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Legionella oakridgensis ATCC 33761 Genome Sequence and Phenotypic Characterization Reveals its Replication Capacity in Amoebae

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Abstract

Legionella oakridgensis is able to cause Legionnaires` disease, but is less virulent compared to *L. pneumophila* strains and very rarely associated with human disease. *L. oakridgensis* is the only species of the family legionellae which is able to grow on media without additional cysteine. In contrast to earlier publications, we found that *L. oakridgensis* is able to multiply in amoebae. We sequenced the genome of *L. oakridgensis* type strain OR-10 (ATCC 33761). The genome is smaller than the other yet sequenced *Legionella* genomes and has a higher G+C-content of 40.9%. *L. oakridgensis* lacks a flagellum and it also lacks all genes of the flagellar regulon except of the alternative sigma-28 factor FliA and the anti-sigma-28 factor FlgM. Genes encoding structural components of type I, type II, type IV Lvh and type IV Dot/Icm, Sec- and Tat-secretion systems could be identified. Only a limited set of Dot/Icm effector proteins have been recognized within the genome sequence of *L. oakridgensis*. Like in *L. pneumophila* strains, various proteins with eukaryotic motifs and eukaryote-like proteins were detected. We could demonstrate that the Dot/Icm system is essential for intracellular replication of *L. oakridgensis*. Furthermore, we identified new putative virulence factors of *Legionella*.

Introduction

L. oakridgensis was first reported in 1983 after isolation from industrial cooling towers, however, meanwhile *L. oakridgensis* was also found in ground water or compost facilities (Orrison *et al.*, 1983, Costa *et al.*, 2005; Casati *et al.*, 2010). *L. oakridgensis*, unlike all other *Legionella* species, does not require additional L-cysteine for growth in media or on agar plates (Orrison *et al.*, 1983). Later, it was demonstrated that *L. oakridgensis*, in contrast to *L. pneumophila*, is positive for serine O-acetyltransferase and cysteine synthase activity that may explain this ability (Ewann and Hoffman, 2006). *L. oakridgensis* (ATCC 33761) was reported as a non-flagellated species and no flagellin gene or protein could be detected by Southern or Western blot analysis, respectively (Orrison *et al.*, 1983; Heuner *et al.*, 1995). In addition, it was shown that *L. oakridgensis* is alkaline phosphatase and secreted protease negative. *L. oakridgensis* is less susceptible to erythromycin compared to *L. pneumophila* (Orrison *et al.*, 1983). By the same authors it was also mentioned, that *L. oakridgensis* exhibits uncharacterized extracellular material and granula of poly-3-hydroxybutyrate (PHB).

It was demonstrated that *L. oakridgensis* is pathogenic for guinea pigs and therefore a potential human pathogenic species (Orrison *et al.*, 1983; Fields *et al.*, 1986). In 1985 a case of human pneumonia caused by *L. oakridgensis* was mentioned for the first time, but in this case a *L. pneumophila* strain Sg6 was also identified within the patient sample (Tang *et al.*, 1985b). However, in a case report from the year 2000, two cases of Legionnaires` disease by *L. oakridgensis* were diagnosed in France and the bacteria could be isolated from the patients (Lo Presti *et al.*, 2000). In one case the disease was hospital acquired whereas in the second case it was community acquired, both with pleural effusion as reported earlier (Tang *et al.*, 1985b; Chereshsky *et al.*, 1986; Lo Presti *et al.*, 2000). Both patients had a previous history of immunological disorders treated with steroids, possibly rendering them more susceptible to infection. It was suggested that *L. oakridgensis* may have virulence factors favouring tropism for pleural tissue (Lo Presti *et al.*, 2000).

It was reported previously that *L. oakridgensis* is not able to replicate in amoebae (*Acanthamoeba castellanii*) (Neumeister *et al.*, 1997), but in Mono Mac 6 cells, in U937 cells and in co-culture with *Tetrahymena pyriformis* (Barbaree *et al.*, 1986; Fields *et al.*, 1986; O'Connell *et al.*, 1996; Neumeister *et al.*, 1997). Later, it was demonstrated that *L. oakridgensis* is also able to replicate in epithelial cells (Vero cells), showing characteristic serpentine chains with clusters in the centre of microcolonies. Furthermore, the authors showed that mitochondria are recruited around the bacteria-containing endosome, a feature well known from *L. pneumophila* (Ogawa *et al.*, 2001, Takekawa *et al.*, 2012). Recently it was published, that *L. oakridgensis* proliferated both inside membrane structures (endoplasmic reticulum) and in the cytoplasm (Takekawa *et al*., 2012).

