 0.3 | 45.2 ± 2.0 | 46.4 ± 1.4 | 7.1 ± 0.6 | 6.7 ± 0.3 | 3.0 ± 0.4 | 0.2 ± 0.0 | 0.1 ± 0.0 | 3.3 ± 0.7 | 1.1 ± 0.1 |
| YP3 (∆rovA) | 0.31 ± 0.03 | 7.1 ± 0.2 | 44.9 ± 2.8 | 56.1 ± 4.6 | 8.2 ± 0.8 | 6.9 ± 0.5 | 2.6 ± 0.1 | 0.2 ± 0.0 | 0.1 ± 0.0 | 4.0 ± 0.7 | 1.5 ± 0.1 |
| YP53 (∆csrA) | 0.14 ± 0.03 | 3.1 ± 0.3 | 45.0 ± 0.9 | 52.5 ± 2.4 | 3.5 ± 0.2 | 3.0 ± 0.1 | 3.0 ± 0.4 | <0.1 | <0.1 | 0.2 ± 0.4 | 0.9 ± 0.1 |
| YP99 (∆crp) | 0.11 ± 0.03 | 1.5 ± 0.3 | 65.1 ± 3.3 | <0.1 | 1.0 ± 0.1 | 0.2 ± 0.1 | 0.8 ± 0.1 | <0.1 | <0.1 | 2.0 ± 0.5 | 0.9 ± 0.0 |
| YP253 (∆pykF) | 0.26 ± 0.02 | 5.5 ± 0.0 | 46.4 ± 1.4 | 36.5 ± 2.8 | 16.2 ± 1.3 | 3.5 ± 0.4 | 0.4 ± 0.1 | 0.8 ± 0.1 | 0.6 ± 0.0 | 3.8 ± 0.8 | 1.4 ± 0.1 |
| YP274 (∆dpdh) | 0.23 ± 0.01 | 6.4 ± 0.4 | 35.9 ± 2.9 | 64.5 ± 4.1 | 3.4 ± 0.5 | 5.4 ± 0.3 | 0.5 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.0 | 5.8 ± 0.3 | 1.7 ± 0.1 |
| YP49 (∆arcA) | 0.31 ± 0.01 | 6.3 ± 0.3 | 32.3 ± 1.5 | 70.7 ± 5.4 | 2.6 ± 0.5 | 16.4 ± 1.1 | <0.1 | <0.1 | 0.2 ± 0.0 | 3.4 ± 0.4 | 1.3 ± 0.1 |

The carbon flux (Figs. 4, 5) resulted in a significant perturbation of the Y. pseudotuberculosis carbon flux (Figs. 4, B–D, and 5). The lack of the most specific virulence regulator RovA decreased the flux through the PPP and the TCA cycle. Both pathways deliver NADPH, and the TCA cycle also provides NADH and FADH. Overall, this change resulted in a decreased amount of reducing power. The reduced TCA cycle flux originated from the increased pyruvate secretion, which withdrew carbon from the core

tion of pyruvate. The growth, substrate uptake, and spectrum of other by-products were approximately maintained at the values of the wild type (Table 4). The loss of CsrA resulted in a 56% reduction in the growth and substrate uptake. The pyruvate excretion increased by 13%, whereas the formation of all other by-products was much weaker or even diminished. The crp mutant YP89 exhibited a further reduction of fitness. This mutant secreted almost no by-products and showed enhanced anabolism (Table 4). In summary, various fluxes, ranging from glucose to biomass and to extracellular products, were affected in all three regulator mutants. The extent of the cellular response clearly matched the hierarchy of the control of proteins within the virulence cascade (Fig. 1).

Intracellular Fluxes of Glucose-grown Y. pseudotuberculosis Strongly Differ from Those of Its Relative E. coli—For metabolic flux analysis, Y. pseudotuberculosis was grown on [1-13C]glucose. The specific growth rate (0.32 h⁻¹), the biomass yield (46 g (mol glucose)⁻¹), and yields for pyruvate (0.46 mol (mol glucose)⁻¹), lactate (0.06 mol (mol glucose)⁻¹), and acetate (0.08 mol (mol glucose)⁻¹) of the isotopic culture agreed well with the three parallel incubations on naturally labeled glucose. This allowed the use of the stoichiometric mean values and standard deviations from the three replicates (Table 4) and the corresponding precursor demand for anabolism (Table 5) as input for the flux calculation. At different time points during the exponential phase, cells were collected from the tracer cultivation followed by GC-MS analysis of mass isotopomer distributions of amino acids from the cell protein (Table 6). Generally, mass isotopomers were quantified with high precision. The cultures exhibited metabolic steady state, inferred from constant growth kinetics (Fig. 2) and stoichiometry (Fig. 3), and isotopic steady state, demonstrated by the constant 13C-labeling of protein-bound amino acids over time (Fig. 3), fulfilling important pre-requisites for the chosen flux methodology (20). On the basis of the experimental data, intracellular carbon fluxes were calculated using a 13C flux model that involved metabolite and isotopomer balancing. Fifty-fold repetition of the flux estimation with statistically varied starting values for the free flux parameters led to identical solutions, which ensured the identification of the global minimum. Concerning the obtained fit, excellent agreement between experimentally determined and calculated mass isotopomer ratios was achieved (Table 6), indicating high consistency of the determined fluxes. All intracellular fluxes were estimated with high precision, as shown by narrow 95% confidence intervals (Fig. 4). The in vivo carbon flux distribution revealed that Y. pseudotuberculosis channeled 33% of the consumed glucose into the pentose phosphate pathway (PPP) (Fig. 4A). This flux exceeded the requirement for PPP-derived anabolic precursors. Carbon was channeled back from the PPP into the EMP pathway at the level of fructose 6-phosphate and glyceraldehyde 3-phosphate. The Entner-Doudoroff pathway was found to be inactive, and ~67% of the glucose uptake was catalyzed by the EMP pathway. Although this part of the metabolism upstream of the PEP node is similar to that of the related gut bacterium E. coli (44, 45), the fluxes through the downstream pathways differ significantly between the two microorganisms for growth under aerobic conditions on glucose. Y. pseudotuberculosis showed a high flux through the TCA cycle. The flux through the entry step, which is catalyzed by citrate synthase, was 63% in Y. pseudotuberculosis, whereas only 27% has been reported for E. coli (44).

