International Journal of Medical Microbiology 309 (2019) 151341

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Screen for fitness and virulence factors of *Francisella* sp. strain W12-1067 using amoebae

Kristin Köppen[a], Fan Chen[b], Kerstin Rydzewski[a], Rosa Einenkel[a], Thea Böttcher[a], Clara Morguet[b], Roland Grunow[c], Wolfgang Eisenreich[b], Klaus Heuner[a,*]

[a] Working Group: Cellular Interactions of Bacterial Pathogens, Centre for Biological Threats and Special Pathogens, Highly Pathogenic Microorganisms (ZBS 2), Robert Koch Institute, Berlin, Germany
[b] Department of Chemistry, Chair of Biochemistry, Technische Universität München, 85748 Garching, Germany
[c] Centre for Biological Threats and Special Pathogens, Highly Pathogenic Microorganisms (ZBS 2), Robert Koch Institute, Berlin, Germany

ARTICLE INFO

Keywords:
Francisella sp. W12-1067
Scatter screen
Acanthamoeba
Persistence
Virulence
Allofrancisella

ABSTRACT

Francisella tularensis is the causative agent of the human disease referred to as tularemia. Other *Francisella* species are known but less is understood about their virulence factors. The role of environmental amoebae in the life-cycle of *Francisella* is still under discussion. *Francisella* sp. strain W12-1067 (F-W12) is an environmental *Francisella* isolate recently identified in Germany which is negative for the *Francisella* pathogenicity island, but exhibits a putative alternative type VI secretion system. Putative virulence factors have been identified *in silico* in the genome of F-W12. In this work, we established a "scatter screen", used earlier for pathogenic *Legionella*, to verify experimentally and identify candidate fitness factors using a transposon mutant bank of F-W12 and *Acanthamoeba lenticulata* as host organism. In these experiments, we identified 79 scatter clones (amoeba sensitive), which were further analyzed by an infection assay identifying 9 known virulence factors, but also candidate fitness factors of F-W12 not yet described as fitness factors in *Francisella*. The majority of the identified genes encoded proteins involved in the synthesis or maintenance of the cell envelope (LPS, outer membrane, capsule) or in the metabolism (glycolysis, gluconeogenesis, pentose phosphate pathway). Further ^{13}C-flux analysis of the Tn5 glucokinase mutant strain revealed that the identified gene indeed encodes the sole active glucokinase in F-W12. In conclusion, candidate fitness factors of the new *Francisella* species F-W12 were identified using the scatter screen method which might also be usable for other *Francisella* species.

1. Introduction

Francisella tularensis is a Gram-negative zoonotic bacterium which is able to cause tularemia in a wide range of animals and in humans (Ellis et al., 2002; Sjostedt, 2011). The clinically most relevant subspecies is *F. tularensis* ssp. *tularensis* which is exclusively found in North America and the less virulent subspecies *holarctica* which is distributed over the whole northern hemisphere (Ellis et al., 2002). In Germany, *F. tularensis* ssp. *holarctica* is the only *Francisella* species known to cause tularemia in animals and humans (Faber et al., 2018). We recently identified a new *Francisella* species (*Francisella* sp. strain W12-1067 [F-W12]) in Germany whose genome DNA sequence is 89% identical to the respective nucleotide sequence of the recently published strain *F. guangzhouensis*, now renamed *Allofrancisella* (Qu et al., 2013, 2016; Rydzewski et al., 2014). However, the identification of new *Francisella* species and further research will reveal whether the proposal for a new genus can be confirmed (Challacombe et al., 2017; Vallesi et al., 2018). While F-W12 is not identical to *F. guangzhouensis*, both strains were isolated from the water reservoir of cooling towers (Qu et al., 2016; Rydzewski et al., 2014). F-W12 is able to persist in a mouse-derived macrophage-like cell line, but its virulence for mice or humans is not yet known. The draft genome was annotated, and *in silico* analysis identified the presence of various virulence genes common to the genus *Francisella*, but the *Francisella* pathogenicity island (FPI) was absent. However, another putative alternative type-VI secretion system (aT6SS) was identified within the genome of strain F-W12 (Rydzewski et al., 2014).

The metabolic capability of *Francisella* is crucial for the ability to replicate in a host cell and, as expected, there are differences in the metabolic usages of specific substrates in various *Francisella* species (Barel et al., 2012, 2015; Brissac et al., 2015; Chen et al., 2017; Meibom

* Corresponding author at: Cellular Interactions of Bacterial Pathogens, Highly Pathogenic Microorganisms (ZBS 2), Centre for Biological Threats and Special Pathogens, Robert Koch Institute, Seestraße 10, 13353 Berlin, Germany.
E-mail address: HeunerK@rki.de (K. Heuner).

https://doi.org/10.1016/j.ijmm.2019.151341
Received 29 April 2019; Received in revised form 17 July 2019; Accepted 18 August 2019

and Charbit, 2010). More specifically, amino acids as well as glucose and glycerol are metabolized at specific rates by *F. tularensis* ssp. *holarctica* (*Fth*) and *F. novicida* (*Fno*) (Alkhuder et al., 2009; Barel et al., 2015; Brissac et al., 2015; Brown et al., 2014; Chen et al., 2017; Gesbert et al., 2014, 2015; Raghunathan et al., 2010; Ramond et al., 2015). Using ^{13}C-labeled substrates, it has recently been demonstrated that glucose is a major carbon source metabolized mainly through the EMP pathway and that glycerol is mainly used by *Fno* but, at lower rates, also by *Fth* as a gluconeogenetic substrate (Brissac et al., 2015; Chen et al., 2017; Huber et al., 2010). Experiments using serine as a substrate indicated that there may be a bipartite metabolism in *Fth*, as described earlier for *Legionella pneumophila* and other pathogens (Eisenreich et al., 2013; Eisenreich and Heuner, 2016; Gillmaier et al., 2016).

The environmental reservoir for *Francisella* spp. has not yet been identified. The persistence of *Francisella* within aquatic habitats has been shown (Buse et al., 2017). In addition, Santic et al. and Abd et al. reported the replication of *Francisella* strains within amoeba (*Acanthamoeba*, *Vermamoeba* [*Hartmanella*]) (Abd et al., 2003; El-Etr et al., 2009; Santic et al., 2011; Verhoeven et al., 2010), but persistence for a long time or intracellular replication, as known for *L. pneumophila*, has not been demonstrated for *Francisella* (Abd et al., 2003; Buse et al., 2017; El-Etr et al., 2009; Verhoeven et al., 2010). However, since *Francisella* spp. is able to persist for a while in co-culture with amoeba, we established a "scatter screen" method for F-W12 in this work. This method was used earlier for *L. pneumophila* to identify virulence factors involved in intracellular replication in amoebae (Aurass et al., 2009; Brzuszkiewicz et al., 2013). Since we successfully used *Acanthamoeba lenticulata* in a scatter screen with *L. oakridgensis* and pretests with F-W12 identified *A. lenticulata* as a potential 'host cell', we used this amoeba to adapt the scatter screen to *Francisella* (Brzuszkiewicz et al., 2013). The aim of this study was to adapt the scatter screen method to *Francisella* and to use it for further characterization of the new F-W12 species, especially to identify new candidate fitness factors. Using this methodology, we identified known as well as new putative fitness factors of F-W12. It can be assumed that this screening method is an effective and useful tool also for other *Francisella* spp..