In the present report, *L. oakridgensis* was chosen for comparative analysis, since *L. oakridgensis* is far less pathogenic than *L. pneumophila*, but is still able to infect immunocompromised humans. For comparative analysis, we sequenced the genome of *L. oakridgensis* type strain ATCC 33761 and the nearly complete genome sequence (draft genome) of *L. oakridgensis* RV-2-2007 (cooling tower isolate, England). The clinical isolates (Lo Presti *et al.*, 2000) were not available for genome sequencing, because the strains could not be regrown from the frozen samples (Jerome Etienne, Lyon, France, personal communication). However, we analyzed the three different *L. oakridgensis* strains (ATCC 33761 [*Loa*], RV-2-2007 [*Lor*] and W09-391-2 [*Low*, cooling tower isolate, Germany]) for various virulence-associated phenotypes. These strains were initially identified as *L. oakridgensis* by *mip* gene sequencing using the *mip* gene sequence database for identification of *Legionella* species available at http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/mip/mip_id.cgi (Ratcliffe *et al*., 1998). Results of genome and phenotypic analysis are presented and discussed within this report.

Materials and methods

Legionella *strains and growth conditions*

Legionella strains used in this study were *L. pneumophila* Corby (Jepras et al., 1985), *L. pneumophila* Corby Δ*icmX*, the cooling tower isolates *L. oakridgensis* ATCC 33761 (Orrison *et al.*, 1983) and *L. oakridgensis* W09-391-2 (Christian Lück, Germany) and the water isolate *L. oakridgensis* RV-2-2007 (Christian Lück, Germany). Bacteria were cultivated in ACES-buffered yeast extract (AYE) broth [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 3M KOH and sterile filtrated] or on ACES-buffered charcoal-yeast extract (BCYE) agar at 37 °C (Edelstein, 1981).

Amoeba strains, cell lines and growth conditions

A. castellanii ATCC 30010, *A. castellanii* ATCC 30234, *A. castellanii* 50739 (growth media: PYG and ATCC medium 712), *A. lenticulata* 45 ATCC 50703, *A. lenticulata* 118 ATCC 50706, *Hartmannella vermiformis* OS101 (growth medium Chang), *Hartmannella vermiformis* ATCC 50256 and *Naegleria gruberi* (growth medium PYNFH ATCC 1034) and the U937 human macrophage-like cell line ATCC CRL-1593.2 (growth medium RPMI 1640 + 10% FCS purchased from PAA, Pasching, Austria) were tested for multiplication of *L. oakridgensis*. All amoebae were grown at room temperature whereas the U937 cell-line was cultivated at 37 °C and 5% CO_2.

Phenotypic assays

Enzyme activity tests were done on agar plates containing the same ingredients as BCYE agar but without charcoal. Alpha-amylase activity plates contained 0.1% starch or glycogen and cellulose-activity plates were supplemented with 0.1% carboxymethyl cellulose (CMC, Sigma-Aldrich, St.-Leon-Roth, Germany). Experiments were carried out as described previously (Herrmann *et al.*, 2011). Protein degrading activity was visualized on agar

containing 0.9% casein (C8654, Sigma-Aldrich, St.-Leon-Roth, Germany) and 0.14% starch (Roth, Karlsruhe, Germany).

Isotopologue profiling

Isotopologue profiling using [U-$^{13}C_6$]glucose (2 g/l) or [U-$^{13}C_3$]serine (0.3 g/l) was done as described previously (Eylert et al., 2010). In brief, 250 ml of the supplemented AYE Medium was inoculated with 2 or 1 ml of an overnight culture of *L. oakridgensis* ATCC 33761 or *L. pneumophila* Corby, respectively. Incubation was carried out at 37 °C and 220 rpm. Cultures became stationary at $OD_{600=}$ ~1.5 or at $OD_{600=}$ ~2.0, respectively. Before harvesting, a culture aliquot was plated on LB agar plates to rule out the possibility of contamination. The bacteria were killed with sodium azide at a final concentration of 10 mM and pelleted at 5500 g and 4 °C for 15 min. The pellet was washed twice with 200 ml of water and then once with 2 ml of water. The supernatant was discarded, and the bacterial pellet was autoclaved at 121 °C for 20 min. Hydrolysis of proteins and PHB and conversion into volatile derivates for GC/MS analysis, dichloromethane extraction, isolation of amino acids and analysis by mass and NMR spectroscopy were done as described before (Eylert *et al.*, 2010).