In addition, the anaerobic fluxes in Y. pseudotuberculosis were generally very low. The pathogen did not exhibit any activity of the glyoxylate shunt, although E. coli has shown a carbon flux of ~10% (44). Similarly, the flux through PEP carboxylase was also lower in Y. pseudotuberculosis (17%) compared with the value of 32% for E. coli (44). Moreover, Y. pseudotuberculosis exhibited a high flux of almost 50% toward extracellular pyruvate, whereas E. coli typically does not secrete this metabolite under similar respiratory conditions. The formation and secretion of large amounts of pyruvate are surprising because this metabolic route does not provide additional ATP, in contrast to the acetate pathway preferred by E. coli (46).
metabolism. In addition, *Y. pseudotuberculosis* YP53 (ΔcsrA) and YP89 (Δcrp) showed a substantial difference in the metabolic flux distribution compared with that of the wild type. In ΔcsrA and Δcrp, the TCA cycle flux was significantly up-regulated by ~10 and 45%, respectively, coinciding with a reduced formation of pyruvate-derived by-products (Figs. 4, C and D, and 5). Overall, the deletion of the global regulator Crp led to the most extensive change in flux. This deletion

**FIGURE 3.** Quantitative physiological characteristics of glucose-grown *Y. pseudotuberculosis* YPIII (A) and the single-gene deletion mutants *Y. pseudotuberculosis* YP3 (ΔrovA) (B), YP53 (ΔcsrA) (C), and YP89 (Δcrp) (D). The linear correlation between glucose consumption, growth, and product formation, respectively, indicates metabolic steady state of the cultures (left panel). The constant labeling pattern of amino acids from hydrolyzed cell protein harvested at different time points during the exponential growth phase verifies isotopic steady state (right panel). The amino acids shown here exemplarily stem from different parts of the metabolic network, whereby Mₐ (nonlabeled), M₁ (single labeled), and M₂ (double labeled) denote relative fractions of the corresponding mass isotopomers.
also affected additional pathways, including the PPP and EMP pathways, and several anabolic reactions, and most strikingly, it resulted in the disappearance of all overflow fluxes (Figs. 4D, 5).

Mutants Deficient in RovA, CsrA, and Crp Reveal an Altered Expression Pattern of Virulence-associated, Stress Adaptation, and Metabolic Genes—To gain a more comprehensive understanding of how the virulence regulators interact with metabolism and mediate flux adaptations, we aimed to correlate changes in the fluxome with alterations in the gene expression pattern. For this purpose, microarray analyses were performed using total RNA isolated from the *Y. pseudotuberculosis* YPIII

| Precursor                | Demand [μmol g⁻¹] | G6P | F6P | R5P | E4P | GAP | PGA | PEP | PYR | AcCoA | OAA | AKG | NADPH |
|-------------------------|-------------------|-----|-----|-----|-----|-----|-----|-----|-----|-------|-----|-----|-------|
| Alanine                 | 604               |     |     |     |     |     |     |     |     |       |     |     |       |
| Arginine                | 277               |     |     |     |     |     |     |     |     | 1     |     |     | 4     |
| Aspartate/Asparagine    | 572               |     |     |     |     |     |     |     | 1   |       |     |     |       |
| Cysteine                | 92                |     |     |     |     |     |     |     | 1   |       |     |     | 5     |
| Glutamate/Glutamine     | 557               |     |     |     |     |     |     |     |     |       |     |     | 1     |
| Gucose                  | 483               |     |     |     |     |     |     |     |     |       |     |     | 1     |
| Histidine               | 98                |     |     |     |     |     |     |     |     |       |     | 1   |       |
| Isoleucine              | 236               |     |     |     |     |     |     |     | 1   |       |     |     | 5     |
| Leucine                 | 463               |     |     |     |     |     |     |     |     | 2     |     |     | 2     |
| Lysine                  | 340               |     |     |     |     |     |     |     |     |       |     |     | 4     |
| Methionine              | 155               |     |     |     |     |     |     |     |     |       |     | 1   | 8     |
| Phenylalanine           | 180               |     |     |     |     |     |     |     |     |       |     |     | 2     |
| Proline                 | 297               |     |     |     |     |     |     |     |     |       |     |     | 3     |
| Serine                  | 275               |     |     |     |     |     |     |     |     |       |     | 1   |       |
| Threonine               | 270               |     |     |     |     |     |     |     |     |       |     |     | 3     |
| Tryptophan              | 57                |     |     |     |     | 1   |     |     |     |       |     |     | 3     |
| Tyrosine                | 91                |     |     |     |     |     |     |     | 2   |       |     |     | 2     |
| Valine                  | 333               |     |     |     |     |     |     |     |     |       |     |     | 2     |

| Protein | 155 | 328 | 850 | 598 | 2,772 | 463 | 1,573 | 1,131 | 11,940 |

| RNA      | 448 | 257 | 191 | 307 |

| DNA      | 208 | 104 | 104 | 421 |

| Lipid    | 129 | 129 | 2,116 | 3,870 |

| LPS components | 51 | 16 | 24 | 24 | 24 | 329 | 470 |

| Peptidoglycan | 55 |     | 28 | 83 | 55 | 28 | 28 | 193 |
| Glycogen     | 154 |
| C1-units     | 49 | |
| Polyamines   | 59 | 178 | 178 |

| Total       | 205 | 71 | 834 | 328 | 129 | 1,142 | 649 | 2,855 | 2,963 | 1,895 | 1,218 | 17,428 |
The most representative changes in the metabolic genes are given in Fig. 6. Additional transcriptional changes and more details are provided in supplemental Table S1. Of all of the protein-encoding chromosomal (4172) and virulence plasmid genes (92), the following changes (1.7-fold, \( p < 0.01 \)) were observed compared with the wild type: 316 genes were affected in the \( \text{rovA} \) mutant (128 up-regulated/188 down-regulated); 911 genes were affected in the \( \text{csrA} \) mutant (475 up-regulated/438 down-regulated), and 729 genes were affected in the \( \text{crp} \) mutant (336 up-regulated/393 down-regulated) (supplemental Table S1). Classification according to the genome annotation of \( Y. \) pseudotuberculosis YPIII showed that the altered genes belonged to the following categories: virulence, motility and chemotaxis, stress adaptation, information storage and processing, and metabolism. The lack of each regulator caused a rather specific change in expression, i.e. only a subset of genes responded.

### TABLE 6

Relative mass isotopomer fractions of \( t \)-butyl-dimethylsilyl-derivatized amino acids from the cell protein of \( Y. \) pseudotuberculosis YPIII and the virulence mutants YP3 (\( \Delta \text{rovA} \)), YP53 (\( \Delta \text{csrA} \)), and YP89 (\( \Delta \text{crp} \)) cultures, which were also used for the fluxome analyses.