2. Materials and methods

2.1. Strains, media and growth conditions

Strains used in this study were the environmental *Francisella* sp. isolate F-W12 (Rydzewski et al., 2014) found recently and Tn5 mutant strains of F-W12. *Francisella* strains were cultivated in medium T (MT) (Becker et al., 2016; Pavlovich and Mishan'kin, 1987) (1% brain heart infusion broth [Difco Laboratories, Inc., Sparks, MD, USA], 1% bacto tryptone [Difco], 1% technical casamino acids [Difco], 0.005 g of $MgSO_4$, 0.01% $FeSO_4$, 0.12% sodium citrate, 0.02% KCl, 0.04% K_2HPO_4, 0.06% L-cysteine and 1.5% glucose), on MT-based agar plates (Tlapak et al., 2018) or on ACES-buffered charcoal–yeast extract (BCYE) agar ([1% N-(2-acetamido)-2-aminoethanesulfonic acid (ACES), 1% yeast extract, 0.04% L-cysteine and 0.025% ferric pyrophosphate, adjusted to pH 6.8 with 3 M potassium hydroxide (KOH) and sterile filtrated], (Edelstein, 1981). Kanamycin was used at a concentration of 12 μg ml^{-1} for *Francisella*. All experiments with F-W12 were performed under BSL2 condition.

Analysis of growth in MT and MT without glucose (MT-Glc^{-}) was performed at 37 °C and 5% CO_2 in a volume of 25 ml at 220 rpm for up to 24 h and OD600 was measured using a photometer (Genesys 10 Bio, Thermo Scientific).

Acanthamoeba lenticulata strains 45 (ATCC 50703) and 118 (ATCC 50706) were cultivated in Peptone Yeast Glucose broth (PYG; for 1 L; 20 g of Proteose Pepton [Oxoid], 1 g of Yeast Extract [Oxoid], 1 g of sodium citrate × 2 H_2O, 10 ml of 0.4 M $MgSO_4$ × 7 H_2O, 10 ml of 0.25 M Na_2HPO_4 × 7 H_2O, 10 ml of 0.25 M KH_2PO_4, 8 ml of 0.05 M $CaCl_2$ × 2 H_2O, 10 ml of 0.005 M $Fe(NH_4)_2(SO_4)_2$ × 6 H_2O, 50 ml of 2 M glucose) at room temperature. The infection/persistence assays were performed in an infection buffer (IB; for 1 L: 1 g of sodium citrate × 2 H_2O, 10 ml of 0.4 M $MgSO_4$ × 7 H_2O, 10 ml of 0.25 M Na_2HPO_4 × 7 H_2O, 10 ml of 0.25 M KH_2PO_4, 8 ml of 0.05 M $CaCl_2$ × 2 H_2O, 10 ml of 0.005 M $Fe(NH_4)_2(SO_4)_2$ × 6 H_2O, 5 g of NaCl, 10 g of glucose).

2.2. Generation of a Francisellasp. strain F-W12 Tn5 clone bank

The EZ-Tn5^{Tm} < KAN-2 > Tnp Transposome Kit (Epicentre) was used for the generation of Tn5 mutant strains of *F*-W12. According to manufacturer's instructions, the Tn5 transposome was inserted by electroporation into F-W12. For the generation of electrocompetent F-W12 cells, bacteria were grown overnight, pelleted (4.500 g for 15 min) and washed twice in 0.5 M sucrose. Then, 1 μl of the EZ-Tn5 < KAN-2 > Tnp Transposome was added to electrocompetent cells. The mixture was transferred into a 0.2 cm gap cuvette and electroporation was performed using standard settings (2.5 kV, 25 μF, 600 Ω). Afterwards, cells were incubated in the absence of selection pressure in MT without antibiotics for 3 to 4 h (at 37 °C and 350 rpm). Then, cells were plated onto agar plates containing kanamycin and incubated at 30/37 °C and 5% CO_2 until bacterial colonies were visible on agar plates (3 days). Colonies were washed off by adding 2–3 ml of PBS per plate and colonies from distinct agar plates were mixed. Glycerin stocks (20%) were prepared from mixed bacterial suspension and stored at −80 °C.

2.3. Screen for co-infection defects of a Francisellasp. strain W12-1067 Tn5 clone bank (scatter screen)

The scatter screen was performed with small deviations as described earlier for *L. pneumophila* by Aurass et al. in 2009 (Aurass et al., 2009). Briefly, a cryostock of F-W12 Tn5 clone bank was cultivated on agar plates supplemented with kanamycin. Bacteria grown overnight (F-W12 WT and Tn5) were adjusted to 10^4 bacteria/ml in IB and mixed with 1 × 10^6 amoebae/ml which were grown in PYG at RT for 3–4 days. This mixture was incubated at 37 °C and 5% CO_2 for 30 min and plated onto agar plates and incubated at 37 °C and 5% CO_2 until bacterial colonies appeared (2–3 days). Then, plates were incubated at different temperatures: RT (20–22 °C), 25, 30 and 37 °C. Colonies were monitored for the development of the so-called scatter phenotype (Aurass et al., 2009). Here, colonies seem to disappear and scatter; the first scatter clones were collected after 10 days. Scatter clones were isolated for further analysis until the wild-type F-W12 began to scatter.

2.4. Infection (Persistence) assay using A. lenticulatastrains 45 and 118

A. lenticulata 45 and 118 were mechanically detached from the flask bottom, transferred into 50-ml tubes and centrifuged at 800 g for 10 min. After cell counting in a Neubauer counting chamber, amoebae were adjusted to 5 × 10^5 cells/ml in IB, transferred into wells of a 24-well plate (1 ml/well) and incubated for 2 h at 25 °C. Bacteria grown overnight were adjusted to OD600 = 1 (corresponding to ˜10^9 CFU/ml), diluted in IB, and the infection was done with a multiplicity of infection (MOI) of 10 or 100 for 2 h at 25 °C. After removing supernatant and washing, cells were incubated with 50 μg/ml of gentamycin for 1 h to kill extracellular bacteria. Then, cells were washed again and covered with 1 ml of IB and incubated for up to 7 days at 25 °C. For colony-forming unit (CFU) determination at various time points of infection (3 h, 3 days, 7 days), cells were harvested by mechanical disruption of amoebae and plated on agar (Whitley Spiral plater, Dw Scientific Ltd., Bingley, UK). Grown bacterial colonies were counted with a colony counter (aCOLyte, Synbiosis, Cambridge, UK) and calculated as bacteria per ml. To evaluate the persistence/survival of scatter clones in comparison to the wild type, the survival rate was determined. Therefore, cell count changes from day 0 to day 7 of a scatter clone were calculated in proportion to cell count changes of the wild type during the assay.

2.5. Determining the Tn5 integration site at the genome of Francisellasp. strain F-W12

The Tn5 insertion into the genome of Tn5 scatter clones was identified using inverse PCR (TopTaq DNA Polymerase, Qiagen, Hilden, Germany). To this end, chromosomal bacterial DNA was digested with HindIII (New England, Biolabs, Frankfurt a.M., Germany), circulated and used in an inverse PCR amplifying the sequences flanking the Tn5 insertion site by using the primer pair KAN-2 FP-1 (ACCTACAACAAA GCTCTCATCAACC) and KAN-2 RP-1 (GCAATGTAACATCAGAGATTTT GAG) (EZ-Tn5™ < KAN-2 > Tnp Transposome™ Kit, Epicentre). Obtained PCR products were extracted and used for Sanger sequencing. Sequences were analyzed using Geneious 11.1.5.

