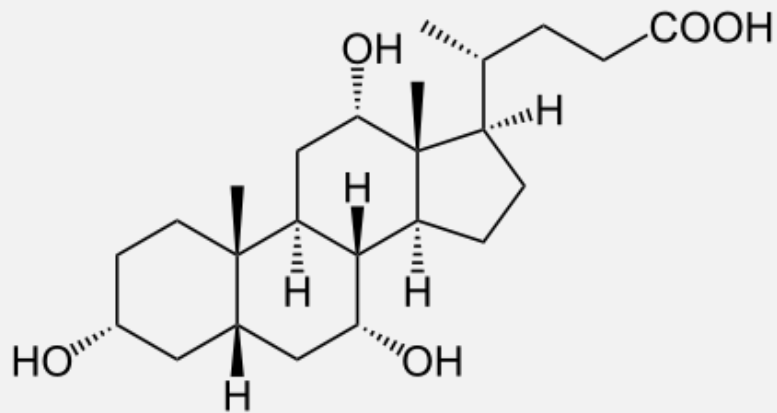
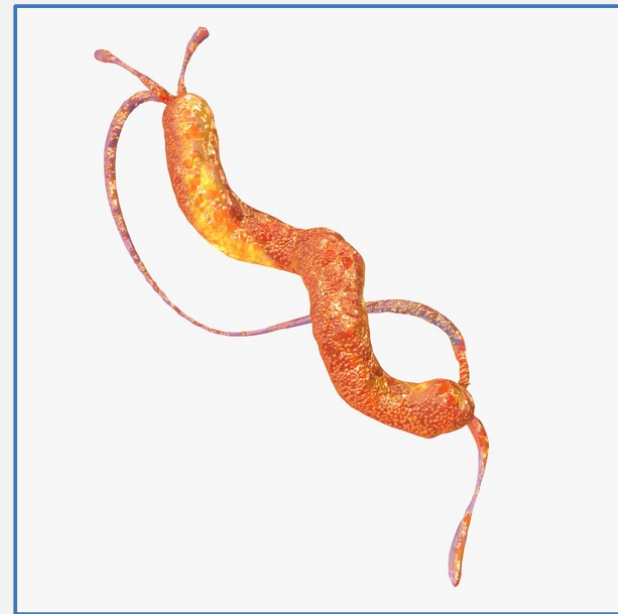


Der Einfluß von Gallensäuren Auf das *C. jejuni*-Proteom und die Wirtszellinvasion

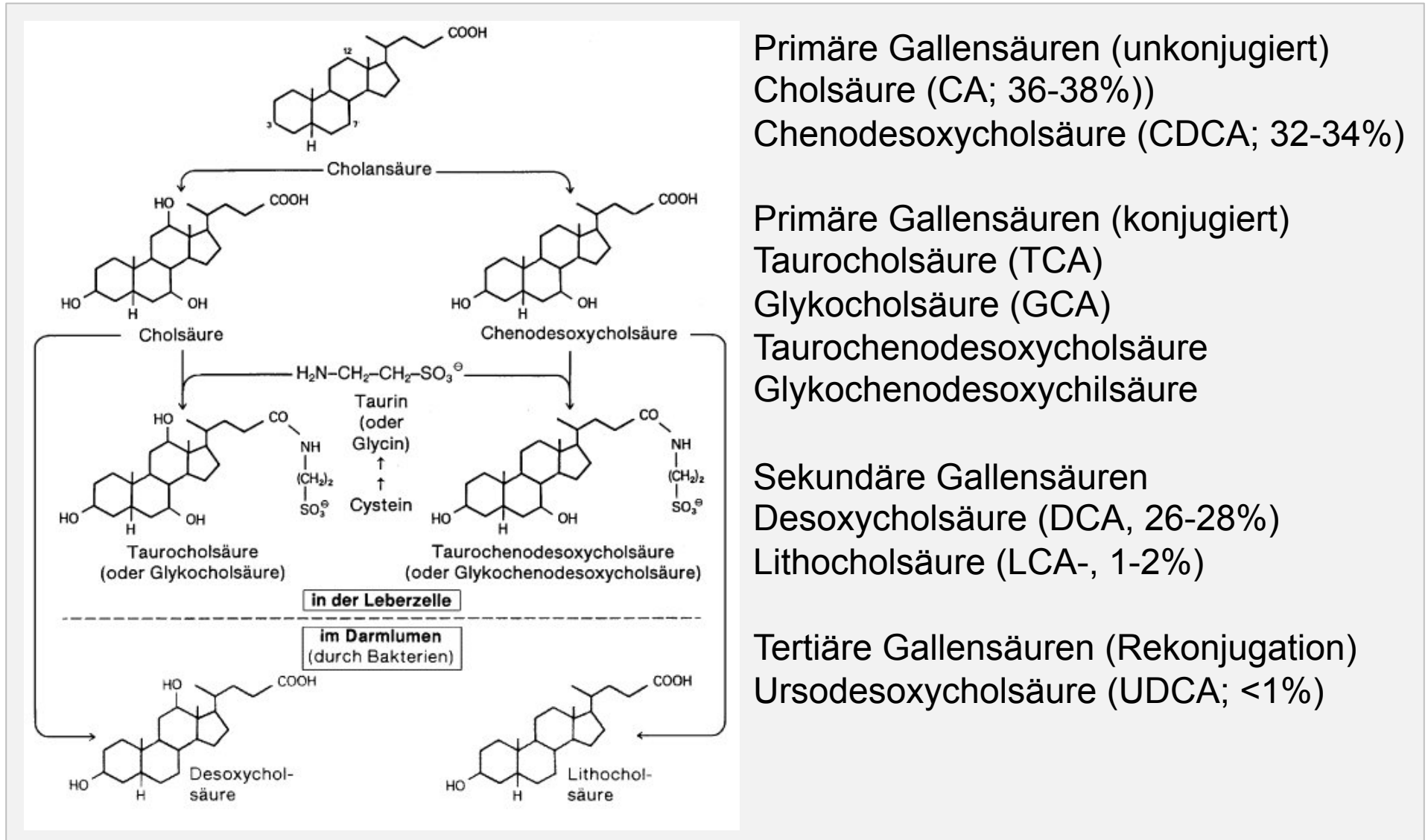
Campylobacter-Proteom-Analyse unter Galleexposition



PD Dr. Andreas Zautner
Institut für Medizinische Mikrobiologie
Universitätsmedizin Göttingen



Die Synthese der Gallensäuren



Der Enterohepatische Kreislauf

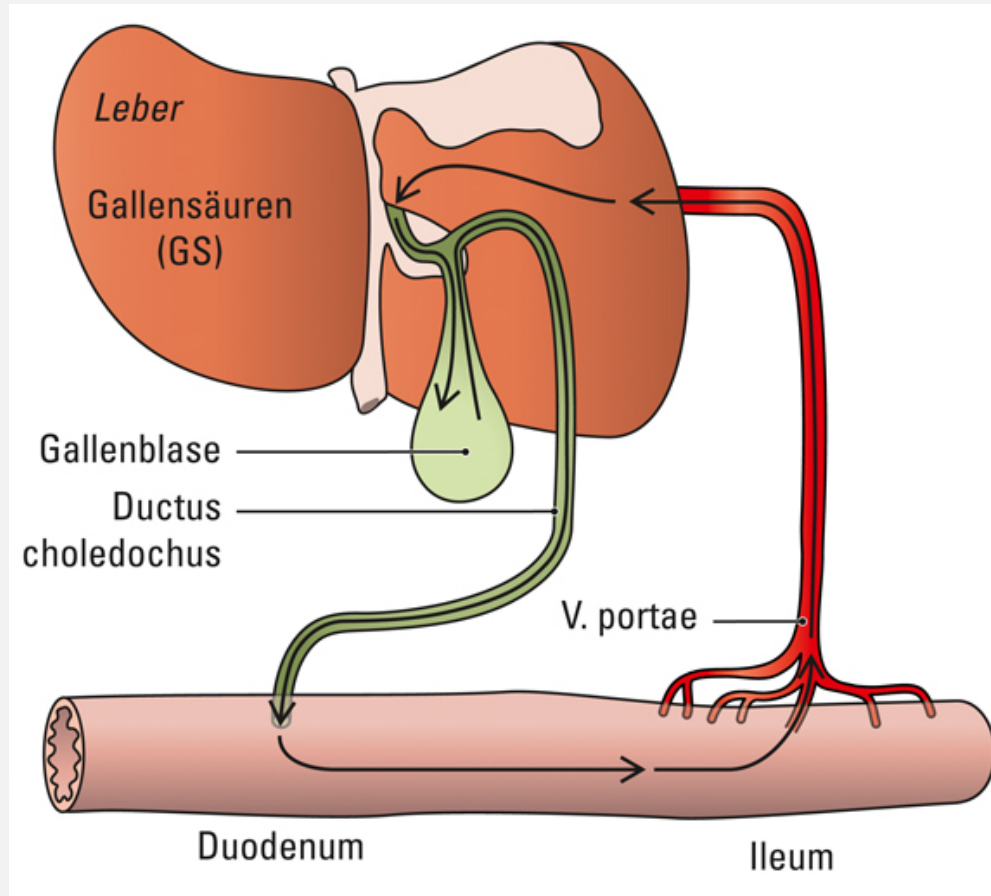
Gallensäurepool gesamt = 3-5 g

Cholesterin
 ↓
 freie Gallensäuren
 0,8g/d
 ↑
 Chenodesoxy-
 cholsäure

$C_{GS} = 0,2-2\%$

$C_{GS}(\text{Duodenum}) =$
 13-46 mM (pp.)
 2,5-10mM (noc.)

$C_{GS}(\text{Ileum}) = 1\text{mM}$
 $C_{GS}(\text{Blut}) = 10\mu\text{M}$



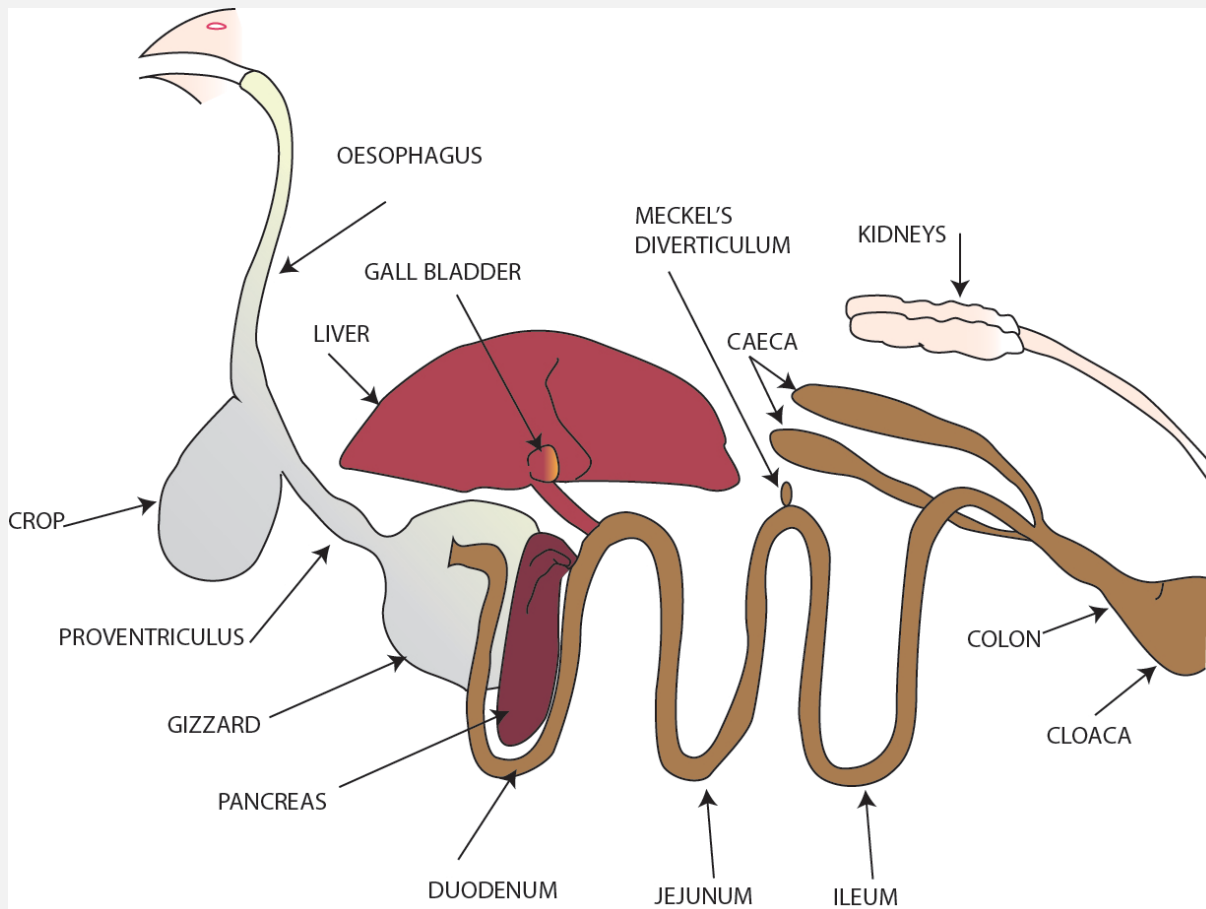
6-8 x tgl.
 Sekretion &
 Rückresorption
 = 20-30 g/d