L. oakridgensis*/ amoebae replication screen on agar plates*

To determine which amoeba strain is suitable to be used for replication assays, *L. oakridgensis* (~10^{10} cells) was suspended in 1 ml dH_2O and 100 µl were plated onto NN-agar plates (14 g/l agar in dH_2O). The amoeba strain (15 µl) was dropped on the centre of the plates and incubated at 23 or 30 °C for 7 days. The plates were inspected daily for movement and replication of the amoeba. *A. lenticulata* 45 and 118 showed no movement and were lysed whereas *A. castellanii*, *H. vermiformis* and *N. gruberi* were motile and not killed by *L. oakridgensis*.

Intracellular multiplication of L. oakridgensis *in amoebae*

Infection assays using *A. castellanii*, *A. lenticulata*, *H. vermiformis* and *N. gruberi* for *in vivo* growth of *L. pneumophila* Corby and *L. oakridgensis* were done as described before with

modifications (Brüggemann *et al.*, 2006, Eylert *et al.*, 2010). In brief, 3-day-old cultures of the amoeba strain were washed in AC buffer (PYG 712 medium without proteose-peptone, glucose and yeast extract), adjusted to 1 x 10^5 cells per ml and incubated in 24-well plates for 2 hours at 37 °C and 5% CO_2. Stationary phase *Legionella* bacteria grown for 3 days on BCYE agar were diluted in AC buffer and mixed with the amoeba strain at a multiplicity of infection (MOI) of 1. After invasion for 2 h at 37°C, the amoeba cell layer was washed once with AC buffer, defining the start point of the time course experiment. The CFU of legionellae was determined at 24, 48, 72 and 96 hours by plating on BCYE agar. Unless otherwise stated, each infection was carried out in duplicates and was done at least three times.

Intracellular multiplication of L. oakridgensis *in U937 cells*

Transformation and infection of U937 cells was done as previously described with modifications (Liles *et al.*, 1999, Cianciotto *et al.*, 1992, Flieger *et al.*, 2004). For differentiation into macrophage-like cells, U937 cells were adjusted to 3 x 10^5 cells/ml and transferred in 100 ml fresh RPMI medium containing 10% fetal calf serum (10% FCS) and PMA (phorbol-12-myristate-13-acetate, Stock 1 mg/ml in dH_2O [P-8139; Sigma-Aldrich]) was added in a concentration of 1:20000. After incubation for 36 h at 37 °C and 5% CO_2, the supernatant was discarded and adhered cells were washed once with 10 ml 0.2% EDTA in PBS. Cells were detached from the flask bottom with RPMI + 10% FCS, transferred in 50 ml tubes and centrifuged at 800 g for 10 min. All cells were counted after trypanblue staining in a Neubauer counting chamber and adjusted to 5 x 10^5 cells/ml with RPMI + 10% FCS. To each well of a 24-well plate 1 ml of the cell suspension was added and incubated for adhesion during 2 h at 37 °C and 5% CO_2. Stationary phase *Legionella* bacteria grown for 3 days on BCYE agar were diluted in plain RPMI medium and the infection was done with a MOI of 1 (time point 0 h) for 2 h at 37 °C and 5% CO_2. Cells were washed 3 times with RPMI and covered with 1 ml RPMI + 10% FCS. For CFU determination at various time points of

infection, coincubations of U937 cells and legionellae were lysed by addition of 10 µl 10% Saponin (S4521, Sigma-Aldrich) for 5 min and serial dilutions were plated on BCYE agar.

Electron microscopy (EM)

Negative staining EM: Bacteria were grown for 1 day in AYE media at 30 °C, harvested by gentile centrifugation at 1,000 rpm for 20 min and suspended in distilled water. The suspension was fixed with an equal volume of 4% paraformaldehyde in HEPES buffer (0.1 M). Concentrated bacteria were adsorbed on Pioloform-F coated, carbon stabilised and glow-discharged copper grids, washed three times with distilled water and negative stained with 0.5% uranyl acetate (pH 4 - 4.5).