2.6. Glucose metabolism of scatter clone #50 analyzed by ^{13}C-analysis

Labeling of *Francisella* F-W12 and scatter clone #50 was done using 250 ml of growth medium (MT) supplemented with 0.5 g of [U-$^{13}C_6$] glucose (11 mM), 37 °C and 220 rpm until stationary growth (approx. 26 h). For further details of the workup of *Francisella* cells including extraction of carbohydrates and amino acids, see Chen et al. (Chen et al., 2017).

GC–MS and ^{13}C-isotopolog analyses were done using a GCMS-QP 2010 Plus spectrometer (*Shimadzu*, Duisburg, Germany) as described earlier (Hauslein et al., 2016). All data were collected using LabSolution software (*Shimadzu*). The samples were analyzed three times as technical replicates. The overall ^{13}C-excess (mol-%) and the relative contributions of isotopomers (%) were computed by an Excel-based in-house software package (Eylert et al., 2008) according to Lee et al. (Lee et al., 1991). For further details see Chen et al. (Chen et al., 2017).

3. Results

3.1. Establishment of the scatter screen and screening for non-persisting/discontinuous clones of the F-W12 Tn5 clone bank

For a first test of the scatter screen (negative control), we used *A. lenticulata* strain 45 and 118 as host species and *Francisella* sp. strain F-W12 wild-type to identify time-dependent scattering at room temperature (RT, 20–22 °C), 25 °C and 37 °C for 25 days (data not shown). RT and 25 °C were chosen as screening temperatures for further experiments, and amoeba-sensitive clones were picked up to 25 days until no amoeba-sensitive wild-type clone was detectable any longer (Fig. 1A). In this assay, colonies of bacteria are “attacked” by the mobile amoebae and, if the bacteria are not able to resist phagocytosis or degradation within the phagosome, the bacterial colony is eaten by the amoebae, generating the so-called scatter phenotype (Aurass et al., 2009). The morphology of amoeba-sensitive (scattered) clones is shown exemplarily in Fig. 1B. Using this protocol, the screening was performed with a Tn5 clone bank of F-W12 (see Materials and methods). F-W12 exhibits ˜1541 protein encoding genes. In total, approximately 7000 clones of the Tn5 clone bank were investigated in the scatter screen. 79 amoeba-sensitive clones (RT: 50 clones; 25 °C: 29 clones; Table 1, columns 1–3) were sampled from 10 to 25 days of co-incubation.

3.2. Infection (Persistence) assay and growth in medium T (MT)

All 79 amoeba-sensitive *Francisella* clones obtained were subsequently tested for reduced fitness or virulence by using an infection assay. At first, we checked the influence of time (up to 10 days) and MOI (10 and 100) on the persistence of F-W12, using five different scatter clones indicating that an incubation time of 7 days and an MOI of 10 is useful for further analysis (supplemental Fig. S1). From the 79 clones tested, 21 showed a reduced persistence in comparison to the wild-type strain in the infection assay, indicated by a survival rate

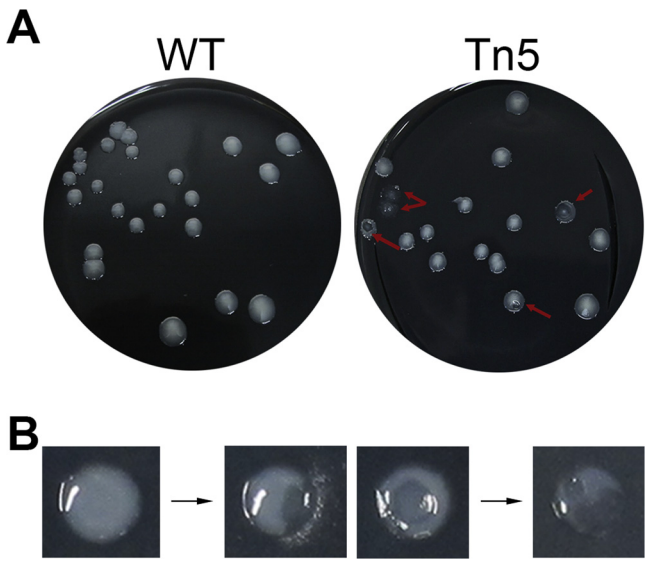

Fig. 1. Habitus of amoebae-sensitive colonies (scatter phenotype) of F-W12 Tn5 clones. Bacteria grown overnight (10^4/ml) were mixed with amoebae (10^6/ml), cultivated and plated onto agar plates which were initially incubated at 37 °C until bacterial colonies appeared and then transferred to RT or 25 °C for further incubation (for more details see Materials and Methods section). A. Overview of scatter agarose plates containing F-W12 wild-type bacteria (WT, left side) or bacteria of the Tn5 mutant bank (Tn5, right side). Colonies showing the scatter phenotype are indicated by red arrows. B. Development of the scatter phenotype. From left to right: intact/untouched colony at the beginning (day 3), beginning of colony’s digestion by amoeba (either from edge or from the middle, as from day 10) and (almost) completely digested colony.

below 0.5 (Table 1, column 4). All clones showing reduced or increased persistence were analyzed for growth in MT. Growth of most of the scatter clones was similar in comparison to growth of the wild-type strain (Table 1, column 5 and Fig. 2A). Only clones #2, #7 and #50 showed a reduced growth in MT (Fig. 2A). In the infection assay, 10 clones (#2, #7, #14, #16, #17, #19, #20, #37, #39 and #49) exhibited a highly reduced persistence (survival rate < 0.1) (Fig. 2B-D), whereas 11 clones (#4, #6, #21, #28, #30, #32, #40, #46, #51, #52 and #65) displayed a moderately reduced persistence (survival rate 0.1–0.5) (Table 1, column 4 and Fig. 2B-D). Further 22 clones showed a moderately but inconsistently reduced persistence (Tab. S1). Two clones (#33, #50) seemed to have an increased persistence (survival rate > 1.5) in the infection assay (Table 1, column 4 and Fig. 2B and D). All data of the 79 scatter clones identified are given in supplemental Table S1.

3.3. Determination of the Tn5 integration site at the genome of F-W12 mutant strains

All clones with a confirmed reduced persistence phenotype in *A. lenticulata* (see Table 1) were selected for the determination of the Tn5 transposon insertion site (intergenic region or gene) in the genome sequence of F-W12. Therefore DNA sequencing was used as described in the Materials and Methods section. Identified protein-encoding genes (peg) and respective DNA clusters are also given in Table 1 (columns 6–8) and Fig. 3. The putative function of the identified genes was determined by their % of identity (on amino acid level) to known proteins; % identity to proteins of *Fno* are given in Table 1. The majority of the identified genes exhibiting the Tn5 element encode genes involved in LPS, cell wall or outer membrane synthesis (cell envelope) or in the metabolism of *Francisella;* some are known virulence genes of *Francisella* (Table 1, column 7, marked with +; and Fig. 3), providing evidence of the validity of the method.