Dekonjugation diverser Metabolite

↘
 fäkale
 Ausscheidung
 0,8g/d

Gallensäurenkreislauf in Vögeln

(in hühnerartigen (gallinaceus) Vögeln)



Zusammensetzung:

Huhn und Pute:

Chenodeoxycholytaurine
Cholytaurine
Allocholytaurine

Enten und Gänse:

Chenodeoxycholytaurine
Phocaecholytaurine

Cremers 2014

$C_{GS} = 0.01 - 0.7\%$
 $C_{GS}(\text{Blut}) < 15\mu\text{M}$

Hypothese

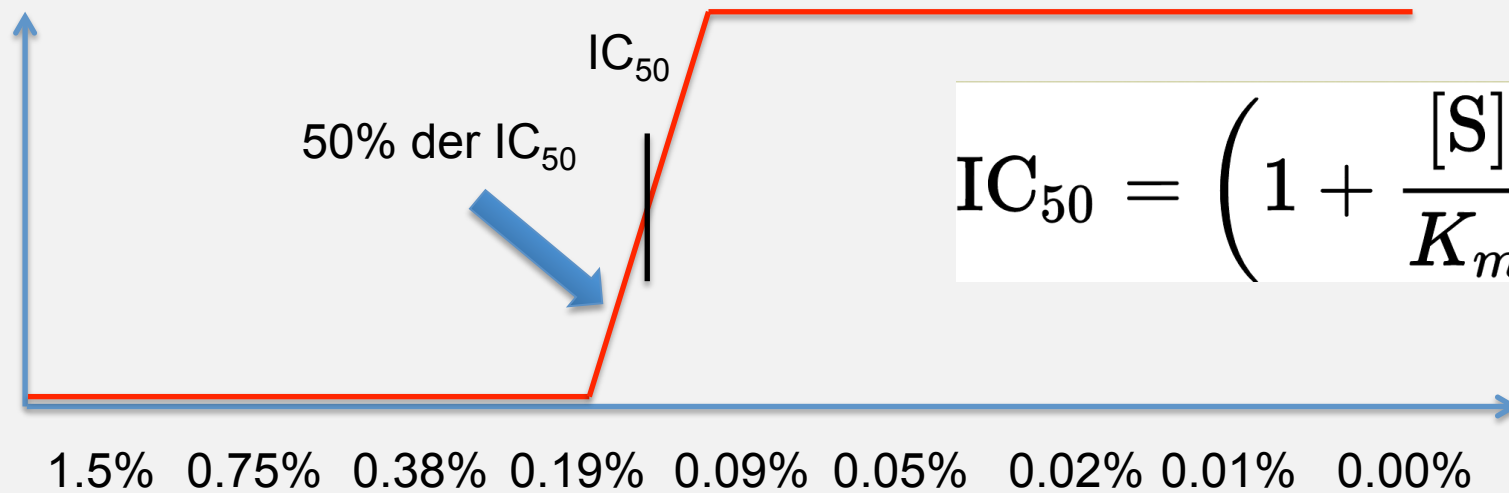
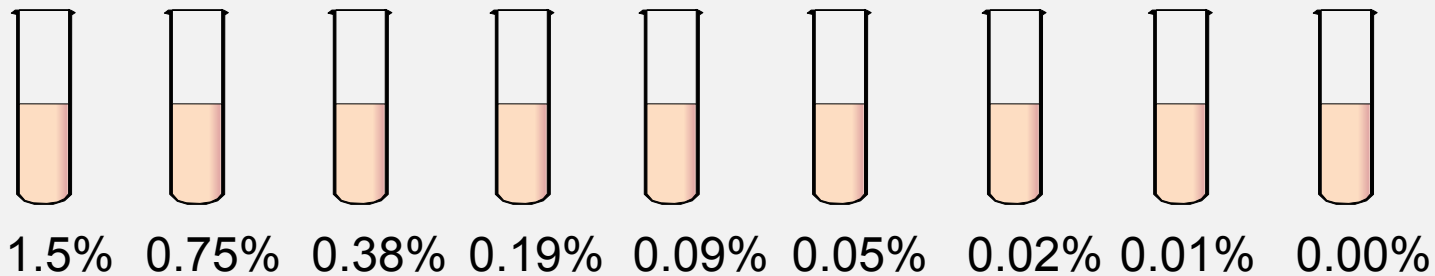
Die erhöhten Gallensäurekonzentrationen im menschlichen Intestinum stellen einen signifikanten Stressor für *Campylobacter jejuni* dar.

→ Es sollte eine spezifische Stressantwort für Gallensäuren in der Bakterienzelle induziert werden.

Methode: Quantitative Proteomik

Toxische Wirkung der Gallensäuren auf *C. jejuni*

Bestimmung der IC_{50} mittels Verdünnungsreihe

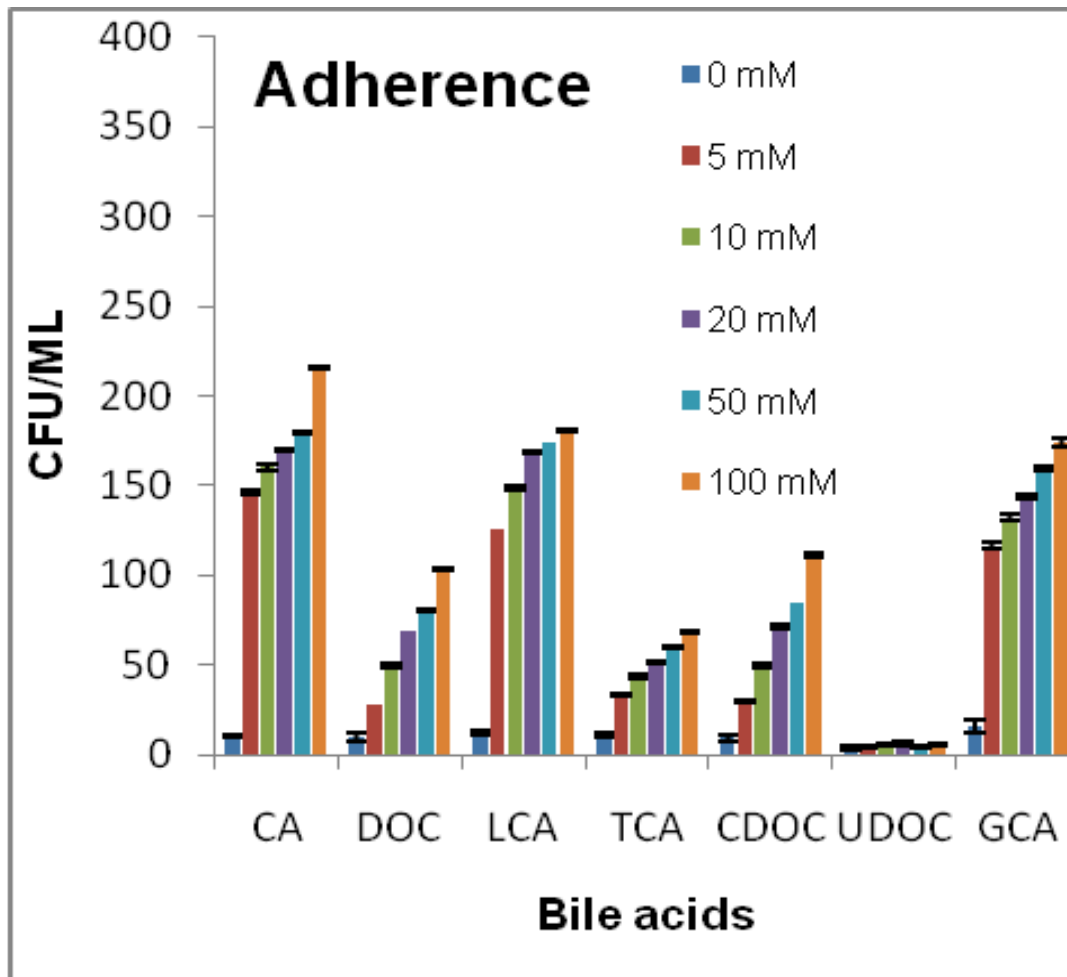


$$IC_{50} = \left(1 + \frac{[S]}{K_m} \right) \cdot K_i$$

IC₅₀ der Gallensäuren für *C. jejuni* 81-176

Gallensäure	IC ₅₀ ± SD % (mM)	Half IC50 % (mM)
CA	0.15% ± 0.05 (3.48)	0.075% (1.74)
CDCA	0.10% ± 0.10 (2.41)	0.050% (1.21)
TCA	0.96% ± 0.23 (18.04)	0.485% (9.02)
GCA	0.74% ± 0.19 (15.18)	0.370% (7.59)
DCA	0.06% ± 0.04 (1.45)	0.030% (0.72)
LCA	1.00% ± 0.25 (26.56)	0.500% (13.28)
UDCA	0.970% ± 0.22 (24.71)	0.485% (12.35)

Der Einfluß von Gallensäuren auf die Zelladhärenz von *C. jejuni* 81-176



Legende:

Cholsäure (CA)

Desoxycholsäure (DCA)

Lithocholsäure (LCA)

Taurocholsäure (TCA)

Chenodesoxycholsäure (CDCA)

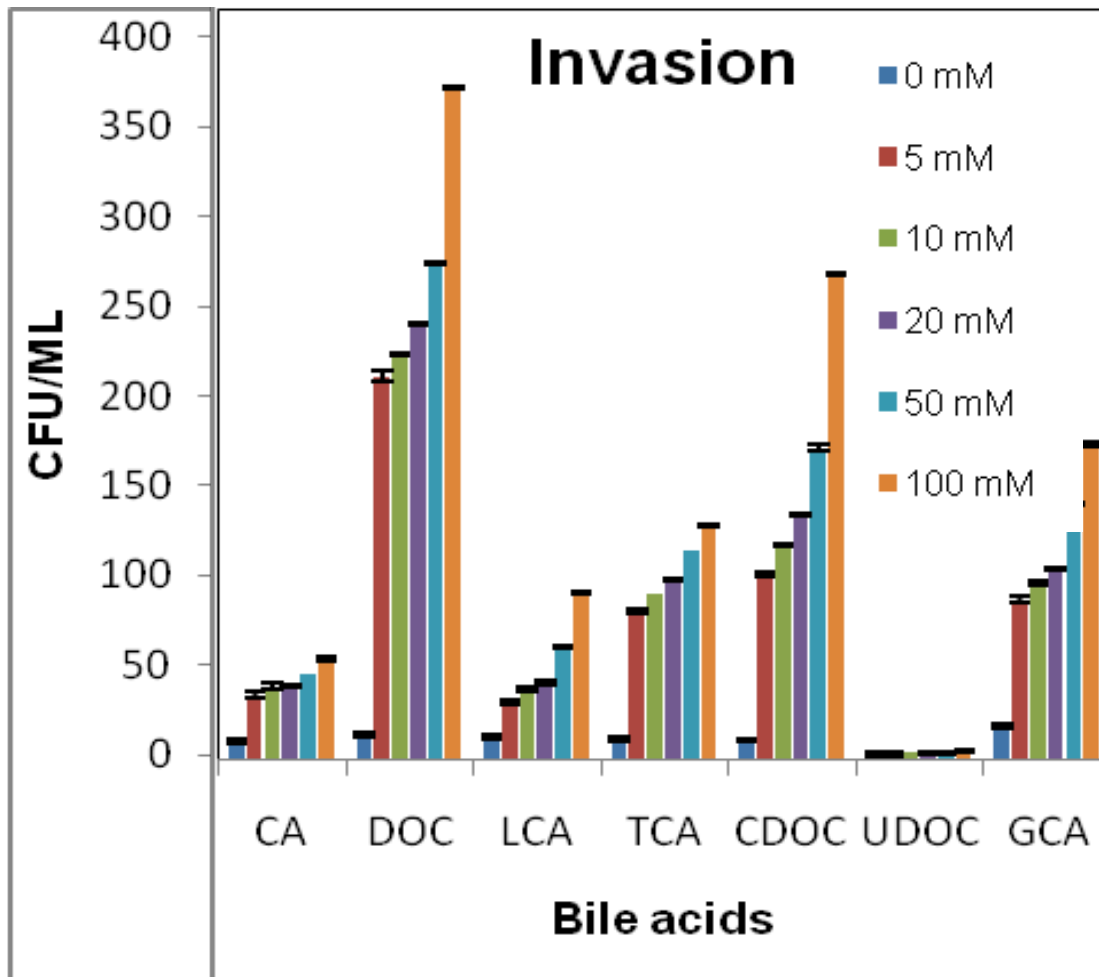
Ursodesoxycholsäure (UDCA)