Thin section EM: *A. lenticulata* 45 or U937 cells were infected with *L. oakridgensis* ATCC 33761 (MOI of 10) at 37 °C as described above. U937 cells or amoebae were fixed 48 h p.i. with 2.5% glutaraldehyde in 0.05 M HEPES buffer. Samples were post-fixed with osmium tetroxide (1% in distilled water), and uranyl acetate (2 % in distilled water), dehydrated stepwise in a graded ethanol series and embedded in LR White resin (Science Services, Munich, Germany) which was polymerized at 60 °C over night. Thin sections were prepared with an ultramicrotome (UC-T; Leica, Vienna, Austria) and counterstained with uranyl acetate (2% in distilled water) and lead citrate (Reynolds 1963).

All samples were examined using a TEM 902 (Carl Zeiss Microscopy GmbH, Oberkochen Germany) at 80 kV, and the images were digitized using a slow-scan charge-coupled-device camera (Pro Scan; Scheuring, Germany).

SDS-PAGE and immunoblotting

Flagellin detection was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed as described previously (Laemmli, 1970). Equal amounts of *Legionella*, grown on BCYE agar plates to stationary phase for 3 days were diluted in PBS boiled for 10 min in Laemmli buffer and loaded onto a 12% SDS polyacrylamide gel. Western blotting was carried out by using

polyclonal anti-FlaA antisera diluted in 1% milk/TBS (1:1000) (Schulz *et al.*, 2012). A horseradish peroxidase-conjugated goat anti-rabbit antibody was used as secondary antibody (1:1000). FlaA was visualized by incubation of the blot with 50 ml colour reaction solution (47 ml TBS, 3 ml 4-chloro-1-naphthol and 80 ml H_2O_2), and the reaction was stopped with distilled water. Results were carried out in at least two independent experiments.

Generation and screening of a L. oakridgensis *ATCC 33761 Tn5 mutant collection*

The EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (Epicentre, Madison, WI, USA) was used for generation of a *L. oakridgensis* ATCC 33761 Tn5 mutant collection according to manufacturer´s instructions and as described previously (Aurass *et al.*, 2009). Here, 5 µl of the transposon-transposase complexes were introduced in 80 µl competent *L. oakridgensis* ATCC 33761 and after 6 h of incubation with shaking at 220 rpm and 37 °C, 100 µl of the suspension were plated on BCYE plates containing kanamycin (12.5 µg/ml). Received clones were analyzed in a Scatter Screen as described previously (Aurass *et al.*, 2009). Differing from the protocol, we used *A. lenticulata* as host because *L. oakridgensis* wild type is digested by *A. castellanii*. Therefore, several modifications were necessary. For the Scatter Screen assay, 7 x 10^6 *A. lenticulata* cells/ml were incubated with 1 x 10^5 *L. oakridgensis* EZ-Tn5 <KAN-2> transposon mutants/ml on BCYE agar plates at 37 °C and 5% CO_2. Colonies with scattered phenotype were transferred to BCYE agar plates containing. Transposon insertion site analysis was done as reported previously to define the obtained mutants (Aurass *et al.*, 2009).

Genome sequencing, ORF finding and annotation

A pyrosequencing approach was used for whole-genome sequencing of *L. oakridgensis* ATCC 33761. It was performed by Eurofins MWG Operon. For this purpose, either commercially available standard techniques, proprietary developments or a combination of both were used. Genomic DNA of *L. oakridgensis* ATCC 33761 and RV-2-2007 were sequenced by using a fraction of nebulized chromosomal DNA and Roche FLX sequencing

was done according to the manufacturer's protocols (Roche Applied Science, Mannheim, Germany). The 454 reads were assembled into contigs by using the Newbler Assembler 6.1 (454; Life Science, Roche). Editing of shotgun sequences and 454 sequences was done by using GAP4 as part of the Staden software package (Staden *et al*., 1998). To solve problems with misassembled regions caused by repetitive sequences and to close remaining sequence gaps of the genome sequence of *L. oakridgensis* ATCC 33761, PCR and primer walking were used. PCR has been carried out with the BioXact Kit (Qiagen, Hilden, Germany) as described by the manufacturer.