The Tn5 element in scatter clone #2 (Fig. 2A and B) is integrated in

Table 1
Selected amoeba-sensitive Tn5 clones of *Francisella* sp. strain W12-1067.

Sc#	*A. lenticulata*	temp.	survival rate[1]	growth (max. OD_{600})[2]	Tn5 insertion site peg.# [3]	gene; function	homolog in *Fno*	% identity to *Fno* (aa)	
2	118	RT	0.07 ± 0.07	1.315	631	*degT/wbtQ* [ı] ; LPS biosynthesis	FTN_1430	90.03%	strongly reduced
7	118	RT	0.01 ± 0.02	1.392	1522	*ftsH* [ı] ; starvation survival	FTN_0668	88.68%	
14	118	RT	0.08 ± 0.08	1.827	413	*galU* [ı] ; LPS and capsule biosynthesis	FTN_0729	87.46%	
16	118	RT	0.05 ± 0.04	1.812	413	*galU* [ı] ; LPS and capsule biosynthesis	FTN_0729	87.46%	
17	118	RT	0.06 ± 0.1	1.856	1387	*mlaA/vacJ* [ı] ; OM asymmetry	FTN_0322	65.52%	
19	118	RT	0 ± 0	1.793	1447	*gtrB* ; cell wall biosynthesis	FTN_1403	82.26%	
20	118	RT	0 ± 0	1.775	1387	*mlaA/vacJ* [ı] ; OM asymmetry	FTN_0322	65.52%	
37	118	RT	0 ± 0	1.834	266	*lptE* ; LPS assembly	(FTL_1211)	55.90%	
39	45	25 °C	0.05 ± 0.06	1.743	249	*talA* ; pentose-phosphate pathway	FTN_0781	73.21%	
49	118	RT	0.09 ± 0.06	1.847	1272	*spoT/relA* [ı] ; starvation survival	FTN_1518	81.49%	
4	118	RT	0.49 ± 0.06	1.788	1424	Enoyl-CoA hydratase ; FA metabolism	FTN_1438	77.72%	reduced
21	118	RT	0.12 ± 0.14	1.791	1224	*mlaD* [ı] ; OM asymmetry	FTN_0326	77.27%	
28	118	RT	0.39 ± 0.24	1.865	1387	*mlaA/vacJ* [ı] ; OM asymmetry	FTN_0322	65.52%	
30	118	RT	0.32 ± 0.23	1.822	1096	*htrB* ; lipid A biosynthesis	FTN_0071	80.78%	
32	118	RT	0.19 ± 0.32	1.804	1387	*mlaA/vacJ* [ı] ; OM asymmetry	FTN_0322	65.52%	
46	45	25 °C	0.11 ± 0.16	2.016	266	*lptE* ; LPS assymmetry	(FTL_1211)	55.90%	
51	45	25 °C	0.21 ± 0.1	1.963	816	chitinase [ı]; carbohydrate metabolism	(Fphi_0512)	69.26%	
52	45	25 °C	0.17 ± 0.03	2.032	102	Ornithine cyclodeaminase	FTN_1444	85.80%	
62	118	RT	0.19 ± 0.18	1.743	792	*glpD* [ı] ; glycerol metabolism	FTN_1584	85.46%	
65	45	25 °C	0.14 ± 0.1	1.802	1387	*mlaA/vacJ* [ı] ; OM asymmetry	FTN_0322	65.52%	
33	118	RT	3.77 ± 2.71	1.705	228	*putA* ; proline metabolism	FTN_1131	84.06%	slightly increased
50	118	RT	1.62 ± 0.56	1.131	721	*glk* ; glucose metabolism	FTN_0462	86.39%	
6	118	RT	0.53 ± 0.43	1.581	828	alpha/beta hydrolase	FTN_0297	70.75%	inconsistent
22	118	RT	1.28 ± 0.91	1.881	1091	*ppdK* [ı] ; gluconeogenesis	FTN_0064	91.78%	
24	118	RT	0.95 ± 0.94	1.938	1091	*ppdK* [ı] ; gluconeogenesis	FTN_0064	91.78%	

[1]ratio of CFU/ml during infection (from day 0 to day 7) of a scatter clone in proportion to the CFU/ml ratio of F-W12 during infection.
[2]max. OD_{600} of F-W12: 1.933.
[3]Reference (Rydzewski et al., 2014).
[ı] known virulence factors.
(aa), on amino acid level; FA, fatty acid; Fphi, *F. philomiragia*; *Fno*, *F. novicida*; OM, outer membrane; *gtrB*, glycosyl transferase family protein; *talA*, transaldolase A; *ppdk*, pyruvate phosphate dikinase; *putA*, proline dehydrogenase; *wbtQ*, glycosyl transferase. Further abbreviations, see text.

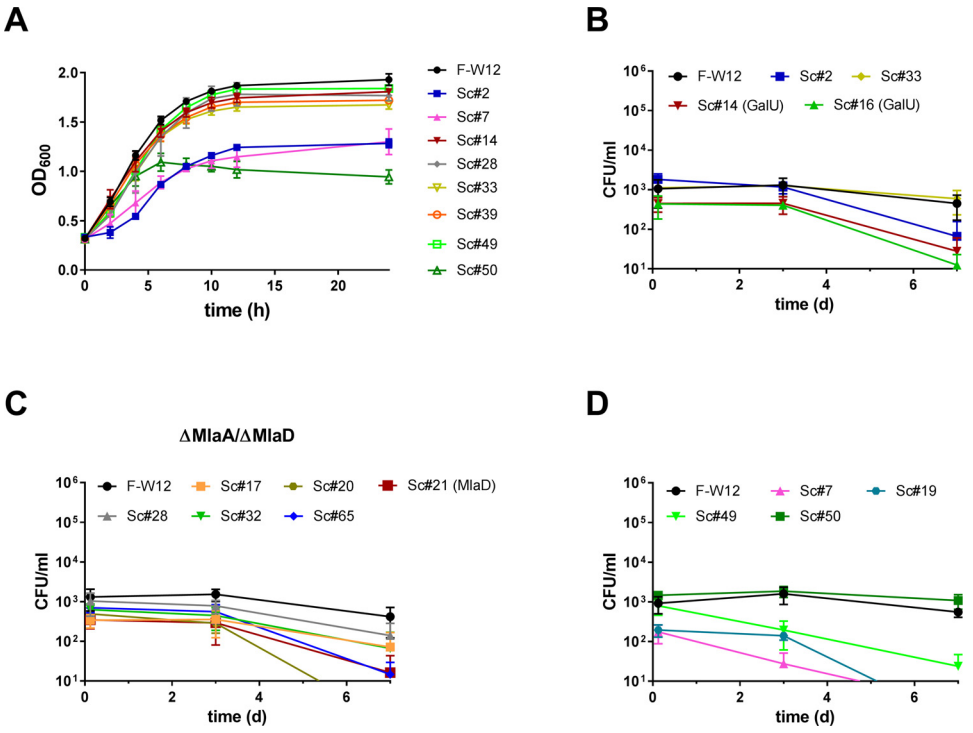

Fig. 2. Growth of Tn5 Sc clones in MT (A) and *A. lenticulata* 118 (B–D). A. Bacteria grown overnight were adjusted to OD_{600} = 0.3 and cultivated for up to 24 h hours at 37 °C and 220 rpm. OD_{600} was measured at indicated time points. Results are mean standard deviations of two independent experiments. **B-D**. Infection/persistence assay of F-W12 and F-W12 Tn5 Sc clones using *A. lenticulata* 118. Amoebae were infected with bacteria at an MOI of 10 for 2 h, followed by 1 h of gentamicin treatment and incubation for up to 7 days at 25 °C. CFU was determined at 3 h, 3 days and 7 days post infection by plating onto agar plates. Results are mean standard deviations of three independent experiments.

a homolog of *degT* (WbtQ, peg0631), encoding a 4-keto-6-deoxy-N-acetyl-D-hexosaminyl-(lipid carrier) amino-transferase putatively involved in the biosynthesis of LPS.