Glykocholsäure (GCA)



**Primäre und sekundäre
Gallensäuren erhöhen die
Zelladhärenz von *C. jejuni***

Der Einfluß von Gallensäuren auf die Zellinvasion von *C. jejuni* 81-176



Legende:

Cholsäure (CA)

Desoxycholsäure (DCA)

Lithocholsäure (LCA)

Taurocholsäure (TCA)

Chenodesoxycholsäure (CDCA)

Ursodesoxycholsäure (UDCA)

Glykocholsäure (GCA)



**Primäre und sekundäre
Gallensäuren erhöhen die
Invasivität von *C. jejuni***

Vorhergehende Studien

Cia Proteins (Konkel et al. 1999, Rivera- Amill et al. 1999, River-Amill et al. 2001, Konkel et al. 2004; Malik-Kale et al. 2008)

CmeABC multidrug efflux pump (Lin et al. 2005)

CmeB, CmeF, CmeR und **Cj0561c** induziert durch DOC (Dzieciol et al. 2011, Mavri et al. 2013)

Gallesalz und Erythromycin-Resistenz sind über gemeinsame Effluxmechanismen (RND efflux pumps, CmeABC und CmeDEF) assoziiert (Mavri et al. 2013)

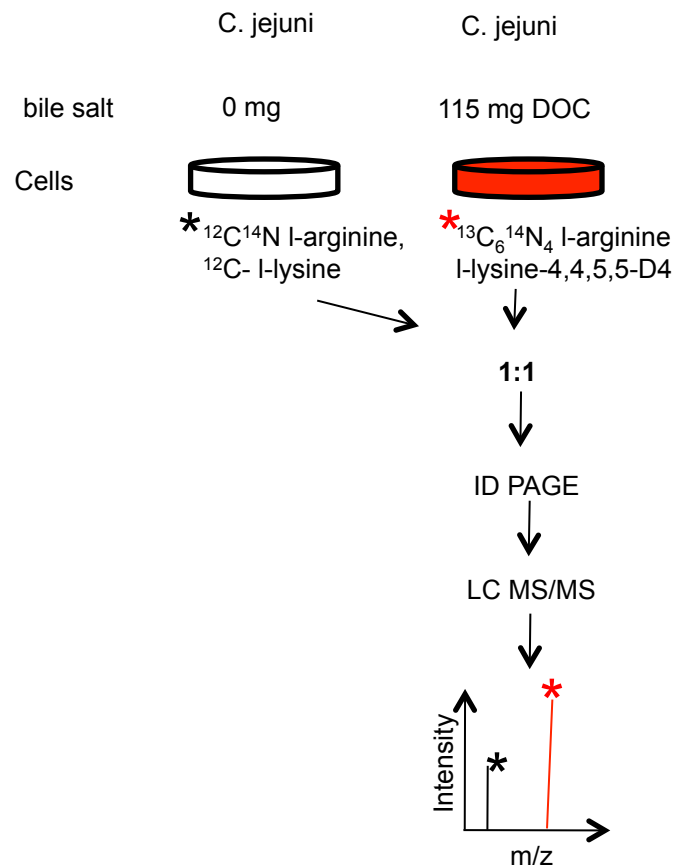
Ein funktionelles Type VI Secretion system (T6SS) erhöht die Suszeptibilität gegenüber 0,2% DOC (Lertpiriyapong et al. 2012)

Negative Chemotaxis vermittelt durch **TLP3 und 4** (Li et al. 2014)

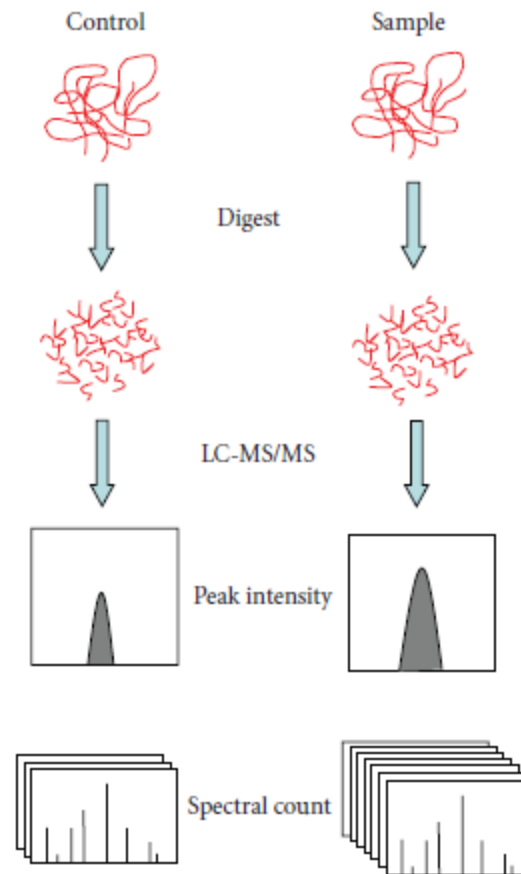
CJIE1 prophage iTRAQ bei 0,1% DOC → 2% changed proteins: (Clark et al. 2014)

Zwei verschiedene massenspektrometrische Ansätze

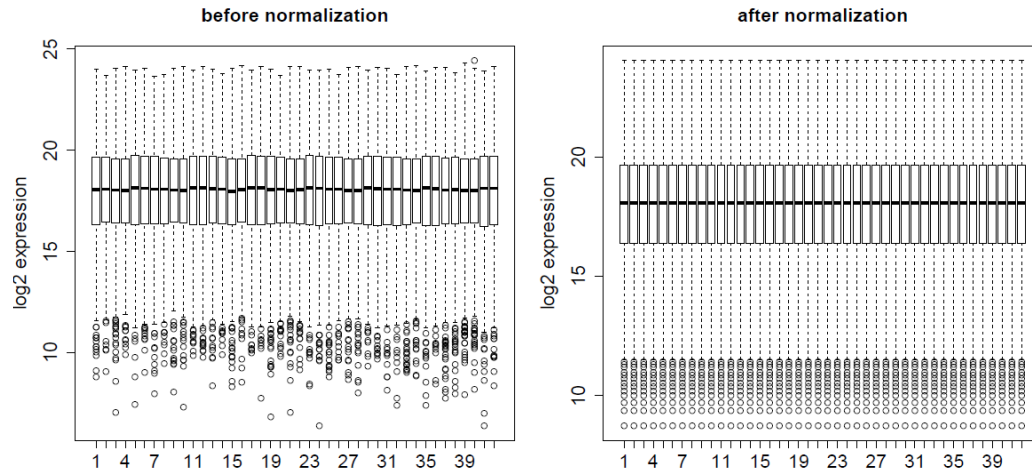
Stable isotope labeling by amino acids in cell culture (SILAC)



Label-free quantitative approach (SWATH-MS)

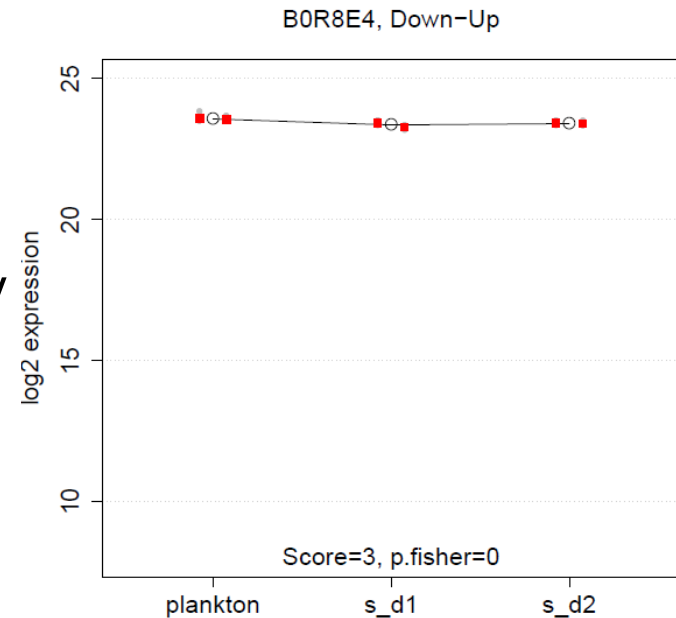


Statistische Analyse (R, Klaus Jung)



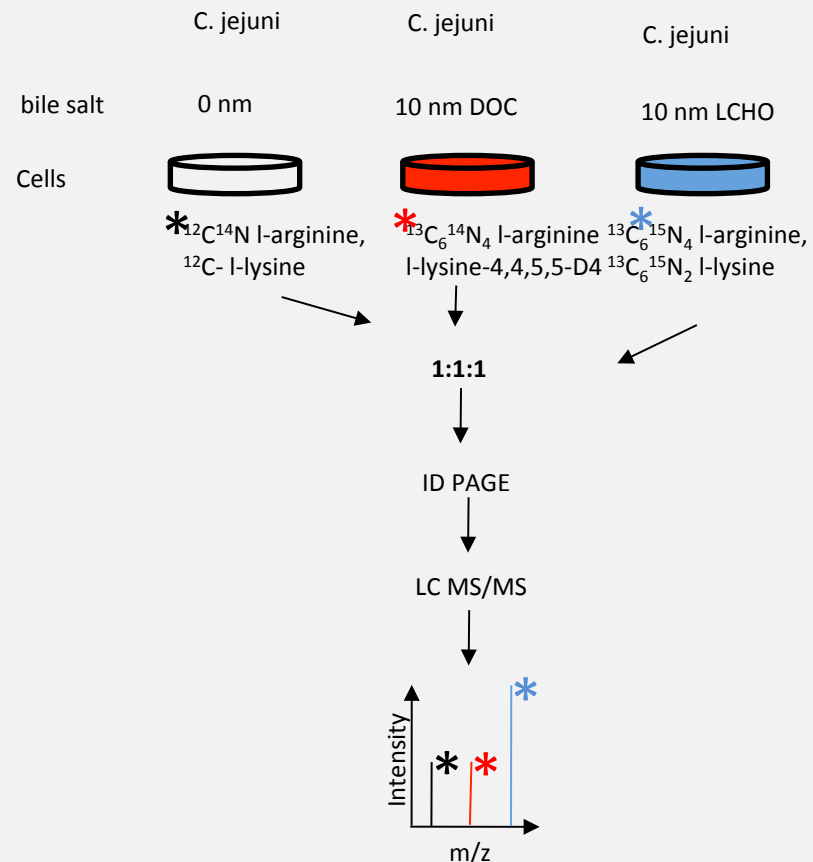
• log₂ transformation and quantile normalization of protein peak areas (Bolstad *et al.*, 2003)

- Pairwise comparison of values using linear models (Smyth *et al.*, 2004) and Benjamini-Hochberg adjustment
- Point scoring of individual protein profiles by log₂ FC and FDR-adjusted confidence intervals (Jung *et al.*, 2009; Jung *et al.* 2011)



Postinfektiöse Komplikationen

- Stable isotope labeling by amino acids in cell culture (SILAC) technique (Ong *et al.*, 2002).
- Identification of suitable *C. jejuni* strain.
- Development of a chemically defined media for the analysis of amino acid nutrition requirements.