Prior to analysis, cultures were grown on minimal medium with \([1-13C]\) glucose and sampled during the exponential growth phase. The data set includes experimental values with deviations from replicate GC/MS measurement (Exp) and calculated values (Calc), predicted by the solution of the mathematical model that corresponded to the optimized set of fluxes (Fig. 4). \( M_0 \) denotes the relative fraction of nonlabeled mass isotopomers; \( M_1 \) denotes the relative fraction of single labeled mass isotopomers; and \( M_2 \) denotes the relative fraction of the double labeled mass isotopomers.
FIGURE 4. *In vivo* carbon flux distribution of glucose-grown *Y. pseudotuberculosis* YPIII (A) and the single gene deletion mutants *Y. pseudotuberculosis* YP3 (ΔrovA) (B), YP53 (ΔcsrA) (C), and YP89 (Δcrp) (D) estimated from the best fit to the experimental results using a comprehensive approach of combined metabolite balancing and 13C tracer experiments with labeling measurements of proteinogenic amino acids. All fluxes are expressed as a molar percentage of the corresponding specific glucose uptake rate (7.1 mmol g−1 h−1, 3.1 mmol g−1 h−1, and 1.5 mmol g−1 h−1, see Table 4), which was set to 100%. The errors represent 95% confidence intervals and were calculated by Monte-Carlo analysis. Significantly altered fluxes (p < 0.01) are marked (*). Arrow thickness and color indicate the extent and the alteration of flux, respectively. The anabolic fluxes from precursors (G6P, F6P, P5P, T3P, PEP, PYR, OAA, AKG, AcCoA) into biomass are shown by gray arrows. Values for these peripheral fluxes, not included in the figure for the sake of clarity, can be calculated by multiplication of the biomass yield (Table 4) with the anabolic demand for the corresponding precursor (Table 5).
similarly in the different mutants (Fig. 6 and supplemental Table S1).

In all of the regulator deletion strains, multiple virulence-associated genes were affected (supplemental Table S1). Y. pseudotuberculosis YP3 (ΔrovA) exhibited specific changes in the expression of genes linked to host-pathogen interactions and serum resistance, including the previously identified RovA-dependent virulence genes of Y. pestis and Y. enterocolitica (47, 48). Y. pseudotuberculosis YP53 (ΔcsrA) and YP89 (Δcrp) showed a broader set of affected genes, e.g. regulators involved in initial colonization of the intestine and other host tissues (e.g. rovA, invA, psaAB, multiple adhesins and fimbrial factors), pathogenicity genes responsible for dissemination and immune defense during an ongoing infection (yadA, ailA, ysc, and yop genes), and type VI secretion systems that support survival in the host (49, 50).

In addition to the classical pathogenicity factors, several virulence-associated physiological processes showed changes in expression. The flhDC operon and several FlhDC-induced genes (motAB, cheAWD, fli, flg, and flh genes) controlling the synthesis of flagella and motility were down-regulated in the crp and csrA mutants. Motility was previously shown to be activated by CsrA and was found to promote the host cell invasion and virulence of Yersinia (9, 11, 51). Several autoinducer systems were also differently expressed in the crp and csrA mutants. This change was complemented by up-regulation of poxB, which encodes pyruvate oxidase as a ubiquinone-reducing alternative pathway for acetate formation. In contrast, NADH-consuming alcohol dehydrogenase (adhE) was significantly down-regulated in the csrA and crp mutants. Furthermore, the expression levels of catabolic enzymes, which are responsible for the uptake and utilization of carbohydrates and amino acids, and glycogen synthesis (glgXAP) were affected in the different mutant strains. Multiple phosphotransferase systems, permeases, and ABC transporters were differentially expressed in the crp and the csrA mutant. In particular, the ptsG

FIGURE 5. Control of the metabolic fluxes of Y. pseudotuberculosis YPIII by the catabolite repressor protein (C rp), the carbon storage regulator (CsrA), and the regulator of virulence (RovA). The data indicate the relative flux changes in the defined virulence mutants YP3 (ΔrovA), YP53 (ΔcsrA), and YP89 (Δcrp), normalized to the wild type flux. The data refer to the intracellular flux distributions (see Fig. 4).
gene encoding the EIIBC subunit of the glucose phosphotransferase system (PTS), the ulaAABC/sgaTBA operon encoding a putative ascorbate PTS system, and glnQPH for polar amino acid transport were strongly altered in the crp mutant and to a smaller extent in the csrA-deficient strain. In agreement with previous results (10), expression of the ptsIH, fruKB, manXZ, glpFK, and mglBAC operons for the uptake of glucose, fructose, mannose/sorbose, glycerol, and methyl galactoside were affected in the absence of the virulence-promoting metabolic regulator Crp, and several amino acid peptide transporter-encoding operons (opp, ddp, and tau) were affected by the loss of Crp and/or CsrA. In addition, several genes involved in the nucleotide and fatty acid uptake and metabolism were regulated by Crp, CsrA, and/or RovA (e.g. nupC1, ndk, pyrD, cdd, gpt, udp, xanP, uhpc, accA, fadBA, and fadLIJ).

Targeted Control of the Metabolic Switch for the TCA Cycle in Yersinia—Taken together, our data strongly support a central role of the virulence-promoting regulator cascade in host-
Interplay between Metabolism and Virulence in Yersinia

adapted re-adjustment of the carbon core metabolism in Y. pseudotuberculosis. In particular, Crp and CsrA seem to tightly coordinate the expression of virulence-associated traits with the operation of the central metabolic switch point, the pyruvate-TCA cycle. To test this hypothesis and investigate the effect of this metabolic control point for virulence, we next focused on targeted perturbations. Different master regulators and enzymes are known to control the flux through this central metabolic switch point in related bacteria (44). Interestingly, several of these candidates were affected in the crp and csrA mutants. For example, the gene encoding the pyruvate dehydrogenase regulator protein (pdhR) was specifically down-regulated in the Y. pseudotuberculosis ∆crp mutant. PdhR acts as a transcriptional regulator in a pyruvate-dependent manner to control central metabolic fluxes, e.g. during the transition from anaerobic to microaerobic conditions (55, 56). The expression of another key regulator of the TCA cycle, i.e. ArcA, was repressed in the ∆csrA strain. This global regulator is involved in the reprogramming of metabolism according to O2 concentration (57). The impact of this transcription factor is further supported by the fact that several genes (cyaAB, ptsG, sucB, ccmE, and fadA), known to be under the control of ArcA in E. coli or Salmonella (58–60), were also controlled by CsrA and/or Crp in Y. pseudotuberculosis (supplemental Table S1).