A first functional annotation was done by Biomax (Planegg, Germany) using the Pendant-Pro™ program. The *L. oakridgensis* strain ATCC 33761 genome was submitted to the Integrated Microbial Genomes- Expert Review (IMG/ER) system (https://img.jgi.doe.gov) that provides support for ORF finding and functional annotation and curation of microbial genomes of interest (Markowitz *et al.*, 2009).

Results and discussion

L. oakridgensis is able to replicate intracellular in *A. lenticulata*

First we analyzed the *in vitro* growth of *L. oakridgensis*. It was reported that *L. oakridgensis* is not able to grow at temperatures higher than 42 °C (Orrison *et al.*, 1983). We analyzed growth of the three *L. oakridgensis* isolates and of *L. pneumophila* Corby at 30, 37, 40 and 42 °C in AYE medium (Fig. 1A). Whereas *L. pneumophila* Corby grew faster than *L. oakridgensis* and reached an optical density $(OD)_{600}$ of ~1.9 at each temperature investigated (Fig. 1A, *Lpc*), *L. oakridgensis* strains only reached maximum OD_{600} of about 1.4 at 30, 37 and 40 °C (Fig. 1A, *Loa*, *Lor* and *Low*). After ~24 h of growth, the bacteria produced a brown pigment, indicating that the bacteria reached post-exponential growth (Warren *et al.*, 1979). Growth of *L. oakridgensis* ATCC 33761 was better than that of strain RV-2-2007 (*Lor*).

However, all three isolates grew very slowly at 42 °C in comparison to *L. pneumophila* Corby (Fig. 1A).

It was reported that *L. oakridgensis* is not able to multiply within amoebae (Neumeister *et al.*, 1997). To prove this result, we tested three different *A. castellanii* strains, two *A. lenticulata* strains, two *Hartmannella vermiformis* strains and *Naegleria gruberi* for their ability to grow on *L. oakridgensis* using an agar plate test. Only *A. lenticulata* 45 and *A. lenticulata* 118 were not able to grow on *L. oakridgensis* (data not shown). Both amoeba strains were then used for infection assays with all three *L. oakridgensis* isolates and *L. pneumophila* Corby as a control. We demonstrated for the first time, that *L. oakridgensis* ATCC 33761 is able to replicate in *A. lenticulata* 45 at 30, 37 and 40 °C (Fig. 1B). All three *L. oakridgensis* isolates investigated were able to replicate within *A. lenticulata* 45 (Fig. 1C). Amplification of the bacteria is detectable after 1 day of co-incubation at 30 °C or 37 °C (Fig. 1B+C). The intracellular replication rate of *L. oakridgensis* was lower than the rate of *L. pneumophila* Corby. Infection assays with *A. castellanii*, *H. vermiformis* and *N. gruberi* demonstrated that *L. oakridgensis* was not able to replicate within one of these strains. However, *L. oakridgensis* survived over a period of 3-5 days within these host cells (Fig. S1B-D). Probably, the main environmental host of *L. oakridgensis* still has to be identified.

In addition, we also performed infection assays with all three *L. oakridgensis* isolates using the human macrophage-like cell line U937. Surprisingly, *L. oakridgensis* strain RV-2-2007 and W09-391-2 replicated nearly as well as the *L. pneumophila* strain, whereas replication of *L. oakridgensis* ATCC 33761 was reduced compared to the other strains investigated (Fig. 1D).

Furthermore, the infection of *A. lenticulata* 45 and U937 cells by *L. oakridgensis* ATCC 33761 was investigated by electron microscopy. *L. oakridgensis* replicates inside a membrane surrounded vacuole (*Legionella*-containing vacuole, LCV) (Fig. 2). In *A. lenticulata* the LCV was partly surrounded by ribosomes (Fig. 2C, arrows) and in U937 cells

mitochondria were found close to the LCV-membrane (Fig. 2F, arrows), indicating that organelle recruitment to the LCV is also present in host cells infected by *L. oakridgensis.* The recruitment of organelles to the LCV is a phenotype which depends on a functional Dot/Icm secretion system, and is a prerequisite for intracellular replication (Horwitz, 1983; Marra *et al.*, 1992; Berger *et al.*, 1993; Sadosky *et al.*, 1993; Kagan *et al.*, 2002). The observed recruitment of mitochondria in U937 cells has also been reported by using Vero cells as host cells (Ogawa *et al.*, 2001, Takekawa et al., 2012). The same group recently published, that *L. oakridgensis* proliferated both inside membrane structures (endoplasmic reticulum) and in the cytoplasm (Takekawa *et al.*, 2012).