Tn5 in scatter clone #7 is integrated within peg1522, encoding a putative metalloprotease (FtsH) which is involved in starvation survival. FtsH is a cytoplasmic membrane protein, containing an ATPase and a Zn^{2+}-metalloprotease domain involved in quality maintenance of membrane proteins (Akiyama et al., 1994a, b; Ito and Akiyama, 2005).

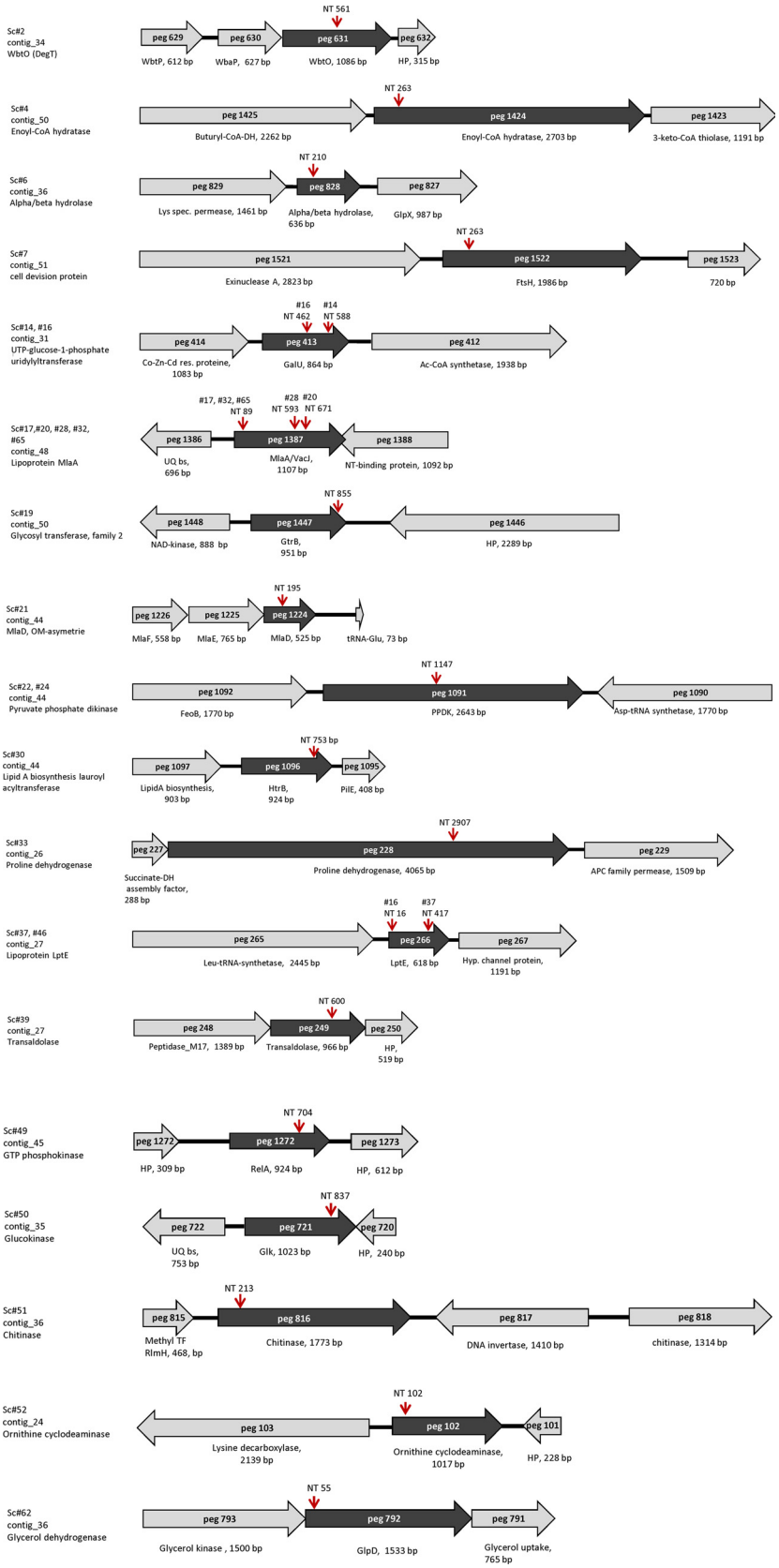

Fig. 3. Chromosomal location of Tn5 insertions within 23 F-W12 Tn5 Sc mutant strains. Tn5 transposon insertion sites (indicated by red arrows) and corresponding nucleotide position (NT) within the gene (black arrows) were identified by inverse PCR and sequencing using Tn5 primes flanking the Tn5 transposon. Genes located up- and downstream are indicated by grey arrows. The peg numbers of shown genes are given within the arrows and gene names and length (in bp) are shown below the arrows. bs, biosynthesis; DH, dehydrogenase; HP, hypothetical protein; res., resistance; TF, transferase; UQ, ubiquinone.