Campylobacter defined media: “Zutaten”

Solution 1	Solution 2	Amino acid mix	Individual components
L-Aspartate	L-Arginine hydrochloride	L-Phenylalanine	L-Cysteine hydrochloride
L-Glutamate	Serine	L-alanine	L-Cystine
NaCl	Solution 3	L-histidine	Oxaloacetate
K ₂ SO ₄	L-Leucine	L-threonine	NaHCO ₃
MgCl ₂ · 6H ₂ O	L-Isoleucine	L-lysine	Biotin
NH ₄ Cl	L-Valine	L-glycine	Thiamine pyrophosphate
EDTA		L-tryptophan	L-Proline
Solution 4	Solution 5		L-Methionine
K ₂ HPO ₄	NAD		CaCl ₂ · 1H ₂ O
KH ₂ PO ₄	Thiamine hydrochloride		Fe(NO ₃) ₃ · 9H ₂ O
	Calcium pantothenate		pH to 6.8

Modified from Catlin B.W. 1973

Auxotypisierung

Auxotypisierung von 291 *C. jejuni* Isolaten

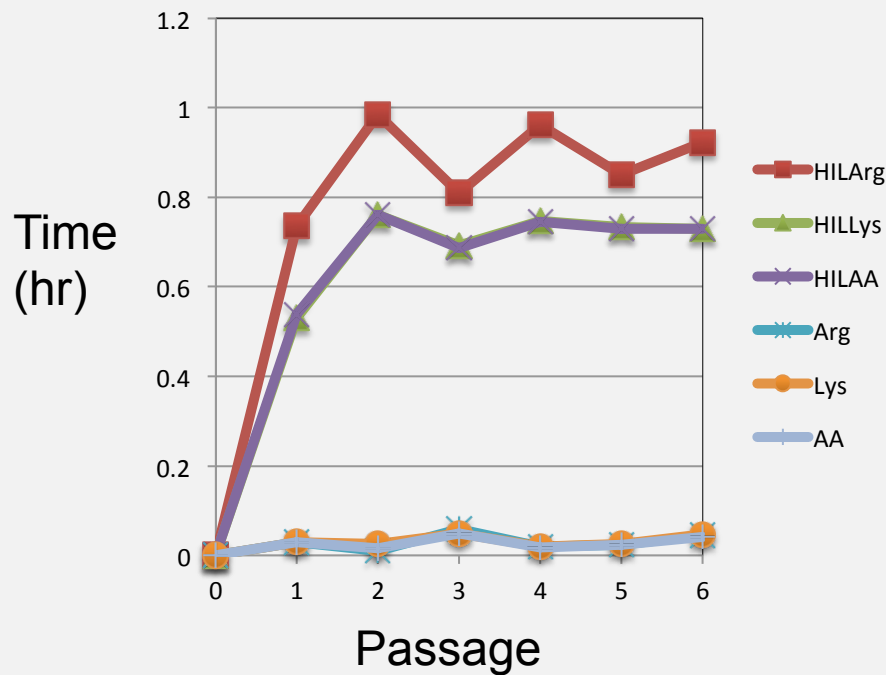
Amino acid requirement	No. of isolates
Prototrophic	285
Met-	17
Arginine-	1
Serine-	1

- Isolat av4258 ist sowohl Arginin als auch Serin auxotroph
- 17 Methionin-auxotrophe Isolate

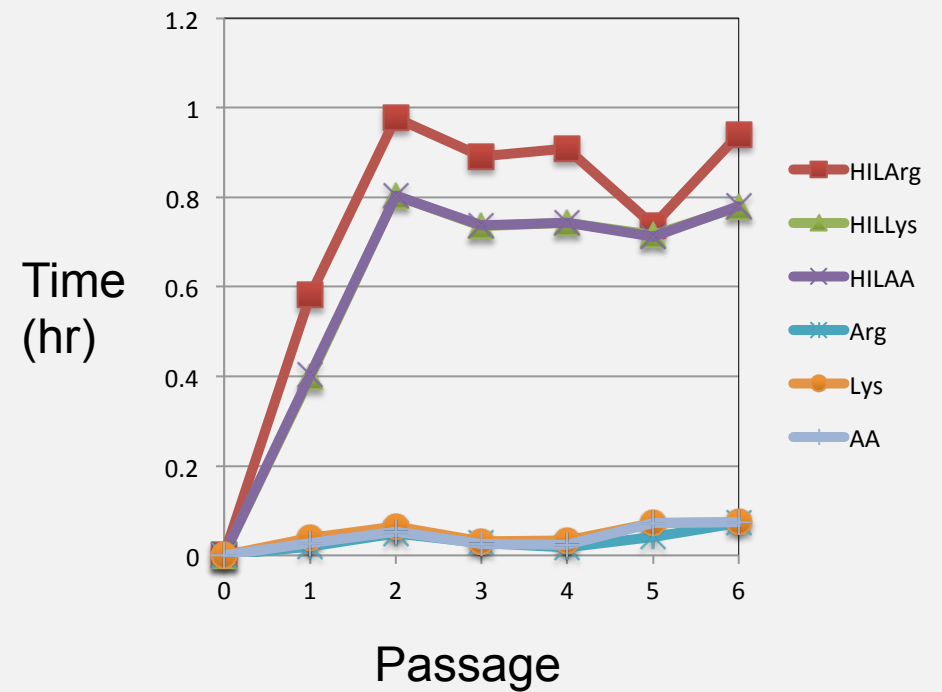


Incorporation of HILAA in CDM broth

gal4258 (Arg-Auxotrophe)



Gal4116 (Prototrophe)

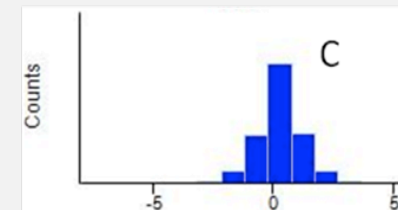
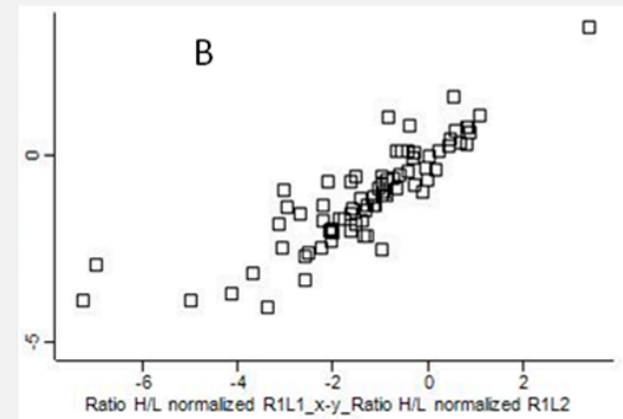
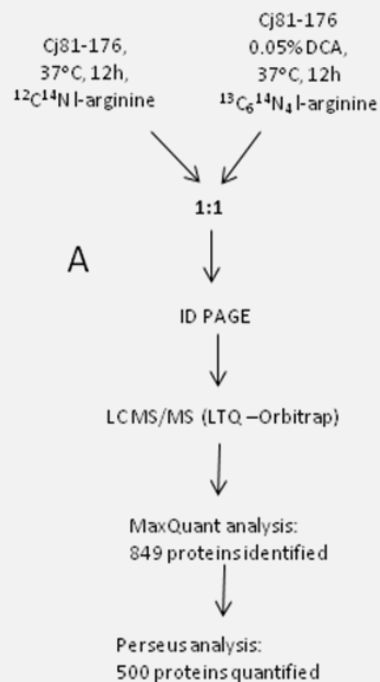


Incorporation of HIL - arginine in other *C. jejuni* prototroph strains in broth

Passage/ strain	4258	B17	81176	11168	4116	av518
P1	86.8	83.4	89.1	84.4	82.8	87.4
P2	95.2	96.2	97.8	95.6	96.4	95.8
P3	99	98.7	99.4	98.8	98.6	98.6
P4	99.7	99.5	99.7	99.5	99.2	98
P5	99.9	99.6	99.8	99.5	99.3	98.4
P6	99.5	99.7	99.4	99.6	99.3	98.7

Ergebnisse SILAC

- SILAC wenig geeignete Methode aufgrund schlechter Lys-Einbaurrate
Verdau mit Arginase ArgC anstelle von Trypsin
- Demonstration des Prinzips mit 0.03% (0,72 mM) DCA bei 37° C über 12 h
- 3 Replikate
- 849 Proteine identifizierbar
- 500 Protein quantifizierbar



Ergebnisse SILAC II

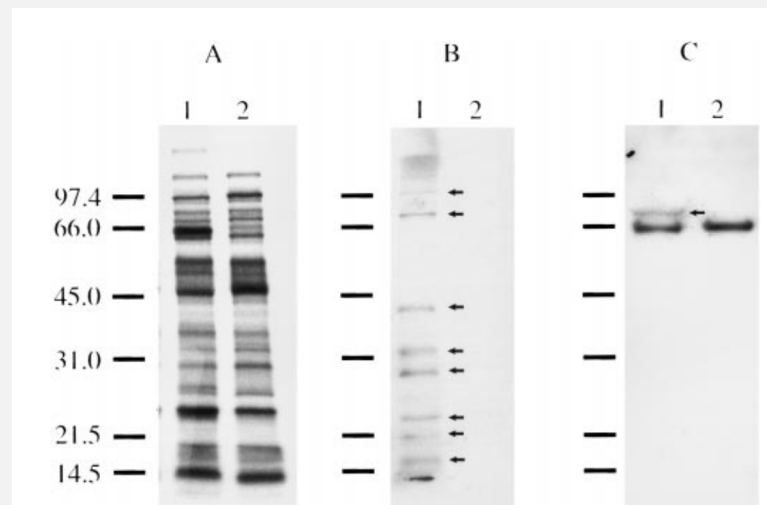
Hoch Regulierte Proteine:

- transcription termination factor Rho (Rho)
- aspartate aminotransferase (aspC)
- GTP cyclohydrolase-2 (ribA)
- dCTP deaminase (dcd)
- methionine aminopeptidase (map)
- succinate dehydrogenase subunit C (sdhC)
- fibronectin-binding protein (cadF)
- 60 kDa chaperonin (groL)

Ergebnisse SILAC II

Unveränderte Proteine (Invasions-assoziiert)

- *Campylobacter* invasion antigen B (ciaB)
- flagellar motor switch protein FliG (FliG)
- paralyzed flagella protein PflA (PflA)
- co-chaperone protein (DnaJ)
- capsular polysaccharide ABC transporter
- periplasmic polysaccharide-binding protein (kpsD)



Konkel et al.
1999

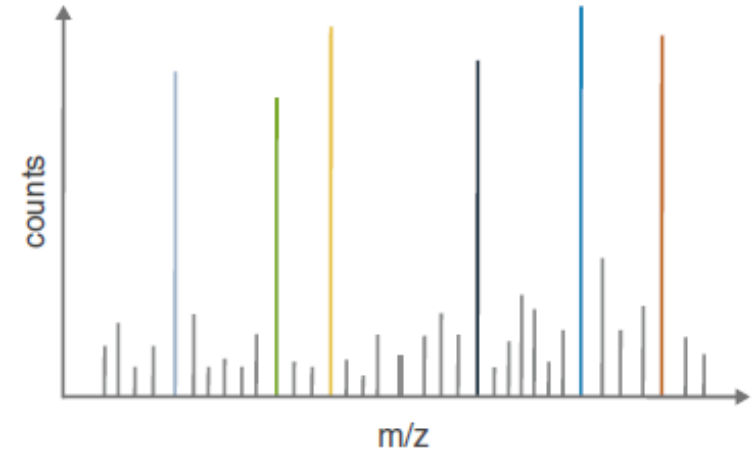
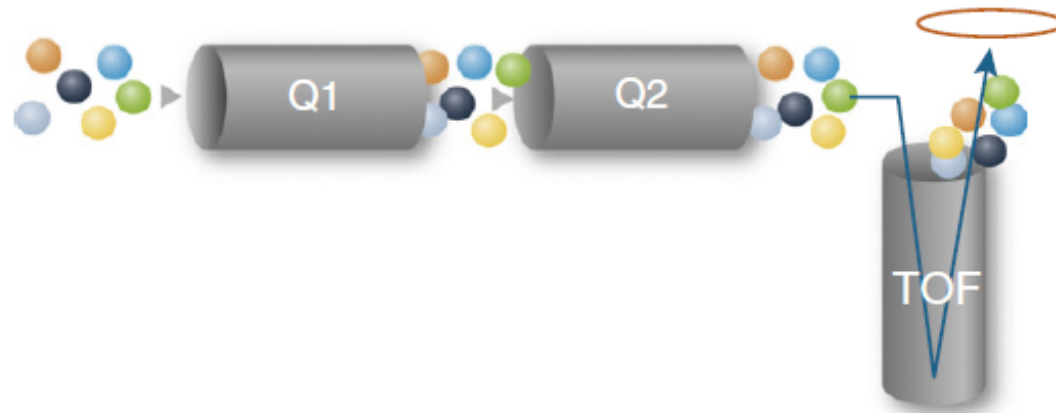
Ergebnisse SILAC III