While the *L. oakridgensis* ATCC 33761 strain was replicating faster in *A. lenticulata* than the RV-2-2007 strain, in U937 cells it was oppositely. Recently we demonstrated that *L. oakridgensis* ATCC 33761, exhibiting the genomic island Trb-1 of *L. pneumophila* Corby, showed no increased fitness in co-culture with U937 cells (Lautner et al., 2013). Yet we can only hypothesize, if the plasmids, the identified genomic islands or genes (e.g. Trb-1 and LorGI-1-like island) not present in *L. oakridgensis* ATCC 33761 are involved in the observed differences in intracellular replication.

Screening for virulence genes of *L. oakridgensis* ATCC 33761 using *A. lenticulata*

Knowing that *L. oakridgensis* is able to multiply in *A. lenticulata*, we generated a Tn5 mutant collection of strain ATCC 33761 and screened it for *in vivo* relevant virulence factors using *A. lenticulata* for a Scatter Screen (Aurass *et al.*, 2009), to detect clones not able to resist degradation by *A. lenticulata* 45. The obtained 33 clones were then tested in co-culture (infection assay) with *A. lenticulata* 45. We identified 30 clones not able to replicate in this assay (data not shown). For 27 clones the insertion site of the Tn5 element has been identified and 24 of them were localized within *dot/icm* genes (*icmE* [8x], *icmB* [7x], *dotA* [4x], *dotD* [2x], *icmX* [1x], *icmV* [1x], *dotB* [1x]), indicating that a functional Dot/Icm system is essential

for intracellular replication of *L. oakridgensis* within host cells (Fig. 3 and data not shown). This is in good agreement with the finding of all components of a functional Dot/Icm system within the genome sequence (see below). Interestingly, both *dotD* mutant strains investigated were not able to replicate in *A. lenticulata*, but they showed only reduced intracellular replication within U937 cells (Fig. 3). In *L. pneumophila*, the *dotD* mutant strain was shown to be unable to replicate in *A. castellanii* and in HL-60-derived human macrophages (Yerushalmi *et al.*, 2005). We identified a lipobox-motif in the N-terminus of DotD ("ITGC"), as well as in the DotC ("LIGC") and the IcmN ("MTGC") protein, shown to be necessary for the function of DotC and DotD in *L. pneumophila* (Yerushalmi *et al.*, 2005). It is yet unknown, why *dotD* mutants of *L. oakridgensis* are still able to replicate within human U937 cells. The generation of specific deletion mutants of *dotD* will proof if DotD is dispensable for intracellular replication in U937 cells.

Yet, three mutant strains exhibiting reduced intracellular replication were identified in which the Tn5 integration site was not located within a gene of the Dot/Icm system (clones #14 [*loa2026*], #20 [*loa2257, gpsA*] and #205 [*loa2771, bipA*]. Gene *loa2026* encodes for a putative sulfotransferase exhibiting a Pfam_sulfotransferase domain. Interestingly, although this strain had no *in vitro* growth defect and was still able to grow on BCYE agar plates without additional cysteine, it was not able to multiply within *A. lenticulata* or U937 cells (data not shown and Fig. 3). We could not identify a homolog of this gene in the yet sequenced *Legionella* strains. Sulfotransferases are able to transfer the sulfate group of 3-phosphoadenosine 5-phosphosulfate (PAPS) to other proteins. *L. oakridgensis* possesses the enzyme complex ATP-sulfurylase (*loa0068/0069; CysN/D*) and an APS-kinase (*loa0068; cysC*) for the synthesis of PAPS from sulfate. Yet, we can only speculate about the function of the sulfotransferase for *L. oakridgensis*, but since the strain is still able to grow on agar plates without additional cysteine, it seems not to be involved in the biosynthesis pathway of cysteine. However, sulfotransferases are known virulence factors in *Mycobacteria* and

Rhizobia (Mougous *et al.*, 2002) and they are involved in cell-cell interactions, host-pathogen interactions, extracellular traffic or in root modulation by *Rhizobia* (Hanin *et al.*, 1997; Negishi *et al.*, 2001; Mougous *et al.*, 2002; Kusche-Gullberg *et al.*, 2003; Del Papa *et al.*, 2007).