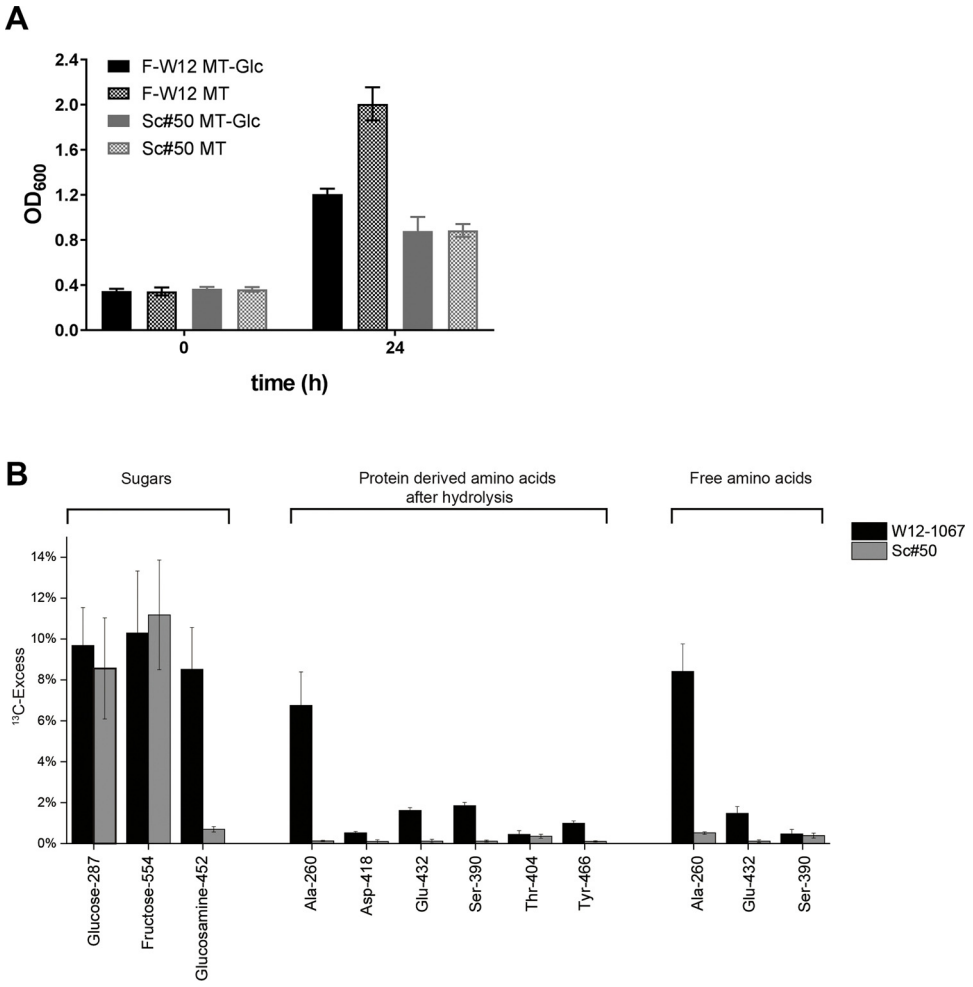

Fig. 4. Analysis of the Sc#50 (*glk*) mutant strain of F-W12. A. Growth of the wild-type and Sc#50 mutant strain in MT and in MT-Glc$^-$ at 37 °C after 24 h. B. ^{13}C-Excess (mol%) values in selected metabolites of wild type (WT) and Sc#50 grown in MT supplied with 11 mM [U-$^{13}C_6$]glucose.

The F-*W12 ftsH* mutant showed a highly reduced fitness in the infection assay (Fig. 2D) and a reduced cell density in the stationary phase of *in vitro* growth (Fig. 2A).

In scatter clone #49, the Tn5 element is integrated the *relA* gene (peg1272), encoding a putative ppGpp synthase I (GTP pyrophosphokinase, EC.2.7.6.5). The *relA* mutant was highly attenuated in the infection assay (Fig. 2D) but grew as well as the wild-type strain in MT (Fig. 2A). In general, this enzyme is involved in the stringent response and, like FtsH, RelA is also involved in starvation survival (during nutrient starvation) of bacteria (Jain et al., 2006; Lithgow et al., 2004).

In the scatter clones #14 and #16, the Tn5 Km^R gene is integrated within peg0413 at two different positions within the gene (see Fig. 3). The gene encodes a putative UTP-glucose-1-phosphate-uridyltransferase (GalU), generally involved in the biosynthesis of carbohydrates of the envelope and also in LPS and capsule synthesis (Bonofiglio et al., 2005; Chang et al., 1996; Mollerach et al., 1998). Both mutant strains showed a highly reduced persistence in the infection assay (Fig. 2B), but they grew well in MT (Fig. 2A). Further genes identified in this assay encoding proteins putatively involved in LPS, envelope or cell wall biosynthesis were: (i) peg1447 (clone #19) encoding a putative glycosyl-transferase-GTA-type (poly glucosamine synthase) required for cell wall biosynthesis, (ii) peg1096 (clone #30) encoding a putative homolog of *htrB* required for lipidA biosynthesis and (iii) peg0266 (clone #37) encoding a putative LptE enzyme generally required for the lipopolysaccharide transport machinery (Lpt), necessary for envelope biosynthesis, assembly and maintenance of lipid asymmetry in the outer membrane (Li et al., 2012). This indicates that the capsule, the envelope and LPS indeed play an important role during the interaction of F-W12 with amoebae.

Interestingly, Peg1387 was identified five times (#17, #20, #28, #32, #65) and at three different insertion sites for Tn5-Kana (see Fig. 3). This gene encodes a putative lipoprotein (also called MlaA/VacJ), exhibiting a signal sequence and a Pfam_MlaA motif. MlaA proteins are involved in the maintenance of the outer membrane asymmetry (Carpenter et al., 2014; Chong et al., 2015; Malinverni and Silhavy, 2009; Suzuki et al., 1994). The peg1387 mutants showed a reduced fitness in the infection assay (Fig. 2C) but a growth in MT comparable to the wild type (Fig. 2A). Like the RelA mutant, the MlaA mutant strains showed a reduced persistence in amoebae (Fig. 2C). In addition, clone #21 encodes a homolog of MlaD and is localized in an operon together with MlaE and MlaF (Fig. 3). MlaD is also involved in the maintenance of the outer membrane asymmetry and is functional in cell wall and envelope biosynthesis (Chong et al., 2015; Malinverni and Silhavy, 2009).

Another part of genes inactivated in the scatter mutant strains encode proteins involved in the metabolism of F-W12. Scatter clone #4 (peg1425) encodes a putative butyryl-CoA-dehydrogenase (fatty acid degradation), #6 (peg0828) a putative alpha/beta hydrolase, #22 and #24 (peg1091) a pyruvate phosphate dikinase (PPDK, gluconeogenesis), #33 (peg0228) a putative proline dehydrogenase, #39 (peg0249) a putative transaldolase (pentose phosphate pathway), #51 (peg0816) a putative chitinase, #52 (peg0102) anputative ornithine cyclodeaminase, #56 (peg0216) a putative fatty acid hydroxylase and #62 (peg0792) a putative glycerol-3 phosphate dehydrogenase (GlpD,

glycerol metabolism). Identified scatter clones #51 (chitinase) and #62 (GlpD) encode enzymes that are already known to be virulence factors in *Francisella* (Asare and Abu Kwaik, 2010; Asare et al., 2010). Both mutants grew well in MT but showed reduced persistence in the infection assay (Table 1, column 4). Scatter clones #33 and #50 showed an increased persistence in the infection assay; however, the significance of these effects has to be confirmed by the analysis of specific gene deletion mutants. The scatter clone #50 will be presented below.