Runterregulierte Proteine (beschrieben als DCA-induziert)

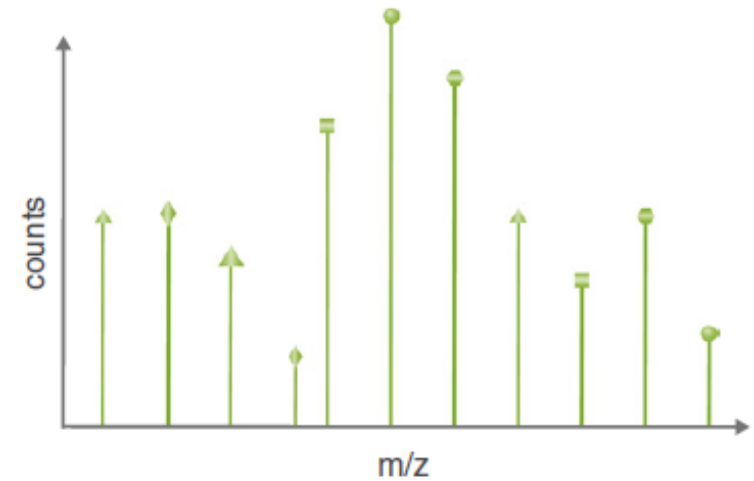
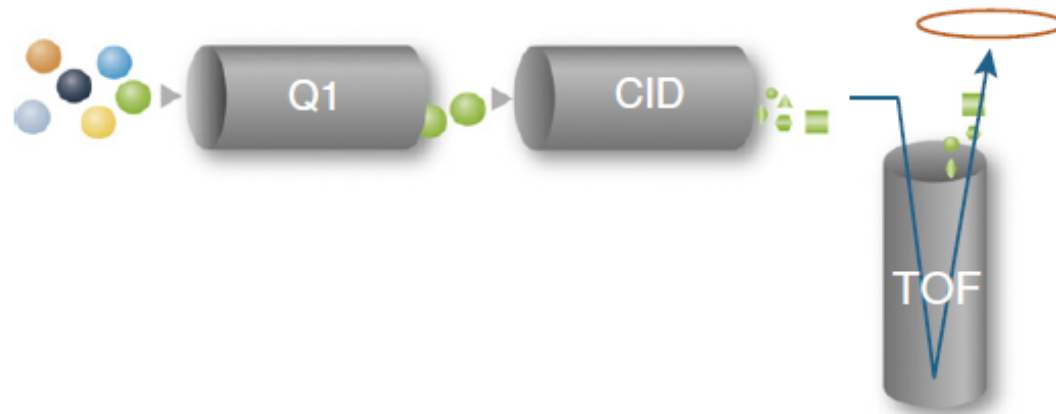
- CmeABC efflux pump proteins
- Catalase A (katA)
- Flagellar protein FlaA (FlaA)
- Flagellar protein FlaG (flaG)
- Flagellar hook protein FlgE

Data-Dependent Acquisition (DDA)

TOF MS

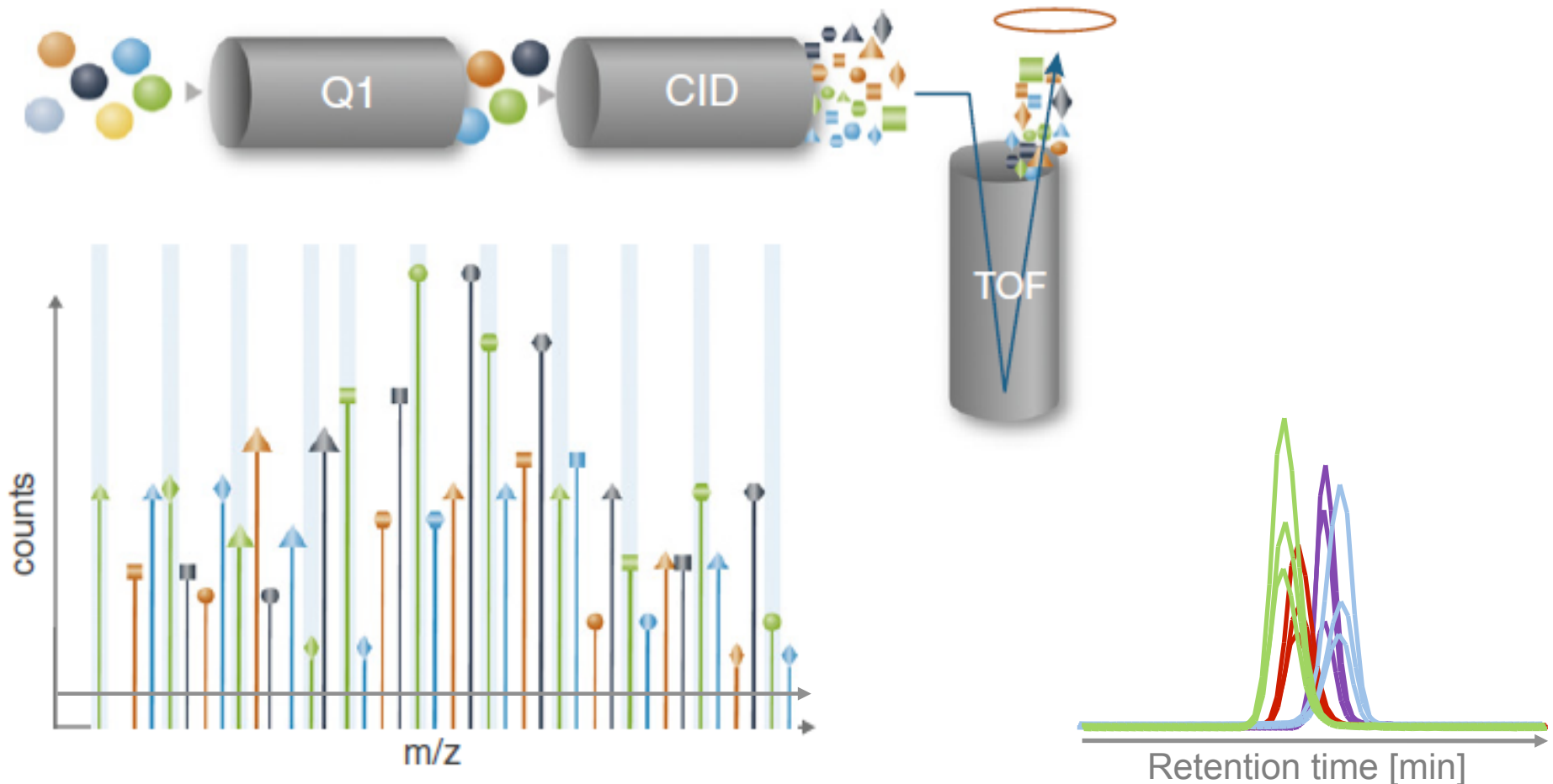


TOF MS/MS

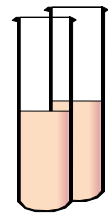


Data-Independent Acquisition (DIA, aka SWATH) on High Resolution (QqToF/Q Exactive) mass spectrometers

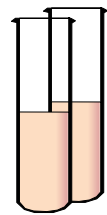
SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra)



37°C for 12 h
50% of IC₅₀



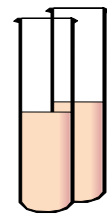
CDM
control



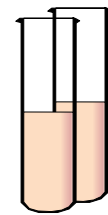
CA



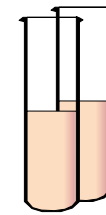
CDCA



TCA



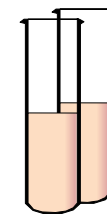
GCA



DCA



LCA

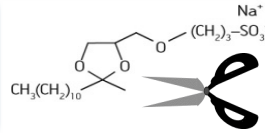


UDCA



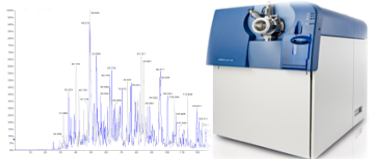
Centrifugation, resuspension in 0.9% NaCl, Sonification,
second Centrifugation, SDS-PAGE, acetone precipitation

Sample
preparation

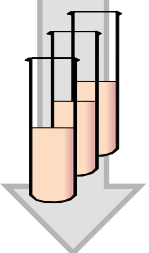
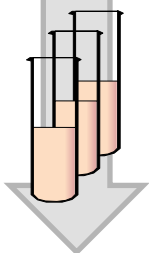
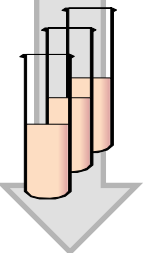
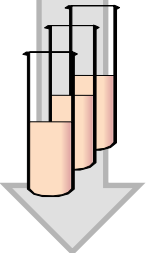
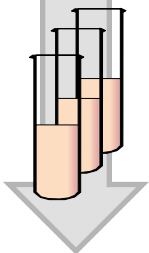
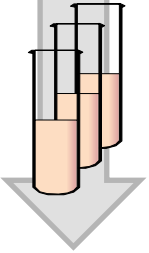
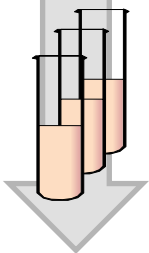
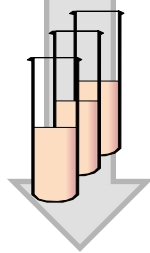


40 µg/sample, Rapigest solubilization
Trypsin digest in solution

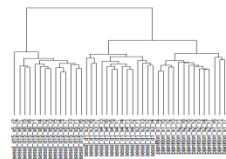
nLC/MS/MS
analysis



nUPLC 0.075x250 mm, 150 min
TT5600+ DDA-Top25 // DIA-SWATH



Data
analysis



ProteinPilot 5.0 (ID)
PeakView 2.1 (Library/Peak Extraction)
R, Perseus 1.5.0.15 (Statistics)



Quantitative Proteomics of Intracellular *Campylobacter jejuni* Reveals Metabolic Reprogramming

Xiaoyun Liu, Beile Gao, Veronica Novik, Jorge E. Galán*

Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut, United States of America

LC-MS/MS-Analysen

in vitro (Agar) vs 2h in Infektionsmedium

→ Keine signifikanten Proteomveränderungen

2 h intrazellulär vs 20 h intrazellulär (COS-1-Zellen)

→ 225 Proteine überwiegend herunterreguliert

→ Kein intrazellulärer Stress

→ Keine intrazelluläre Zellteilung

→ Reprogrammierung der Zelle:

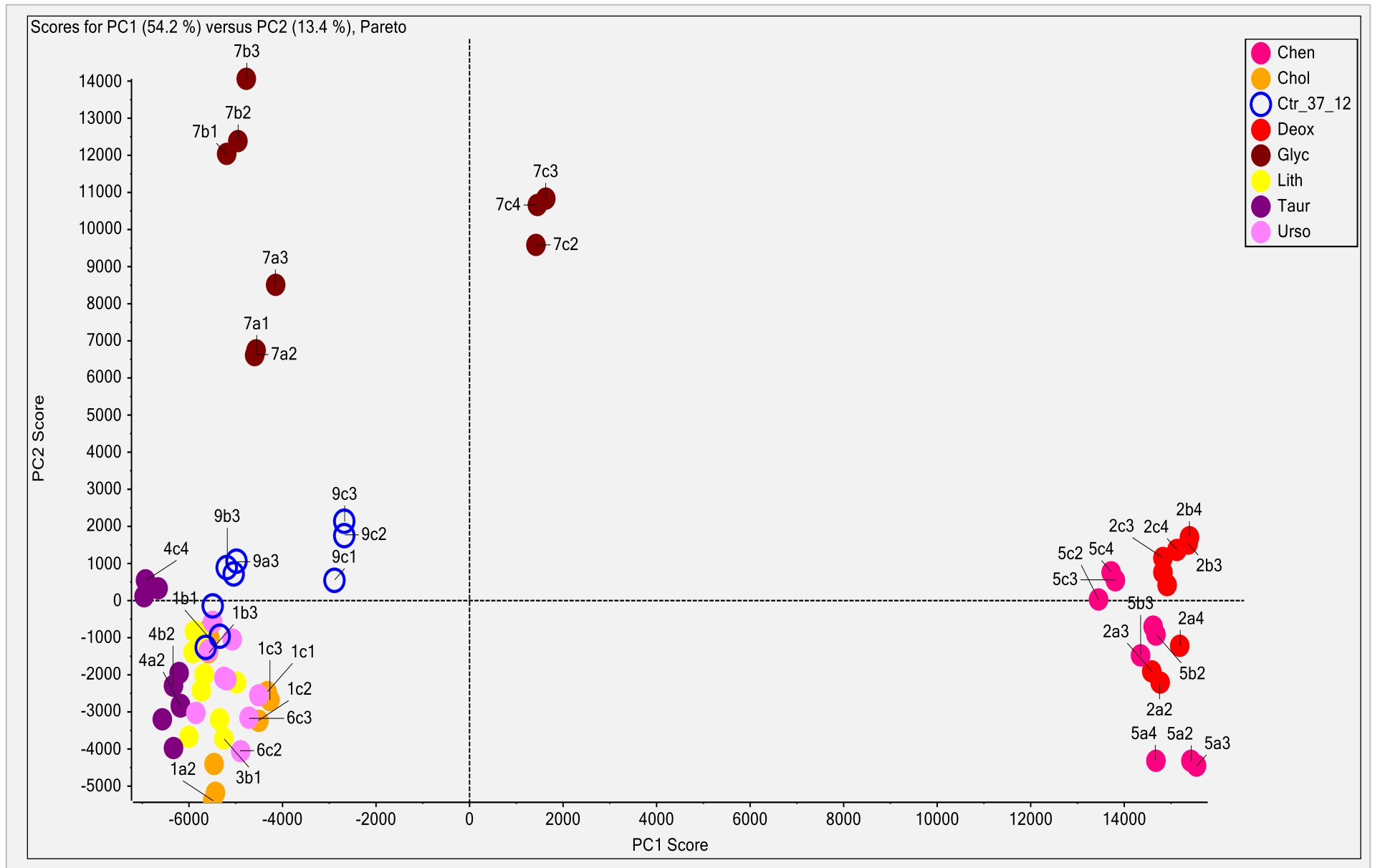
Herunterregulation des Metabolismus
Fumarat-Respiration

Protein identification by nanoLC/MS/MS

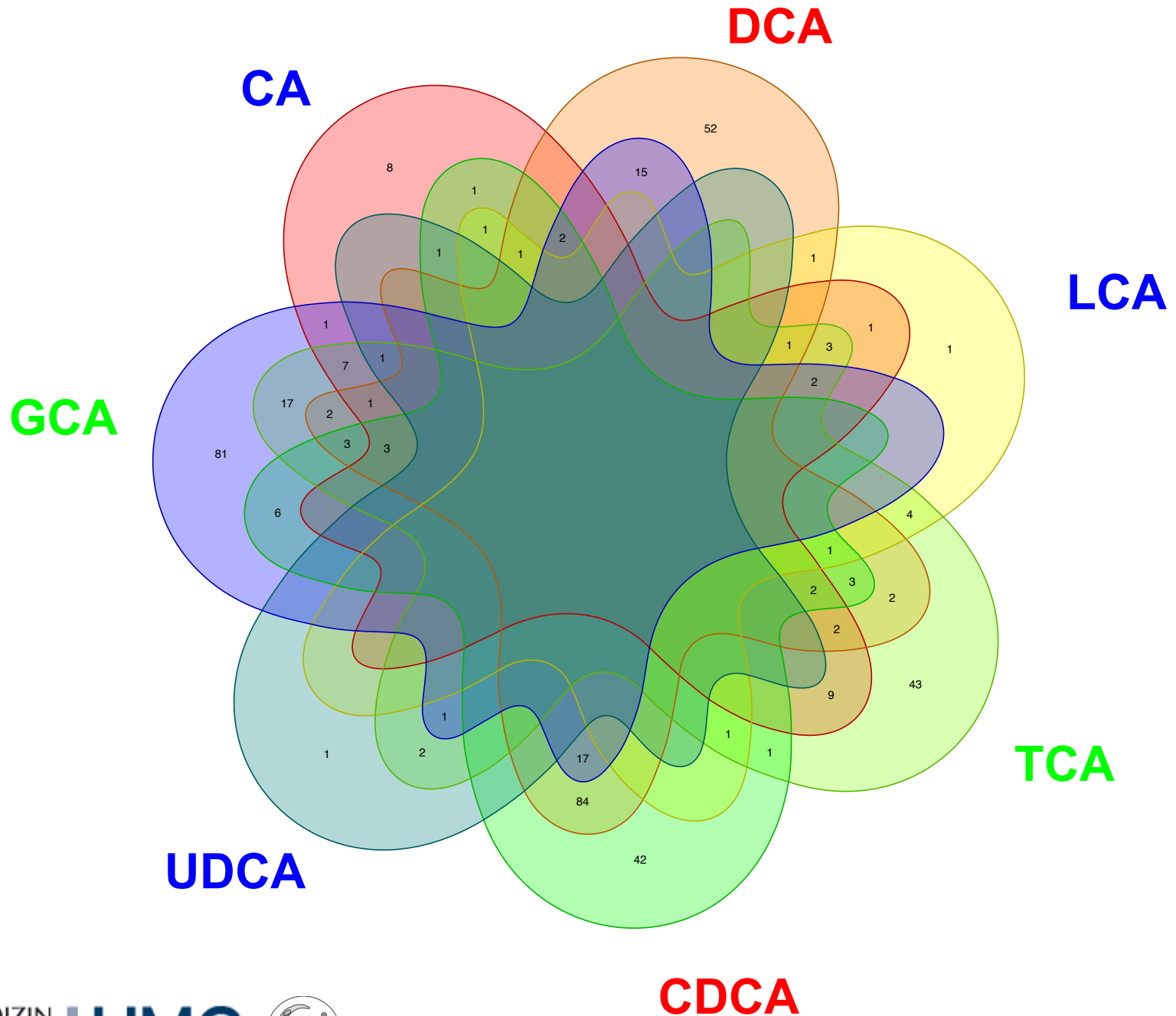
SWATH-MS

Anz. Proteine	1079	(SILAC 849) (Liu et al. 1428)
(Max Quant/Protein Pilot 5.0)		
Quant. Proteine	957	(SILAC 500)
Anz. Peptide	14,644	
Proteome coverage	58.9%	(Liu et al. 86%)
Signifikant Verändert	700	(Liu et al. 225)
bekannt	459	
hypothetisch	241	

Principal component analysis (PCA).



Venn-Diagramm aller getesteten Gallensäuren



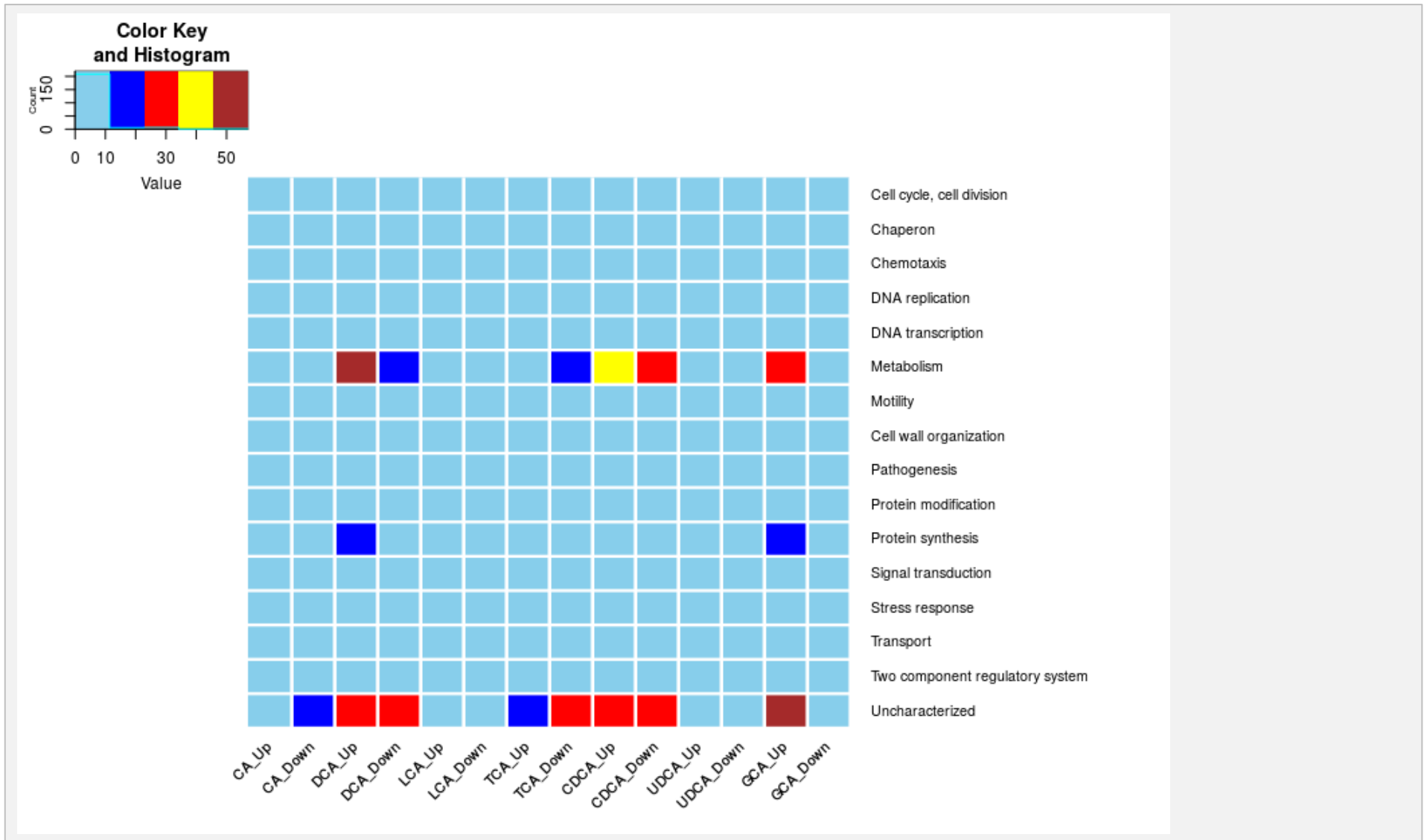
Ergebnisse

Bile acid (mM)	No. of significantly upregulated proteins ¹	No. of significantly downregulated proteins ²	Total no. Significantly changed
CA (1.74)	19	28	47
CDCA (1.21)	89	80	169
TCA (9.02)	51	60	111
GCA (7.59)	139	20	159
DCA (0.72)	113	79	192
LCA (13.28)	4	13	17
UDCA (12.35)	2	4	6