Gene *loa2257* encodes a glycerol-3-phosphate dehydrogenase (GpsA, NADPH-dependent). This protein generally is involved in the metabolism of glycerol. The mutant (Δ*gspA*) was not able to replicate in *A. lenticulata*, but growth within U937 cells was only reduced (Fig. 3). This is the first experimental hint, that glycerol metabolism in *Legionella* is important for intracellular replication. Recently, it was published that *glpD* encoding for another glycerol-3-phosphate dehydrogenase (NADH-dependent) is upregulated during intracellular growth in human macrophages (Faucher et al., 2011). A *Listeria monocytogenes glpD* deletion mutant shows reduced intracellular growth (Schauer *et al.*, 2010). Together, this indicates that the metabolism of glycerol is also necessary for full *in vivo* fitness of *Legionella*.

Gene *loa2771* encodes for a GTP-binding protein A (BipA) which seems to be necessary for the fitness of *L. oakridgensis* in co-culture with *A. lenticulata* (Fig. 3A). In summary, the data suggest that the identified genes might play an important role for the intracellular multiplication of *L. oakridgensis*.

Phenotypic characterization

Growth without cysteine

L. oakridgensis is described as the sole species of *Legionella* to be able to grow without additional cysteine within the culture media. *L. jordanis* also seemed to be able to grow in such a medium, but growth was very poor (Orrison *et al.*, 1983). We first analyzed all three isolates for their ability to grow on such BCYE agar plates. As expected, we found that the isolates investigated were able to grow, in contrast to *L. pneumophila* (Fig. S2A). We were

able to identify two putative serine-O-acetyltransferases (*loa0042* and *loa2106*) and a cysteine synthase (*loa0120*) within the genome sequence of *L. oakridgensis*, which may explain the ability to grow on agar plates without additional cysteine. To prove the functionality of *loa0042* and *loa2106* further experiments are needed. However, the enzymatic activity of serine-O-acetyltransferase and cysteine synthase was demonstrated in lysates of *L. oakridgensis* ATCC 33761, but not in lysates of *L. pneumophila* (Hoffman, 1984, Ewann *et al.*, 2006). As mentioned above, *L. oakridgensis* ATCC 33761 exhibits the genes to generate PAPS from sulfate, as well as a PAPS-reductase (*loa0054*; *cysH*) and a PAPS-phosphatase (*loa0066*; *CysQ*). However, no sulfite reductase was annotated within the genome.

Cellulose-, glycogen-, starch- and protein-degrading activities

We also analyzed the isolates for their ability to degrade cellulose, glycogen and proteins. We found cellulose-degrading activities in the pellet and supernatant fractions of *L. oakridgensis* in comparable amounts as in the *L. pneumophila* strain (Fig. S2D). In contrast to *L. pneumophila* Corby, all three *L. oakridgensis* isolates were negative for starch- and glycogen-degrading activities (Fig. S2E and data not shown). In the first report it was described, that *L. oakridgensis* exhibits low protease activity (Orrison *et al.*, 1983). *L. oakridgensis* is able to degrade proteins (casein), but the activity found in the supernatant was low compared to *L. pneumophila* (Fig. S2F). In the genome sequence of *L. oakridgensis* we identified homologs of MspA (Loa0778) and LasB (Loa2804), both exhibiting a putative signal sequence and all components of a putative functional T2SS are present.

Properties of the metabolism

We recently demonstrated that *L. pneumophila* is able to metabolize glucose via the Entner-Doudoroff pathway and that this pathway is a fitness factor of *L. pneumophila* (Eylert *et al.*, 2010). We therefore analysed *L. oakridgensis* ATCC 33761 for its ability to metabolize [U-

$^{13}C_6$]glucose and [U-$^{13}C_3$]serine by isotopologue profiling. In contrast to *L. pneumophila* Paris, *L. oakridgensis* was not able to metabolize glucose in detectable amounts. Label from [U-$^{13}C_3$]serine was transferred to some amino acids at amounts which were similar to the corresponding experiment with *L. pneumophila* Paris, with the exception that a higher amount of the added [U-$^{13}C_3$]serine was directly used for protein synthesis (Fig. 4). Similar to *L. pneumophila*, ^{13}C from labelled serine was also found in PHB from *L. oakridgensis*, a general energy and carbon storage compound of bacteria (Fig. 4). Notably, ^{13}C-excess in PHB was significantly higher in *L. oakridgensis*. The features of this section will be further discussed in the next part of the manuscript (see below)