In addition to ArcA and PdhR, which were among the Crp- and CsrA-dependent genes, PtsN and PykF appeared to be promising candidates to modulate the pyruvate-TCA cycle node in Y. pseudotuberculosis based on their impact in E. coli. A recent study showed that the second phosphotransferase system of E. coli, denoted as nitrogen-PTS (PTS^Ntr), controls fluxes in the central metabolism, especially at the pyruvate-TCA cycle node (61, 62). The identified PTS^Ntr-dependent genes include multiple TCA cycle genes, which are differentially expressed in the Y. pseudotuberculosis csrA and/or crp mutant strains (gltAB, sucB, sdhD, and aceA), indicating a close link between these global regulatory systems. In support of this assumption, expression of the kdpFABC operon, a well known target of PTS^Ntr in E. coli (63), was found to be up-regulated in the Crp-deficient mutant. The phosphorylation state of EI^Ntr (PtsN) is influenced by the activity of the sugar PTS through cross-talk (e.g. by competition for PEP); thus, alterations in the PTS^Glc components in the Crp- and CsrA-deficient strains (i.e. repression of ptsG and ptsH in a crp mutant) might also affect the flux through the pyruvate-acetyl-CoA node and the TCA cycle via alteration of the PTS^Ntr activity. Utilization as well as strong up-regulation of the sugar PTS (e.g. ptsG induction in the presence of Crp) might drain the phosphoryl groups of PEP toward the EI^Glc, leading to preferentially dephosphorylated EI^Ntr.

During growth on glucose, pyruvate is generated from PEP as a product of PTS^Glc and by the pyruvate kinase isoenzymes (PykA and PykF). To specifically target the pyruvate-TCA cycle node, it seemed appropriate to also directly perturb a particular enzyme of this metabolic control point. The deletion of pyruvate kinase in E. coli results in rerouting of a local flux through the combined reactions of PEP carboxylase and malic enzyme (64, 65). Of the two pyruvate kinase enzymes (PykA and PykF), PykF was selected because it is highly active during growth on glucose in E. coli, whereas PykA has a much lower activity (65, 66).

In this regard, single mutants of arcA, pykF, ptsN, and pdhR were constructed to study their impact on virulence. The first characterization of their growth physiology under laboratory conditions revealed that the glucose uptake was comparable with that of the wild type, suggesting the absence of any major effect on the biological fitness of the pathogen (Table 4). In contrast, the pyruvate-derivative by-products differed significantly, indicating that the fluxes around the pyruvate-TCA cycle node were affected. Each mutant showed a unique metabolic phenotype.

Perturbations of the Metabolic Core Machinery at the Pyruvate-TCA Cycle Node Reduce Yersinia Virulence—To examine whether the pyruvate-TCA cycle node represents a focal point of virulence control, we performed co-infection experiments with the wild type and the mutant strains YP49 (∆arcA), YP252 (∆ptsN), YP253 (∆pykF), and YP274 (∆pdhR). Groups of BALB/c mice were orally infected with ~10^7 bacteria in an inoculum composed of an equal mixture of the wild type and the isogenic mutant strains. We quantified the number of bacteria present in the PP, the MLN, the liver, and the spleen 5 days post-infection (Fig. 7). The loss of pykF, ptsN, and arcA resulted in a strong reduction of Y. pseudotuberculosis virulence, and a mild effect was observed for the pdhR mutant. The absence of PdhR and ArcA significantly reduced the initial colonization of the PPs early in the infection. The subsequent dissemination of the pathogen into the MLNs was significantly decreased or totally abolished in the arcA, pykF, and ptsN mutants (Fig. 7).

Our experiments further revealed that PykF and PtsN functioning was crucial for colonization and/or persistence in deeper tissues, such as the liver and spleen. These results clearly indicate that the presence of the pyruvate-TCA node-modulating factors ArcA, PtsN, and PykF is particularly important for the pathogenesis of Y. pseudotuberculosis, whereby the individual modulators seem to participate at different stages during the course of the infection. The observed effects are not based on a general growth defect of the investigated mutants because each mutant had an overall glucose uptake rate comparable with that of the wild type (Table 4) and a similar colonization rate in at least one of the tested tissues (Fig. 7).

DISCUSSION

This study, using a systems biology approach that integrates transcriptional control (transcriptome) with functional network operation (fluxome) in the pathogen Y. pseudotuberculosis, elucidated a close link between the metabolic core machinery and pathogenic traits. We show that the virulence-promoting regulators RoVA, CsrA, and Crp strongly affect the intracellular carbon fluxes (Figs. 4 and 5) and the expression of multiple metabolic and virulence genes of Y. pseudotuberculosis (Fig. 6 and supplemental Table S1). In particular, pyruvate metabolism and the TCA cycle emerged as focal points of control (Fig. 8). Among the large set of metabolic flux changes, only a subset can be explained by transcriptional control (Fig. 9). Obviously, the carbon fluxome of Y. pseudotuberculosis is largely under post-transcriptional control. Similarly, the primary metabolism of other bacteria is mainly controlled by post-
In this study, we observed that...cycle. Changing environments that they face during their infection marks the limit of detection. The statistical significances between the wild type and the mutants were determined by a Mann-Whitney test. Bars represent the medians of the competitive index values. A competitive index score of 1 denotes no difference in virulence compared with that of YPIII.

**Y. pseudotuberculosis Secretes Large Amounts of Pyruvate—**

In this study, we observed that *Y. pseudotuberculosis* released large amounts of pyruvate into the medium during growth on glucose. Excretion of pyruvate to this extent (46% of the glucose uptake) under these conditions is uncommon among related bacteria and primarily seems to be a transient phenomenon triggered during specific metabolic shifts, e.g. from aerobic to anaerobic growth (55). Metabolically, the pyruvate secretion is somewhat intriguing at first glance because its synthesis does not yield additional ATP or contribute to redox balancing, as in the case of pyruvate-derived acetate or lactate. Generally, high growth rate aerobic cultivations of related Enterobacteriaceae such as *E. coli* are accompanied by acetyl excretion, achieved by concurrent activation of acetate formation via the phospho-acetylase acetate kinase (Pta-Ack) pathway through increased levels of pyruvate (70) and by decreased re-assimilation of acetate through repression of acetyl-CoA synthetase (Acs) triggered by catabolite repression (46). In contrast, these enzymes do not seem to be controlled by Crp in *Yersinia* (Fig. 8). The observed high fluxes for glucose uptake and glycolysis could induce pyruvate secretion due to a metabolic bottleneck downstream of this node. However, a limitation of the respiratory capacity can be excluded as a trigger for the pronounced pyruvate overflow due to the fully aerobic conditions during growth. Alternatively, the excretion of pyruvate could be important for the bacteria to avoid glucose-phosphate stress. High rates of nutrient uptake, in particular readily metabolizable PTS sugars, provide an advantage in the gut environment because these high rates reduce the availability of carbon nutrients for competing strains and organisms. However, an intracellular accumulation of phosphorylated sugar intermediates due to a metabolic bottleneck is growth inhibitory or bactericidal and induces a specific stress response (71), so that secretion of pyruvate under high growth rate aerobic cultivations on glucose would prevent accumulation of detrimental/stress-inducing phospho-sugars and would enable recycling of the phosphate groups to regenerate the ATP pool. Under this type of phosphate-sugar stress, *E. coli* induces the expression of the small regulatory RNA SgrS, which negatively controls the translation of *ptsG* mRNA, the mRNA that encodes the major glucose transporter EIICB*c* to reduce the substrate influx (72). This mechanism might not function in the *Y. pseudotuberculosis* wild type, which reveals high *ptsG* expression (supplemental Table S1) and glucose influx (Table 4), whereas the *ptsG* transcript was found to be much less abundant in the *Y. pseudotuberculosis* crp mutant (supplemental Table S1), in which significantly lower amounts of pyruvate are secreted and glucose uptake is reduced (Fig. 4 and Table 4). Additionally, pyruvate is a key signal in bacteria to program core metabolism (55, 69); thus, the secretion might contribute to the fine adjustment of the intracellular level of this metabolite. Taken together, these findings indicate that pyruvate metabolism and
export are likely used to control the biological fitness and virulence of *Yersinia*. As shown, pyruvate spilling was maintained as long as glucose was present (Fig. 2). We conclude that this unusual phenomenon occurs in the glucose concentration range found in the gastrointestinal tract of mammals (73) and therefore has an impact during infection.