3.4. The glucokinase mutant strain (Sc#50)

Scatter clone #50 was analyzed in more detail, because it is known that glycolysis and gluconeogenesis are important for the nutrition of intracellular *Francisella* and since our group is interested in the pathometabolism of bacteria. Although the clone exhibited a reduced growth in MT (OD_{600} of 1.1), its persistence in the infection assay was slightly increased compared to the wild-type strain (Fig. 2A and D). Analysis of the Tn5 insertion site revealed that a putative glucokinase (peg0721) was inactivated in clone #50 (Fig. 3). The glucokinase catalyzes the first reaction of glycolysis, the phosphorylation of glucose resulting in glucose-6 phosphate. Capitalizing on this fact, we performed growth experiments in MT with and without glucose. While the wild-type strain showed normal growth in MT until 24 h (OD_{600} = 2.009 ± 0.15) and growth without glucose was reduced (OD_{600} = 1.206 ± 0.05) (Fig. 4A), the glucokinase mutant strain grew similarly in both media (OD_{600} = 0.885 ± 0.06 vs. OD_{600} = 0.881 ± 0.12) (Fig. 4A). This indicates that glucose is important for growth of F-W12 in MT. In contrast, the observed reduced growth of the #50 mutant strain did not depend on the presence of glucose in the growth medium. To corroborate these surprising results further, we performed growth experiments with the wild type and mutant #50 using MT containing [U-$^{13}C_6$] glucose. Labeled bacteria were then analyzed using GC–MS-based ^{13}C-analysis of key metabolites, as described in Materials and Methods. The ^{13}C-incorporation values observed for sugars and amino acids (see supplemental Tabs. S2–S4) clearly indicate that the glucokinase mutant strain is indeed highly reduced in its capacity to use glucose as a carbon substrate (Fig. 4B). Interestingly, the mutant showed a highly reduced carbon flux to glucosamine but not into fructose which was found to be highly labeled in the wild-type as well as in the glucokinase mutant strain (Fig. 4B and Tab. S4). This finding suggests that the conversion of glucose to fructose is possible, even when no glucokinase was present in the #50 mutant strain.

4. Discussion

4.1. The scatter screen

In this report, we successfully adapted the known agar plate-based scatter screen originally using *Legionella* and amoebae as host (Aurass et al., 2009; Brzuszkiewicz et al., 2013) to be used with *Francisella*. As a proof of principle, we tested the newly identified F-W12 strain and *A. lenticulata* as host in this screen. From 79 identified amoeba-sensitive clones, 21 showed also a reduced persistence in the subsequent infection assay, whereas two amoeba-sensitive clones (#33, #50) revealed a slightly increased persistence in the infection assay. In each of these clones, we determined the Tn5 integration site in the genome of F-W12. The results revealed that mainly genes were affected that encoded a function in the biosynthesis of the cell envelope structure (outer membrane, LPS, cell wall, capsule), during starvation or in the core metabolism (Table 1 and below).

Importantly, we received amoeba-sensitive F-W12 strains in which the Tn5 element was integrated into genes encoding previously known virulence factors of *Francisella* (*relA*, *galU*, *degT*, *glpD*, chitinase) or virulence factors yet only known for other bacteria (*ftsH*, *mlaA*, *mlaD*). The identified genes are indicated as "candidate" fitness and virulence factors since the Tn5 mutant strains have not been complemented during this work. However, the results demonstrate that *A. lenticulata* is appropriate as a host for the identification of putative virulence as well as fitness factors of *Francisella* sp. strain W12-1067 using the scatter screen method.

We also obtained two scatter clones (#33 and #50) which showed a slightly increased survival rate, and in contrast to #50, #33 did not exhibit reduced growth in medium. We yet did not know the reason for this phenotype. However, the generation and characterization of a specific deletion mutant and its corresponding complementation may answer this question. In addition, we also obtained various amoeba-sensitive mutant strains which seemed to behave similarly to the wild-type strain in the infection assay. Probably, the inactivated genes of these clones may play a role in the resistance against phagocytosis or intracellular degradation by the amoebae only on a solid surface (agar plate) but not in the interaction with amoeba in the liquid infection buffer. In the infection assay, an additional gentamycin treatment should kill extracellular bacteria, which is not the case for the agar-based scatter screen. Therefore, in the infection assay mainly the persistence of intracellular bacteria is monitored.

4.2. Genes inactivated in amoeba-sensitive clones exhibiting reduced persistence in the infection assay

The 'envelope'

In this study, we identified genes of F-W12 with a role in the infection assay, a general assay used to characterize virulence factors of pathogenic bacteria. The amoeba-sensitive clones showing the highest decrease in persistence (#2, #7, #14, #16, #17, #19, #20, #30, #37) code putatively for DegT (#2), HtrB (#30), Lpte (#37) and MlaA (#17, #20) involved in LPS biosynthesis or assembly, the metalloprotease FtsH (#7), the UTP-Glc-1P-uridyl transferase GalU (#14, #16) and a glycosyl transferase (#19).

In five different amoeba-sensitive clones (#17, #20, #28, #32, #65) the Tn5 transposon integrated into the homolog of a known virulence factor, VacJ of *Shigella flexneria* (Carpenter et al., 2014; Suzuki et al., 1994), at different growth temperatures and in both screens using different amoeba strains (see Table 1). The gene encodes a protein with a signal sequence and a Pfam_MlaA motif and is putatively involved in the maintenance of the outer membrane asymmetry (Chong et al., 2015; Malinverni and Silhavy, 2009). A further gene (*mlaD*) of this pathway was affected by Tn5 transposon in mutant strain #21. MlaD in *M. tuberculosis* is involved in the entry into and survival in macrophages (Arruda et al., 1993; Chitale et al., 2001; Kumar et al., 2003). The metalloprotease FtsH is generally involved in quality maintenance of membrane proteins, protein assembly into and through the cytoplasmic membrane as well as in balancing the phospholipid and LPS synthesis (Akiyama et al., 1994a,b; Ito and Akiyama, 2005; Ogura et al., 1991). FtsH is a virulence factor in *Staphylococcus aureus*, and the deletion results in reduced survival of the bacteria under amino acid and phospho-limiting conditions as well as in a reduced virulence in mice (Lithgow et al., 2004). GalU (affected in Sc#14, #16), like GalE (Weiss et al., 2007), is involved generally in the biosynthesis of carbohydrates of LPS, the envelope and the capsule (Bonofiglio et al., 2005; Chang et al., 1996; Mollerach et al., 1998). The capsule in *Francisella* is a well-known virulence factor and was identified also in a Tn5 mutant screen using *Ftt* SchuS4 and *Fth* LVS (Jayakar et al., 2011; Rowe and Huntley, 2015; Weiss et al., 2007). F-W12 mutant strain #19 encodes a further glycosyl transferase (poly-glucosamine synthesis) putatively involved in cell wall synthesis. Altogether, the clones showing the highest decrease in persistence are associated with the function of the 'envelope' of the bacterium, and the maintenance of the outer membrane asymmetry seems to be an important factor for the persistence of F-W12 in co-culture with amoebae. Genes involved in the function or biosynthesis of the cell envelope were also identified in some other published works looking for virulence genes using different hosts (cell lines, mice and flies) and different *Francisella* strains (Asare

et al., 2010; Brunton et al., 2015; Pechous et al., 2009; Ahlund et al., 2010; Akimana et al., 2010; Akimana and Kwaik, 2011; Moule et al., 2010).

Starvation. In mutant strain #49, the virulence gene (*relA*) encoding the GTP pyrophosphokinase RelA was affected by the Tn5 transposon, indicating that the stringent response is also an important factor for the bacteria–host interaction in F-W12. RelA is a well-known virulence factor in various bacteria and is involved in the regulation of the stringent response of bacteria under nutrient starvation (Dalebroux et al., 2009; Hammer and Swanson, 1999; Haralalka et al., 2003; Jain et al., 2006; Moule et al., 2010; Muller et al., 2012; Ojha et al., 2000). In *L. pneumophila*, RelA together with SpoT (ppGpp synthase II) also regulates the biphasic life-cycle and the expression of the flagellum (Appelt and Heuner, 2017; Dalebroux et al., 2010; Hammer and Swanson, 1999; Oliva et al., 2017). The *relA* gene is also a known virulence factor in *Francisella*; stress and ppGpp are involved in the expression of the genes encoding the T6SS located on the FPI (Cuthbert et al., 2017; Dean et al., 2009). The *relA* and *spoT* genes are annotated in the genome sequences of *Fno*, *Ftt* and F-W12 (Larsson et al., 2005; Rohmer et al., 2007; Rydzewski et al., 2014). In addition, the above-mentioned metalloprotease FtsH (clone #7) is also involved in the process of starvation survival.