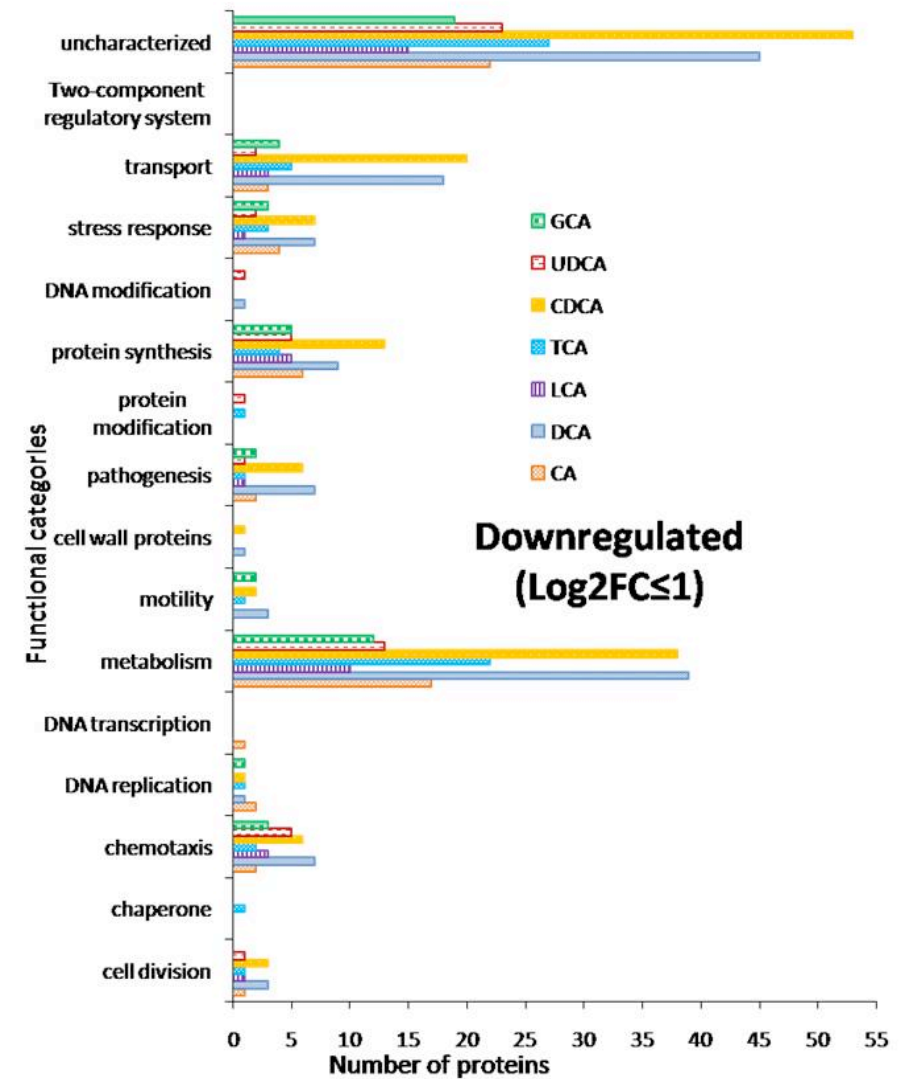
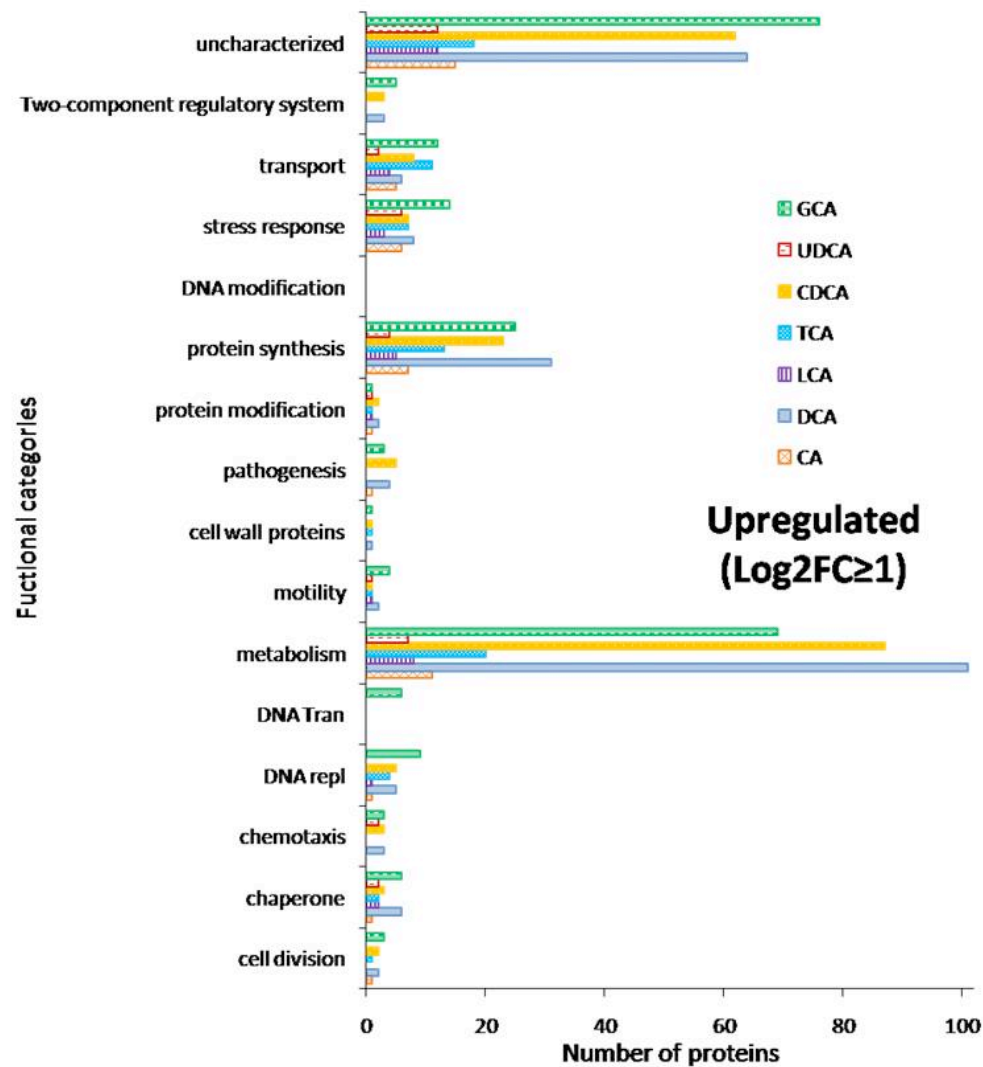
¹significantly upregulated proteins correspond to log₂ Fold Change ≥ 1

²significantly downregulated proteins correspond to log₂ Fold Change ≤ -1

Significantly distinct expressed proteins in cj81-176 on response to low concentration of each individual bile acid



Significantly distinct expressed proteins in cj81-176 on response to low concentration of each individual bile acid



Beschriebene **Virulenz-assoziierte Faktoren** die durch Gallensäuren hochreguliert werden

Protein ID	Gene Name	Protein function	Bile acid
A0A0H3PA52	<i>htrA</i>	serine protease	TCA
A1VZQ5	<i>peb1C</i>	Amino acid transport	TCA, GCA
A1W0U6	<i>pseG</i>	Flagella glycosylation	DCA, CDCA
Q5QKR7	<i>pseC</i>	Flagella glycosylation	DCA
Q939J8	<i>pseI</i>	Flagella glycosylation	DCA, CDCA
A0A0H3PE81	<i>ciaC</i>	Flagella secreted protein	TCA, UDCA, GCA
A0A0H3P9C5	<i>mapA</i>	Lipoprotein	CA
A1W0G0	<i>tatA</i>	Protein secretion	TCA
A0A0H3PAY0	<i>tatB</i>	Protein secretion	GCA
A0A0H3PAN7	<i>secF</i>	Protein transport	DCA, GCA
A0A0H3PEE2	<i>secG</i>	Protein transport	LCA
A0A0H3P9B1	<i>yajC</i>	Protein transport	GCA
A0A0H3PAC3	<i>cjj81176_1161</i>	LOS sialylation	TCA, GCA
A0A0H3PCP5	<i>cdtC</i>	Toxin	GCA

Signifikant hochregulierte Proteins assoziiert mit **Chemotaxis und Motilität**

Protein ID	Gene name	Protein function	Bile acid
A0A0H3P9J9	<i>aer1/cetB</i>	Energy taxis	TCA, GCA
A0A0H3P9P7	<i>aer2/cetC</i>	Energy taxis	GCA
A0A0H3P9T7	<i>tlp4</i>	Sensing external stimuli	GCA
A0A0H3PAG7	<i>cheW</i>	Signal transducer	TCA, GCA
A0A0H3PAM0	<i>cheA</i>	Transferase	GCA
A0A0H3PAN9	<i>tlp9/cetA</i>	Signal transducer	CA, TCA, GCA
A0A0H3PB06	<i>tlp5</i>	Sensing internal stimuli	DCA, CDCA, GCA
A0A0H3PB49	<i>tlp10</i>	Sensing external stimuli	LCA, UDCA
A0A0H3PBN1	<i>cheY</i>	Chemotaxis protein CheY	DCA, CDCA
A0A0H3PEF7	<i>tlp 1</i>	Sensing external stimuli	CA, LCA, TCA, GCA
A0A0H3PEL1	<i>tlp2</i>	Sensing external stimuli	CA, TCA, GCA
A0A0H3PAE1	<i>cheR</i>	Methyltransferase	DCA, CDCA
A0A0H3P9L2	<i>fliM</i>	C-ring protein	GCA
A0A0H3PA78	<i>fliY</i>	Controls flagellar motor direction	DCA, GCA
A0A0H3PAL4	<i>fliG</i>	C-ring protein	CA, DCA
A0A0H3PIF6	<i>fliL</i>	Increases torque movement	CA, TCA, GCA

Allg. **Stressantwort**: DNA-Reparatur, Chaperone, reactive oxygen stress (ROS) defence proteins

Protein ID	Gene name	Protein function	Bile acid
A0A0H3P9V7	<i>cjj81176_1101</i>	DNA repair	DCA, GCA
A0A0H3PAG5	<i>radA</i>	DNA repair	TCA, GCA
A0A0H3PJI4	<i>recN</i>	DNA repair	GCA
A0A0H3PB76	<i>dnaJ-1</i>	Protein folding	CA, TCA
A0A0H3PEB4	<i>nth</i>	Protein folding	GCA
A1VYN0	<i>htpG</i>	Protein folding	GCA
A1VYU6	<i>ligA</i>	Protein folding	GCA
A0A0H3PBJ5	<i>dsbD</i>	Protein folding	TCA
A0A0H3PBY8	<i>ahpC</i>	Regulation of oxidative stress	GCA
A1VXQ2	<i>sodB</i>	Regulation of oxidative stress	DCA, CDCA

Signifikant hochregulierte **Metabolismus-**assozierte Proteine I

Protein ID	Gene name	Function in protein synthesis	Bile acid
A0A0H3P9B1	<i>yajC</i>	Alanyl-tRNA aminoacylation	GCA
A1VXT6	<i>infC</i>	Aminoacyl-tRNA synthetase	GCA
A0A0H3P9K7	<i>metS</i>	Aminoacyl-tRNA synthetase	GCA
A0A0H3PAI4	<i>ileS</i>	Aminoacyl-tRNA synthetase	DCA, CDCA
A0A0H3PB64	<i>trpS</i>	Aminoacyl-tRNA synthetase	DCA, TCA, CDCA, GCA
A0A0H3PBL2	<i>def</i>	Aminoacyl-tRNA synthetase	DCA, GCA
A0A0H3PBY2	<i>cjj81176_0318</i>	Aminoacyl-tRNA synthetase	DCA
A0A0H3PCJ0	<i>cjj81176_0101</i>	Aminoacyl-tRNA synthetase	GCA
A0A0H3PDU5	<i>tyrS</i>	Aminoacyl-tRNA synthetase	DCA, CDCA, GCA
A0A0H3PDX5	<i>rnc</i>	Aminoacyl-tRNA synthetase	CA, TCA, GCA