*Pyruvate Node and the TCA Cycle Are Focal Points of Virulence Control*—In this study, we showed that the virulence-promoting metabolic regulators CsrA and Crp regulate the expression of multiple enzymes implicated in the control of the pyruvate-TCA cycle node (supplemental Table S1 and Fig. 9). Furthermore, we demonstrated that modulators of this control point and, particularly, one of its enzymes (PykF) are crucial for *Y. pseudotuberculosis* virulence (Fig. 7).

With regard to CsrA and Crp, both deletion mutants secreted much less or even no by-products; instead, these mutants channeled the carbon into the TCA cycle. This finding can be explained by the fact that CsrA and Crp act as repressors of multiple TCA cycle enzymes and associated pathway genes, and their deletion results in a moderate and strong derepression of the flux through the TCA cycle (Figs. 5 and 8 and supplemental Table S1). Obviously, the control of the central carbon flow through the TCA cycle toward complete substrate oxidation seems to be important for optimizing overall fitness, reinforcing competitiveness and adjusting the virulence functions of the pathogen. Recent work on the metabolic flux response in the commensal *E. coli* Nissle 1917 also revealed that CsrA has a strong influence on the central metabolism of glucose. However, in this case, strikingly lower fluxes via the TCA cycle were observed, which were accompanied by a 40% increase in acetate production (74). This finding indicates significant differences in the operation of central carbon metabolism between the commensal *E. coli* strain and the invasive enteropathogenic *Yersinia*, which most likely reflect different adaptation techniques of the colonized niches.
Pioneering systems biology studies of pathogenic *Yersinia* species, including *Y. pestis*, have reported that the metabolic core machinery is tightly regulated by virulence-associated environmental parameters in concert with virulence genes (14, 75). The first observations revealed the following: (i) an adjustment of many catabolic pathways for the metabolites available in mammals in response to a temperature shift from 26 to 37 °C (75); (ii) conserved post-transcriptional control of metabolism, and (iii) the translational machinery, including the modulation of glutamate levels in *Yersinia* spp. (14). Here, we discovered that the pyruvate-TCA cycle node is a focal point of virulence control. We demonstrated that regulators and enzymes modulating the fine adjustment of the pathway fluxes at this node affect important cellular components linked to metabolism and virulence in *Y. pseudotuberculosis*. Our mouse infection data indicate that the discovered metabolic control point is of an utmost but previously unrecognized importance for the pathogenicity of this microorganism. Despite a shift in temperature, our *in vivo* and *in vitro* data provide a consistent picture. Pathogenic *Yersinia* species are able to survive in a large variety of environmental reservoirs (e.g., soil, plants, and insects) where they experience moderate temperature. To manage a successful infection, they already express important virulence factors under such moderate temperatures, e.g. the primary cell invasion factor invasin (9, 11, 12), which is controlled by the Crp-CsrA-RovA regulator cascade addressed in this study. Expression of invasin and co-regulated metabolic functions under these growth conditions seem to prime the bacteria to guarantee rapid colonization of the gut in successful competition with the intestinal microbiota. Only during later stages of the infection does the higher temperature lead to a reprogramming of virulence and metabolic functions in a Crp-dependent manner (9), which then allows dissemination and persistence in deeper tissues. An earlier study of *Y. enterocolitica* addressing the influence of two secreted bacterial effector proteins YscM1 and YscM2 also suggested a relevance of the pyruvate and/or PEP carboxylation (76). Both effectors are able to bind the PEP carboxylase and seem to control pyruvate and oxaloacetate formation to maintain an optimal energy balance during the infection of macrophages. Our observation that the pyruvate-TCA cycle node is a focal point of virulence control is further supported by recent studies of the closely related species *Y. pestis*, the causative agent of plague. Adaptation of the bacterium to the environment encountered within infected mouse lungs involves a down-regulation of the TCA cycle (77). In addition, a recent study using a *Y. pestis* mutant library demonstrated that enzymes of the terminal part of the glycolysis are essential for a successful infection of rats, whereas deletions of the TCA cycle genes *fumC*, *gltA*, and *acnA* had no influence (78).

**FIGURE 9.** Quantitative correlation of the fluxome and transcriptome in the central carbon metabolism of *Y. pseudotuberculosis*. The integrated data are given as the relative change in the virulence mutants YP3 (ΔrovA), YP53 (ΔcsrA), and YP89 (Δcrp) compared with the wild type YPIII. The abbreviations used are as follows: *actP*, acetate permease; *adhE*, alcohol dehydrogenase; *pfB*, formate acetyl-transferase; *ldh*, lactate dehydrogenase; *acnB*, aconitase; *icdA*, isocitrate dehydrogenase; *pykF*, pyruvate kinase; A/D, Embden-Meyerhof-Parnas pathway and pentose phosphate pathway; B, tricarboxylic acid cycle; C, fumarase, malate dehydrogenase, and pyruvate kinase; E, pentose phosphate pathway; F, TCA cycle, pyruvate dehydrogenase, and phosphoenolpyruvate carboxylase. Gene names were taken from *Y. pseudotuberculosis* IP23953, KEGG database.
There are other interesting precedents that tempt us to speculate that the co-adjustment of metabolism and virulence through the pyruvate-TCA cycle node is not only crucial for the genus Yersinia but also constitutes a more general strategy in pathogenic bacteria. For instance, deletions in the pyruvate pathway have been shown to alter the SPI1-mediated gene expression and infectivity of the Salmonella enterica serovar typhimurium (79). Furthermore, it has been reported that Salmonella mutants that are unable to convert malate to pyruvate and oxaloacetate are avirulent and immunogenic in BALB/c mice (80) and that an incomplete TCA cycle increases the survival of Salmonella during infection (81). Moreover, in Pseudomonas aeruginosa, mutations in TCA cycle enzymes have been shown to affect the type III secretion system of the pathogen (82), and regulators of Staphylococcus aureus responding to TCA cycle-associated metabolic changes have also been implicated in virulence control (83).