Metabolism. For *F. tularensis* it has been shown that the metabolism of amino acids, of glycerol as well as glycolysis and gluconeogenesis are important pathways for *in vitro* and for the intracytosolic growth (Brissac et al., 2015; Chen et al., 2017; Ramond et al., 2015; Ziveri et al., 2017a, b). Therefore, it is not surprising that we also identified some amoeba-sensitive mutant strains (#4, #6, #22, #24,#33,#39, #50, #51, #52, #56, #62) harboring genes, containing an integrated Tn5 element, involved in the metabolism of F-W12. The amoeba-sensitive strain #4 (putative butyryl-CoA dehydrogenase), #6 (putative alpha/beta hydrolase with unknown function) and #56 (putative fatty acid alpha hydroxylase/peroxygenase) encode proteins with putative functions in the lipid metabolism of *Francisella*, but the effect in the infection assays was rather low. However, mutant strains #51 (chitinase), #22 and #24 (PPDK), #39 (transaldolase, TA) and #62 (GlpD) as well as #33 (proline dehydrogenase) and #52 (ornithine cyclodeaminase) encode putative proteins involved in the metabolism of chitin, carbohydrates, glycerol or amino acids. Chitinase activity has been identified in the supernatant of F-W12 grown in MT (Rydzewski et al., 2014), chitinases have been detected as essential virulence factors in *Fno* and they are involved in biofilm formation (Asare et al., 2010; Margolis et al., 2010; Weiss et al., 2007). PPDK-, TA- and GlpD-encoding genes have been identified earlier as virulence factors in other *Francisella* strains (Asare and Abu Kwaik, 2010; Brissac et al., 2015; Margolis et al., 2010; Weiss et al., 2007). Mutant strain #50 (glucokinase) with its crucial role for glycolysis (EMP pathway) will be discussed below.

The glucokinase of F-W12. In the amoeba-sensitive clone #50, a gene encoding a putative glucokinase (peg0721) was inactivated. Although the mutant showed a reduced *in vitro* growth, its persistence in the infection assay was even slightly increased compared to the WT strain. We did not know yet the reason for this phenotype. Therefore, we decided to analyze the role of glucose metabolism in F-W12 in more detail. The glucokinase (encoded by *glk*) is the first enzyme of the EMP pathway and phosphorylates glucose to glucose-6 phosphate which is then converted into fructose-6 phosphate by a glucose-6 phosphate isomerase (encoded by *pgi*). Therefore, it was not surprising that growth of the mutant in MT was reduced. Interestingly, in contrast to the wild-type strain, in MT-Glc$^-$ supplemented with 2 g/l glucose the *glk* mutant strain grew similarly to the wild-type strain in MT-Glc$^-$. This indicates that *glk* is the only glucokinase present in F-W12 and that F-W12, as shown for other *Francisella* species by *in silico* genome analysis, does not seem to exhibit a PEP phosphotransferase system (PTS) (Meibom and Charbit, 2010; Ziveri et al., 2017a). PTS generates glucose-6 phosphate during the import of glucose and the glucokinase is not necessary for glucose phosphorylation. *Ftt* and *Fth* exhibit a homolog of F-W12 *glk* gene (*glk1*) and they exhibit a second *glk2* gene which is inactivated (pseudogenes, FTT0706, FTL1530) in these strains, but not in *Fno* (Champion et al., 2009). All *Francisella* species are able to metabolize glucose, but only *F. hispaniensis*, *F. philomiragia* and probably some strains of *Fth* are able to metabolize Glc-1 P or Glc-6 P (Gyuranecz et al., 2010; Huber et al., 2010), indicating strain-specific metabolic differences for *Francisella*. *In silico* analysis of the draft genome of F-W12 did not reveal the presence of a second glucokinase (Rydzewski et al., 2014), corroborating the experimental results. In *E. coli*, glucose is taken up mainly by PTS but it exhibits also a glucokinase, and therefore *glk* mutant strains of *E. coli*, in contrast to *Francisella*, grow well on glucose (Fraenkel, 1986).

Experiments using MT supplemented with [U-$^{13}C_6$]glucose indeed showed that the carbon flux from glucose into amino acids and carbohydrates was highly reduced in F-W12. Interestingly, carbon flux into fructose was not reduced in the *glk* mutant strain (Fig. 4). As mentioned above, fructose phosphate is generated from glucose-6 phosphate by the glucose-6 phosphate isomerase, but ^{13}C-labeled glucose-6 phosphate should not be present in the *glk* mutant strain. In *E. coli* an enzyme (XylA, EC.5.3.1.5) is known to convert xylose to xylulose. Xylose isomerase (glucose isomerase) catalyzes the isomerization of D-xylose to D-xylulose, but also D-glucose to D-fructose, an enzymatic activity used in the food industry (Bhosale et al., 1996). Although *in silico* analysis did not reveal a XylA-homolog in the genome of F-W12, the enzyme activity may be present in F-W12, explaining the observed high amount of ^{13}C mol% values in fructose in the *glk* mutant strain. However, further research is necessary to answer this open question.

5. Conclusion

Recently, some putative virulence factors of F-W12 have been identified *in silico* (Rydzewski et al., 2014). To characterize this new *Francisella* species further, we adapted the scatter screen method to *Francisella* in order to be able to identify candidate fitness genes of F-W12 experimentally. We detected genes well known to be virulence factors in *Francisella* or in other bacteria, and one gene (peg_1387) was identified at different culture temperatures and using different amoeba strains. Altogether, this indicates that the scatter screen is applicable to the identification of candidate fitness or virulence factors in F-W12, and therefore potentially also in other *Francisella* species. Interestingly, we found no amoeba-sensitive clone exhibiting a Tn5 insertion within a gene of the putative aT6SS of F-W12, indicating that this system may not play an important role in the F-W12–amoebae interaction. In addition, no host cell has yet been found in which F-W12 is able to replicate equivalently to known pathogenic *Francisella* species.

Furthermore, the metabolism of *Francisella* is important for the ability to replicate in host cells (Ziveri et al., 2017a). It was demonstrated that amino acids as well as glucose and glycerol are metabolized by *Fth* and *Fno* (Brissac et al., 2015; Chen et al., 2017; Ziveri et al., 2017a), and we recently demonstrated that glucose is a major carbon source metabolized mainly through the EMP pathway (Chen et al., 2017). Here, we could corroborate the importance of glucose and glycerol metabolism for the new species F-W12.

Declaration of Competing Interest

All authors declare no conflict of interests.

Acknowledgments

We would like to thank Ursula Erikli for copy-editing. This work received financial support from the Deutsche Forschungsgemeinschaft (EI 384/11) and the Robert Koch Institute. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. No financial conflicts of interest regarding

the contents of the manuscript and its authors exist.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.151341.

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