Signifikant hochregulierte **Metabolismus-**assozierte Proteine II

Protein ID	Gene name	Function in protein synthesis	Bile acid
A0A0H3PHD8	<i>valS</i>	Aminopeptidase	GCA
A0A0H3PHR2	<i>pheT</i>	ATP-binding	GCA
A0A0H3PID1	<i>glyS</i>	Endonuclease	GCA
A1VYL8	<i>alaS</i>	Hydrolase	DCA, GCA
A1VYQ2	<i>proS</i>	Isomerase	DCA, GCA
A1VYU1	<i>rppH</i>	Ligase	DCA, CDCA, GCA
A1VZ00	<i>aspS</i>	Ligase	DCA
A1VZ23	<i>rplI</i>	Ligase	DCA, GCA
A1VZN1	<i>pheS</i>	ATP-binding	GCA
A1W048	<i>gatA</i>	Small protein activating enzyme	DCA, CDCA
A1W011	<i>gatB</i>	Translation	DCA, CDCA
A1W165	<i>truD</i>	Translation	TCA, GCA
A1W1L3	<i>rpsT</i>	Translation	TCA
A1W1U6	<i>rpsH</i>	Translation	DCA
A1W1V3	<i>rplP</i>	Translation	TCA
A1W1V4	<i>rpsC</i>	Translation	TCA, GCA
A1VYB8	<i>gatC</i>	Translation initiation factor	DCA

23 Proteine wurden in SILAC gegensätzlich in Vergleich zu SWATH-MS reguliert

T: Protein IDs	Protein name	N: Razor + unique peptides	N: Q-value
A1VYG6	50S ribosomal protein L28	6	0
A1VZV2	50S ribosomal protein L34	1	0
A0A0H3PD07	C4-dicarboxylate transport protein	1	0
A0A0H3P972	CCP20	1	0
A1VZQ6	Chemotaxis protein methyltransferase	2	0
A0A0H3PJJ8	Chorismate mutase/prephenate dehydratase	6	0
A0A0H3P9Y5	Cpp21	3	0
A0A0H3PA83	Cytochrome C	1	0.0037267
A0A0H3PHE9	Flagellin	3	0
A0A0H3PDZ8	Formate dehydrogenase, iron-sulfur subunit	3	0
A0A0H3P9F9	Mechanosensitive ion channel family protein	1	0.0025773
A0A0H3PAT6	Phosphatase, Ppx/GppA family	3	0
A0A0H3PGN8	Pseudouridine synthase	4	0
A0A0H3P9G2	Putative, Cell division protein FtsH	3	0
A0A0H3PJC4	Putative, Chemotaxis protein MotB	1	0
A0A0H3PAE0	RloH	4	0
A0A0H3PD83	Signal peptidase I	7	0
A0A0H3PGQ9	Signal peptide peptidase SppA, 36K type	2	0
A0A0H3P9Y3	Signal recognition particle receptor FtsY	3	0
A0A0H3PJ87	TPR domain protein	4	0
A0A0H3PEZ9	Uncharacterized protein	3	0
A0A0H3PHD0	Uncharacterized protein	2	0
A0A0H3PI03	Uncharacterized protein	1	0

Fazit

Relevante Gallensäuren Chenodesoxycholsäure, Desoxycholsäure, Glykocholsäure

in vitro Experimente wie Invasion, Adhäsion usw. unter Zusatz von Gallensäuren

SILAC-MS ungeeignet für *Campylobacter spec.*

SWATH-MS (Label free, DIA) geeignet für *Campylobacter spec.*

MS-Studien Neue Techniken → hinterfragen der Ergebnisse „veralteter Studien“ unter Anwendung „veralteter Techniken“

Die wichtigsten Galleinduzierten Stressreaktionen sind Proteinsynthese/Metabolismus, Chemotaxis/Motilität, ROS-Proteine, Chaperone, Sec/Tat-Transport

1/3 der signifikant veränderten Proteine sind unbekannt (Hypothetisch)

Ausblick

Konstruktion von k.o.-Mutanten:

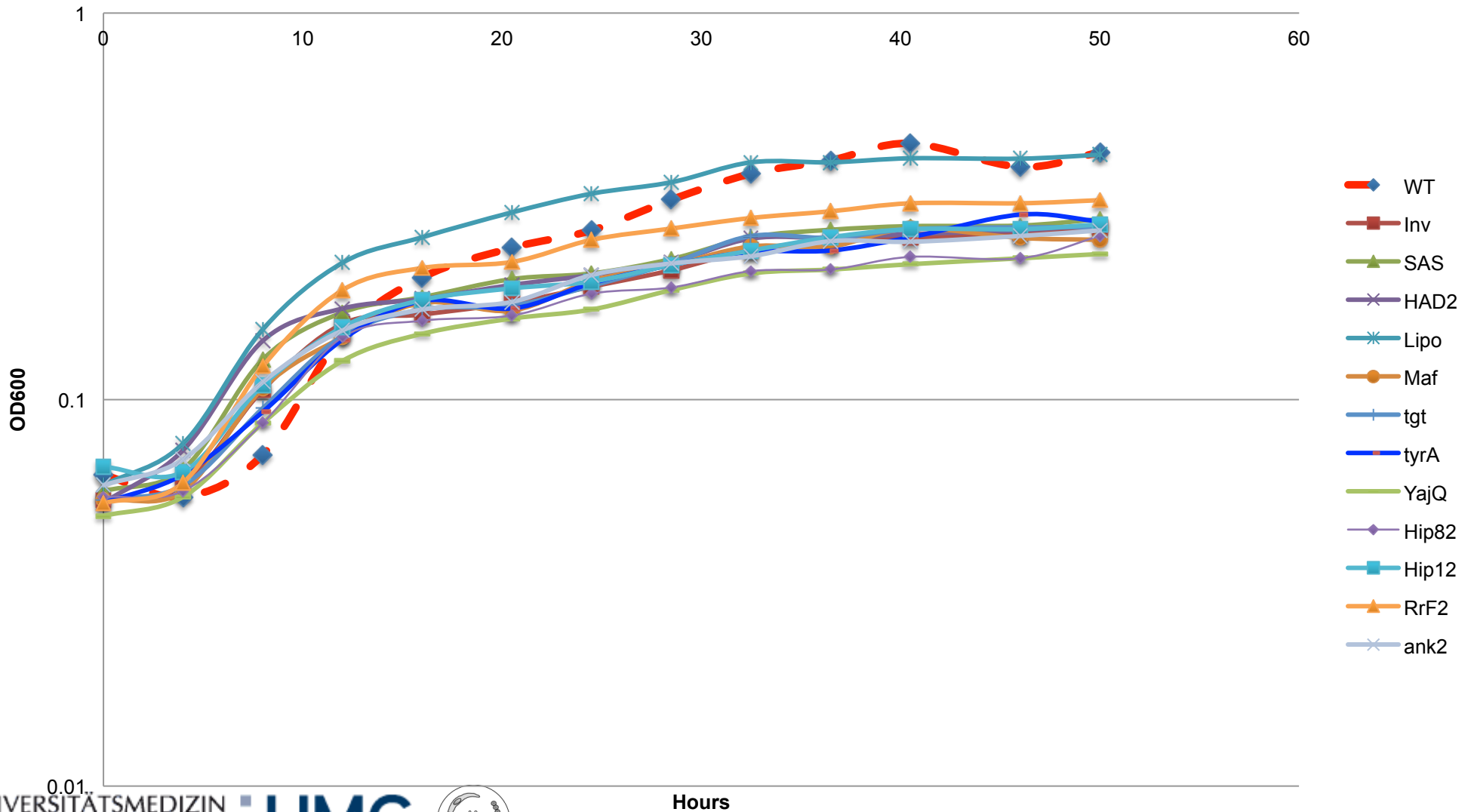
N°	Name	Uniprot	Bile acids Up-regulated	Function
1	Inv (IT-6)	A0A0H3P9Z9	GCA	Invasion phenotype protein
2	SAS (S2)	A0A0H3PA18	GCA	Sodium:alanine symporter
3	HAD2 (1)	A0A0H3PI47	CA, TCA, UDOC	HAD-superfamily hydrolase, subfamily IA, variant 1 family protein
4	Lipo (L1)	A0A0H3PCP8	CA, TCA	Lipoprotein, putative
5	Maf (1)	A1VYL9	CA, DOC, TCA, CDOC, GCA	Maf-like protein CJJ81176_0535
6	tgt (5)	A1VZZ8	LCA	Queuine tRNA-ribosyltransferase
7	tyrA (8)	A0A0H3PAH1	CDOC, GCA	Prephenate dehydrogenase
8	YajQ (Y1)	A1VY95	DOC, CDOC	UPF0234 protein CJJ81176_0398/YajQ family cyclic di-GMP-binding protein
9	Hip82 (1)	A0A0H3PBG0	DOC, CDOC	Hypothetical protein CJJ81176_1382
10	Hip12 (1)	A0A0H3P9A5	DOC	Hypothetical protein CJJ81176_0112
11	RrF2 (1)	A0A0H3PDG2	CA, DOC, LCA, UDOC, GCA (all neg)	RrF2 family protein, putative

→ Drei Effektorproteine für das *C. jejuni/C. coli* Typ-VI-Sekretionssystem

Growth curve

From Liquid medium, with Gaspacks
(opening the flask every time point)

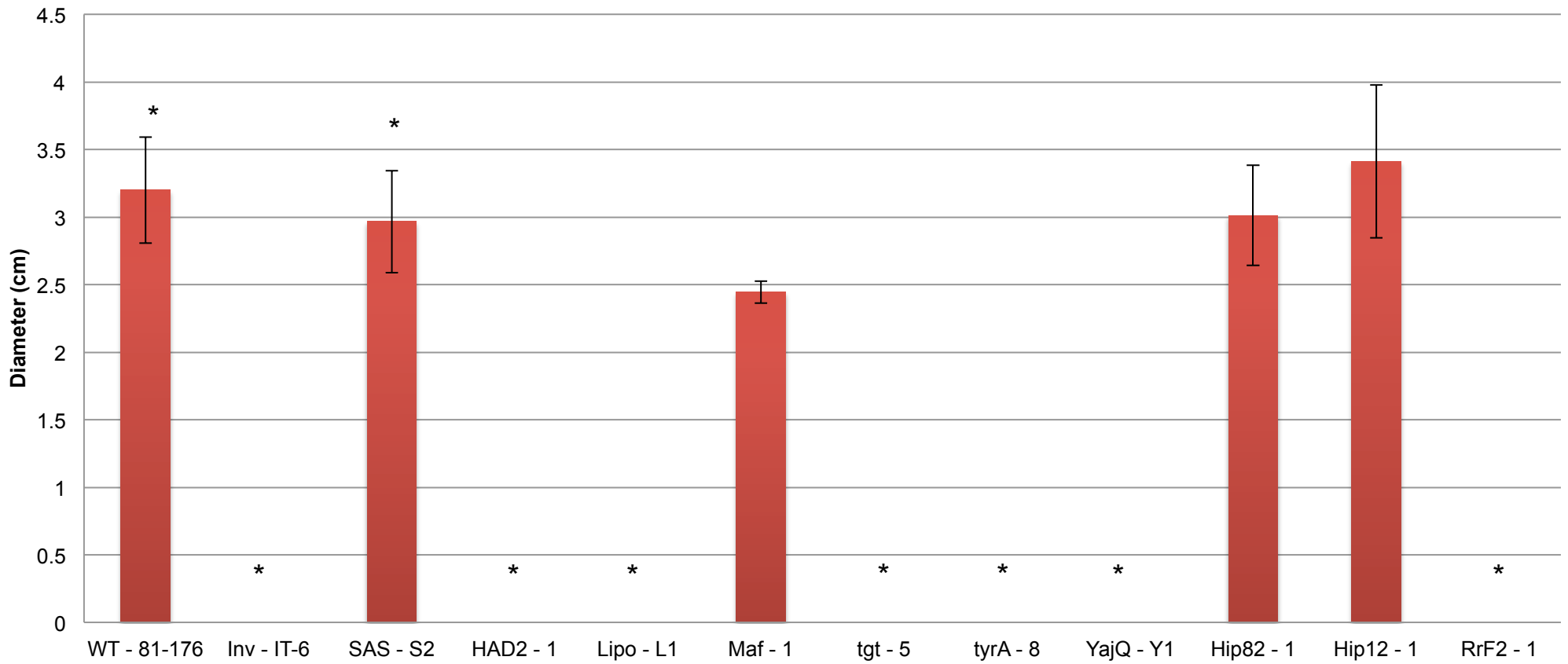
Growth curve (13.09.17)



Motility

OD600 = 0,025
Diameter (cm) after 48hs in 0,4% M.H agar plate

Motility (N=3)



Danksagung

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Christof Lenz (MPI)

Lisa Neuenroth (MPI)

Jan Schrader (MPI)

Uwe Groß (UMG)

Raimond Lugert (UMG)