A more detailed investigation of the interplay between the pyruvate-TCA cycle node and the regulation of virulence factors in enteric and other pathogens promises a better understanding of the complex networks of host-adapted metabolism and may aid in the discovery of novel drug targets and in the design of more effective therapies against bacterial infections.

REFERENCES

1. Rimhanen-Finne, R., Niskanen, T., Hallanvuo, S., Makary, P., Haukka, K., Pajunen, S., Siitonen, A., Ristalainen, R., Pöyry, H., Ollgren, J., and Kuusi, M. (2009) Yersinia pseudotuberculosis causing a large outbreak associated with carrots in Finland, 2006. Epidemiol. Infect. 137, 342–347.

2. Jalava, K., Hakkinen, M., Valkonen, M., Nakari, U. M., Palo, T., Hallanvuo, S., Ollgren, J., Siitonen, A., and Nuorit, I. P. (2006) An outbreak of gastrointestinal illness and Erysiphe nodosum from grated carrots contaminated with Yersinia pseudotuberculosis. J. Infect. Dis. 94, 1209–1216.

3. Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A., and Carniel, E. (1999) Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis. Proc. Natl. Acad. Sci. U.S.A. 96, 14043–14048.

4. Bottone, E. J. (1997) Yersinia enterocolitica: the charisma continues. Clin. Microbiol. Rev. 10, 257–276.

5. Isberg, R. R., and Leong, J. M. (1990) Multiple β1 chain integrins are receptors for invasion, a protein that promotes bacterial penetration into mammalian cells. Cell 60, 861–871.

6. Dube, P. (2009) Interaction of Yersinia with the gut: mechanisms of pathogenesis and immune evasion. Curr. Top. Microbiol. Immunol. 337, 61–91.

7. Chang, D. E., Smalley, D. J., Tucker, D. L., Leatham, M. P., Norris, W. E., Stevenson, S. J., Anderson, A. B., Grissom, J. E., Laux, D. C., Cohen, P. S., and Conway, T. (2004) Carbon nutrition of Corynebacterium glutamicum for Terry Speed. Mol. Microbiol. 50, 44–54.

8. Hofreuter, D., Novik, V., and Galán, J. E. (2008) Metabolic diversity in Campylobacter jejuni enhances specific tissue colonization. Cell Host Microbe 4, 425–433.

9. Heroven, A. K., Böhmke, K., and Dersch, P. (2012) The CrsR/Srm system of Yersinia and related pathogens: a post-transcriptional strategy for managing virulence. RNA Biol. 9, 379–391.

10. Heroven, A. K., Sest, M., Pisia, F., Scheb-Wetzel, M., Steimann, R., Böhmke, K., Klein, J., Müchn, R., Schomburg, D., and Dersch, P. (2012) Crp induces switching of the CrsB and CrsC RNAs in Yersinia pseudotuberculosis and links nutritional status to virulence. Front. Cell. Infect. Microbiol. 2, 158.

11. Heroven, A. K., Böhmke, K., Rohde, M., and Dersch, P. (2008) A Crs-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of Yersinia pseudotuberculosis through RovM. Mol. Microbiol. 68, 1179–1195.

12. Nagel, G., Lahrz, A., and Dersch, P. (2001) Environmental control of invasin expression in Yersinia pseudotuberculosis is mediated by regulation of RovA, a transcriptional activator of the SlyA/Hox family. Mol. Microbiol. 41, 1249–1269.

13. Zhan, L., Han, Y., Yang, L., Geng, J., Li, Y., Gao, H., Guo, Z., Fan, W., Li, G., Zhang, L., Qin, C., Zhou, D., and Yang, R. (2008) The cyclic AMP receptor protein, CRP, is required for both virulence and expression of the minimal CRP regulon in Yersinia pestis biovar microtus. Infect. Immun. 76, 5028–5037.

14. Ansong, C., Schirme-Rutledge, A. C., Mitchell, H. D., Chaun, S., Jones, M. B., Kim, Y. M., McAttee, K., Deatherage Kaiser, B. L., Dubois, J. L., Brewer, H. M., Frank, B. C., McDermott, J. E., Metz, T. O., Peterson, S. N., Smith, R. D., Motin, V. L., and Adkins, J. N. (2013) A multi-omics approach to elucidating Yersinia virulence mechanisms. Mol. BioSyst. 9, 44–54.

15. Sauer, U. (2006) Metabolic networks in motion: 13C-based flux analysis. Mol. Syst. Biol. 2, 62.

16. Kohlstedt, M., Becker, J., and Wittmann, C. (2010) Metabolic fluxes and beyond—systems biology understanding and engineering of microbial metabolism. Appl. Microbiol. Biotechnol. 88, 1065–1075.

17. Miller, J. H. (1992) A Short Course in Bacterial Genetic: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

18. Sandbrook, J., and Russell, D. W. (2001) Molecular Cloning A Laboratory Manual 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

19. Derbise, A., Lesic, B., Dacheux, D., Ghigo, J. M., and Carniel, E. (2003) A rapid and simple method for inactivating chromosomal genes in Yersinia. FEMS Immunol. Med. Microbiol. 38, 113–116.

20. Wittmann, C. (2007) Fluxome analysis using GC-MS. Micro. Cell Fact. 6, 6.

21. Wittmann, C., Kim, H. M., John, G., and Heinzle, E. (2003) Characterization and application of an optical sensor for quantification of dissolved O2 in shake-flasks. Biotechnol. Lett. 25, 377–380.

22. Becker, J., Reinefeld, J., Stellmacher, R., Schäfer, R., Lange, A., Meyer, H., Lalk, M., Zelder, O., von Abendroth, G., Schröder, H., Haefner, S., and Wittmann, C. (2013) Systems-wide analysis and engineering of metabolic pathway fluxes in bio-succinate producing Basfia succinctiproducens. Bio- technol. Bioeng. 110, 3013–3023.

23. Krömer, J. O., Fritz, M., Heinzle, E., and Wittmann, C. (2005) In vivo quantification of intracellular amino acids and intermediates of the methionine pathway in Corynebacterium glutamicum. Anal. Biochem. 340, 171–173.

24. Smith, K. G. (2005) in Bioinformatics and Computational Biology Solutions Using R and Bioc infectious. (Gentleman, R., Carey, V. J., Huber, W., Irizarry, R. A., and Dudoit, S., eds) pp. 397–420, Springer, New York.

25. Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J. Y., and Zhang, J. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.

26. Silver, J. D., Ritchie, M. E., and Smyth, G. K. (2009) Microarray background correction: maximum likelihood estimation for the normal-exponential convolution. Bioinformatics 10, 352–363.

27. Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. 30, e15.

28. Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19, 185–193.

29. Yang, Y. H., and Thorne, N. P. (2003) in Science and Statistics: A Festschrift for Terry Speed (Goldstein, D. R., ed) pp. 403–418, Institute of Mathemat- ical Statistics, Beachwood, OH.

30. Smyth, K. G. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3.

31. Heroven, A. K., Nagel, G., Tran, H. J., Parr, S., and Dersch, P. (2004) RovA is autoregulated and antagonizes H-NS-mediated silencing of invasive and
rowA expression in Yersinia pseudotuberculosis. Mol. Microbiol. 53, 871–888
32. Walker, J. (ed) (2002) The Protein Protocols Handbook, pp. 3–6, Humana Press, Totowa, NJ
33. Neidhardt, F. C., Ingraham, J. L., and Schaechter, M. (1990) Physiology of the Bacterial Cell: A Molecular Approach, pp. 133–173, Sinauer Associates Inc., Sunderland, MA
34. Dauner, M., and Sauer, U. (2001) Stoichiometric growth model for riboflavin-producing Bacillus subtilis. Biotechnol. Bioeng. 76, 132–143
35. Brown, T. A. (2002) Genomes, 2nd Ed., Wiley-Liss, Oxford
36. Wittmann, C., Hans, M., and Heinzle, E. (2002) Metabolic fluxes in Corynebacterium glutamicum during lysine production with sucrose as carbon source. Appl. Environ. Microbiol. 70, 229–239
37. Wittmann, C., Kiefer, P., and Zelder, O. (2004) Metabolic fluxes in Corynebacterium glutamicum during lysine production with sucrose as carbon source. Appl. Environ. Microbiol. 70, 7277–7287
38. Becker, J., Klop Rogge, C., Zelder, O., Wittmann, C. (2004) Comparative metabolic flux analysis of lysine-producing Corynebacterium glutamicum cultured on glucose or fructose. Appl. Environ. Microbiol. 70, 8587–8596
39. Quek, L. E., Wittmann, C., Nielsen, L. K., and Krömer, J. O. (2009) Open-flux analysis of intracellular amino acid labeling by GC/MS. Anal. Biochem. 307, 379–382
40. Kiefer, P., Heinzle, E., Zelder, O., and Wittmann, C. (2004) Comparative metabolic flux analysis of lysine-producing Corynebacterium glutamicum cultured on glucose or fructose. Appl. Environ. Microbiol. 70, 229–239
41. Antoniewicz, M. R., Kelleher, J. K., and Stephanopoulos, G. (2007) Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions. Metab. Eng. 9, 68–86
42. Becker, J., Klop Rogge, C., and Wittmann, C. (2008) Metabolic responses to pyruvate kinase deletion in lysine producing Corynebacterium glutamicum. Micro. Cell Fact. 7, 8
43. Monk, I. R., Casey, P. G., Cronin, M., Gahan, C. G., and Hill, C. (2008) Development of multiple strain competitive index assays for Listeria monocytogenes using pLMC; a new site-specific integrating vector. BMC Microbiol. 8, 96
44. Haverkorn, J., Rijsewijk, B. R., Nanchen, A., Nallet, S., Kleijn, R. J., and Sauer, U. (2011) Large-scale 13C-flux analysis reveals distinct transcriptional control of respiratory and fermentative metabolism in Escherichia coli. Mol. Syst. Biol. 7, 477
45. Sauer, U., Cananoco, F., Heri, S., Perrenoud, A., and Fischer, E. (2004) The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of Escherichia coli. J. Biol. Chem. 279, 6613–6619
46. Valgepea, K., Adamberg, K., Nahku, R., Lahtvee, P. J., Arike, L., and Vilu, R. (2008) Soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of Escherichia coli. J. Biol. Chem. 279, 6613–6619
47. CATHELYN, J. S., CROSBY, S. D., LATHAM, W. W., GOLDMAN, W. E., and MILLER, V. L. (2006) RowA, a global regulator of Yersinia pestis, specifically required for bubonic plague. Proc. Natl. Acad. Sci. USA. 103, 13514–13519
48. CATHELYN, J. S., ELISSON, D. W., HINCHLIFFE, S. J., WREN, B. W., and MILLER, V. L. (2007) The RowA regulons of Yersinia enterocolitica and Yersinia pestis are distinct: evidence that many RowA-regulated genes were acquired more recently than the core genome. Mol. Microbiol. 66, 189–205
49. Cascales, E. (2008) The type VI secretion toolkit. EMBO Rep. 9, 735–741
50. Husemann, J., Sung, A., and Trüeb, K. (2006) Yersinia's stratagem: targetting innate and adaptive immune defense. Curr. Opin. Microbiol. 9, 55–61
51. YOUNG, G. M., BADGER, J. L., and MILLER, V. L. (2000) Motility is required to initiate host cell invasion by Yersinia enterocolitica. Infect. Immun. 68, 4323–4326
52. ATKINSON, S., CHANG, C. C., RYAN, H. L., BUCKLEY, C. M., WANG, Y., SACKETT, R. E., CÁMARA, M., and WILLIAMS, P. (2008) Functional interplay between the Yersinia pseudotuberculosis YpsRI and YtbRI quorum sensing systems modulates swimming motility by controlling expression of flhDC and flaA. Mol. Microbiol. 69, 137–151
53. ATKINSON, S., GOLDSTONE, R. J., JOSHUA, G. W., CHANG, C. C., PATRICK, H. L., CÁMARA, M., WREN, B. W., and WILLIAMS, P. (2011) Biofilm development on Caenorhabditis elegans by Yersinia is facilitated by quorum sensing-dependent repression of type III secretion. PLoS Pathog. 7, e1001250
54. YU, J., MADSEN, M. L., CARRUTHERS, M. D., PHILLIPS, G. J., KAVANAUGH, J. S., BOYD, J. M., HORSWILL, A. R., and MINION, F. C. (2013) Analysis of autotransporter type II secretion systems in Yersinia pseudotuberculosis. Infect. Immun. 81, 4053–4062
55. TROTTER, E. W., ROLFE, M. D., HounsloW, A. M., CRAVEN, C. J., WILLIAMSON, M. P., SANGUINETTI, G., POOLE, R. K., and GREEN, J. (2011) Reprogramming of Escherichia coli K-12 metabolism during the initial phase of transition from an anaerobic to a micro-aerobic environment. PLoS ONE 6, e25501
56. GÖHLER, A. K., KÖKPINAR, O., SCHMIDT-HECK, W., GEFFERS, R., GUTHKE, R., RINAS, U., SCHUSTER, S., JAHREIS, K., and KALETA, C. (2011) More than just a metabolic regulator–elicitation and validation of new targets of PdhR in Escherichia coli. BMC Syst. Biol. 5, 197
57. SPIRO, S., and GUEST, J. R. (1991) Adaptive responses to oxygen limitation in Escherichia coli. Trends Biochem. Sci. 16, 310–314
58. EVANS, M. R., FINK, R. C., VAZQUEZ-TORRES, A., PORWOLLIK, S., JONES-CARSON, J., MCCIELLAND, M., and HASSAN, H. M. (2011) Analysis of the ArcA regulon in anaerobically grown Salmonella enterica sv. Typhimurium. BMC Microbiol. 11, 58
59. LIU, X., and DE WULF, P. (2004) Probing the ArcA-P Modulin of Escherichia coli by whole genome transcriptional analysis and sequence recognition profiling. J. Biol. Chem. 279, 12588–12597
60. Iuchi, S., and LIN, E. C. (1988) ArcA (dye), a global regulatory gene in Escherichia coli mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. U.S.A. 85, 1888–1892
61. JAHN, S., HAFERKORN VON RIJSIEWIK, B. R., SAUER, U., and BETTENROCK, K. (2013) A role for EIAlaS in controlling fluxes in the central metabolism of E. coli K12. Biochim. Biophys. Acta 1833, 2879–2889
62. COMMICHAU, M. F., FORCHHAMMER, K., and STÜLKE, J. (2006) Regulatory links between carbon and nitrogen metabolism. Curr. Opin. Microbiol. 9, 167–172
63. LÜTTMANN, D., HEERMANN, R., ZIMMER, B., HILLMANN, A., RAMPP, I. S., JUNG, K., and GÖRKE, B. (2009) Stimulation of the potassium sensor KdpD kinase activity by interaction with the phosphotransferase protein IIA(S) in Escherichia coli. Mol. Microbiol. 72, 978–994
64. EMMERLING, M., DAUER, M., PONTI, A., FLAUX, I., HOCHULI, M., SYPERTSKI, T., WÜTHRICH, K., BAILEY, J. E., and SAUER, U. (2002) Metabolic flux responses to pyruvate kinase knockout in Escherichia coli. J. Bacteriol. 184, 152–164
65. AL ZAID SIDDIQUEE, A., ARAUZO-BRavo, M. J., and SHIMIZU, K. (2004) Metabolic flux analysis of pykF gene knockout Escherichia coli based on 13C-labeling experiments together with measurements of enzyme activities and intracellular metabolite concentrations. Appl. Microbiol. Biotechnol. 63, 407–417
66. MEZA, E., BECKER, J., BOLIVAR, F., GOSSET, G., and WITTMANN, C. (2012) Consequences of phosphoenolpyruvate-sugar phosphotransferase system and pyruvate kinase isozymes inactivation in central carbon metabolism flux distribution in Escherichia coli. Microbiol. Cell Fact. 11, 127
67. SCHILLING, O., FRICK, O., HERZBERG, C., EHRENREICHT, A., HEINZLE, E., WITT-}
68. SCHILLING, O., FRICK, O., HERZBERG, C., EHRENREICHT, A., HEINZLE, E., WITT-
Interplay between Metabolism and Virulence in Yersinia

73. Ferraris, R. P., Yasharpour, S., Lloyd, K. C., Mirzayan, R., and Diamond, J. M. (1990) Luminal glucose concentrations in the gut under normal conditions. *Am. J. Physiol.* 259, G822–G837

74. Revelles, O., Millard, P., Nougayrède, J. P., Dobrindt, U., Oswald, E., Lé-tisse, F., and Portais, J. C. (2013) The Carbon Storage Regulator (Csr) system exerts a nutrient-specific control over central metabolism in *Escherichia coli* strain Nissle 1917. *PLoS ONE* 8, e66386

75. Motin, V. L., Georgescu, A. M., Fitch, J. P., Gu, P. P., Nelson, D. O., Mabery, S. L., Garnham, J. B., Sokhansanj, B. A., Ott, L. L., Coleman, M. A., Elliott, J. M., Kegelmeyer, L. M., Slezak, T. R., Brubaker, R. R., and Garcia, E. (2004) Temporal global changes in gene expression during temperature transition in *Yersinia pestis*. *J. Bacteriol.* 186, 6298–6305

76. Schmid, A., Neumayer, W., Tru¨lzsch, K., Israel, L., Imhof, A., Roessler, M., Sauer, G., Richter, S., Lawu, S., Eyert, E., Eisenreich, W., Heesemann, J., and Wilharm, G. (2009) Cross-talk between type three secretion system and metabolism in *Yersinia*. *J. Biol. Chem.* 284, 12165–12177

77. Lathem, W. W., Crosby, S. D., Miller, V. L., and Goldman, W. E. (2005) Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17786–17791

78. Pradel, E., Lemaître, N., Merchez, M., Ricard, I., Reboul, A., Dewitte, A., and Sebbane, F. (2014) New insights into how *Yersinia pestis* adapts to its mammalian host during bubonic plague. *PLoS Pathog.* 10, e1004029

79. Abernathy, J., Corkill, C., Hinojosa, C., Li, X., and Zhou, H. (2013) Deletions in the pyruvate pathway of *Salmonella typhimurium* alter SPI1-mediated gene expression and infectivity. *J. Anim. Sci. Biotechnol.* 4, 5

80. Mercado-Lubo, R., Leatham, M. P., Conway, T., and Cohen, P. S. (2009) *Salmonella enterica* Serovar *typhimurium* mutants unable to convert malate to pyruvate and oxaloacetate are avirulent and immunogenic in BALB/c mice. *Infect. Immun.* 77, 1397–1405

81. Bowden, S. D., Ramachandran, V. K., Knudsen, G. M., Hinton, J. C., and Thompson, A. (2010) An incomplete TCA cycle increases survival of *Salmonella typhimurium* during infection of resting and activated murine macrophages. *PLoS ONE* 5, e13871

82. Dacheux, D., Epaulard, O., de Groot, A., Guery, B., Leberre, R., Attree, I., Polack, B., and Toussaint, B. (2002) Activation of the *Pseudomonas aeruginosa* type III secretion system requires an intact pyruvate dehydrogenase aceAB operon. *Infect. Immun.* 70, 3973–3977

83. Somerville, G. A., and Proctor, R. A. (2009) At the crossroads of bacterial metabolism and virulence factor synthesis in staphylococci. *Microbiol. Mol. Biol. Rev.* 73, 233–248

84. Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* 172, 6557–6567

85. Bölin, I., Norlander, L., and Wolf-Watz, H. (1982) Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. *Infect. Immun.* 37, 506–512

86. Chang, A. C., and Cohen, S. N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* 134, 1141–1156

87. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6640–6645

88. Becker, J., Klopprogge, C., Schröder, H., and Wittmann, C. (2009) Metabolic engineering of the tricarboxylic acid cycle for improved lysine production by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 75, 7866–7869