Whole genome sequence analysis

General features of the genome

Sequencing of the genome of *L. oakridgensis* ATCC 33761 revealed that it consists of 2.77 x 10^6 bp and has an average G+C content of 40.86% (Tab. 1). The genome contains 2887 protein encoding genes and a further 45 ORFs on an IncF plasmid (50245 bp, 39% G+C content). The genome contains three copies of the 23, 16 and 5S rRNAs and 42 tRNA genes, respectively. The contigs (draft genome, 487 contigs) of the sequenced genome of *L. oakridgensis* RV-2-2007 consist of approximately 2.86 x 10^6 bp and has an average G+C content of 40.8%. The alignment of both genome sequences revealed high overall identity (Fig. S3A). The comparison of the *L. oakridgensis* ATCC 33761 genome with the yet available genomes of *L. pneumophila* indicated that *L. oakridgensis* genome has a higher G+C content (40.86%) and is substantially smaller (2.77 Mb) (Tab. 1). The supplemental Fig. S3B shows that the *L. oakridgensis* ATCC 33761 genome has very limited synteny with *L. pneumophila*, indicating the evolutionary distance between both species, whereas the genome of strain ATCC 33761 is highly similar to the draft genome of strain RV-2-2007.

As shown for *L. pneumophila*, *L. oakridgensis* also exhibits genomic islands encoding an Lvh-T4ASS (*L. oakridgensis* ATCC 33761), a Trb1-T4ASS (*L. oakridgensis* RV-2-2007), a LorGI-1-like element (*L. oakridgensis* RV-2-2007) encoding an additional putative T4SS and a strain specific genomic region (*L. oakridgensis* ATCC 33761, ~22 kb, *loa0025* to l*oa0060***)** encoding proteins putatively involved in capsule and cell wall biogenesis. The gene loci of the islands will be discussed below.

Approximately 1.4% of the genome sequence of RV-2-2007 is absent from the genome of ATCC 33761 (Fig. S3A). The major part of this DNA encodes for non-ribosomal peptide synthases and three different Tn/IS elements (data not shown).

L. oakridgensis ATCC 33761 and RV-2-2007 exhibit a similar IncF-like plasmid (*par* and *tra* region, 93 and 88 % DNA identity, respectively) but the plasmids are different within region II (Fig. S4). The plasmid of strain ATCC 33761 (50245 bp, 43 ORFs) contains a replication site, a *tra* region and genes encoding proteins putatively involved in metal ion resistance (Fig. S4A). The IncF plasmid of strain RV-2-2007 (64418 bp, 51 ORFs) contains a replication site and a *tra* region (Fig. S4B). Furthermore, it encodes an additional type-I modification-restriction system not present at the plasmid of *L. oakridgensis* ATCC 33761. Similar plasmids have also been described for *L. pneumophila* Paris and Lens (Cazalet *et al.*, 2004). In general, plasmids are described for *L. micdadei*, *L. longbeachae*, *L. gormanii* and *L. dumoffii*, but less is known about the role of plasmids in legionellae (Heuner *et al.*, 2002b).

Protein secretion systems

L. pneumophila genomes encode multiple protein secretion systems (DeBuck et al 2007; Lammertyn *et al.*, 2004). As in *L. pneumophila*, all genes of the general secretion system (Sec), the twin arginine translocation (Tat) system, T1SS Lss, T2SS Lsp and the Dot/Icm T4BSS are present at the genome of *L. oakridgensis*, exhibiting an overall DNA identity of

about 70% to the corresponding systems of *L. pneumophila*. The T1SS Lss encodes homologs of the *lssXYZA* genes with a similar gene order and composition up- and downstream compared to the *lss* operon of *L. pneumophila* (Jacobi *et al.*, 2003). However, the genes *lssBDE* are absent from the genome sequence (Fig. 5A). Similar results have been reported previously for other non-*L. pneumophila* species (Jacobi *et al.*, 2003; Kozak *et al.*, 2010). There is also a complete Sec and Tat system, as well as all components of the T2SS and the Dot/Icm secretion system present in *L. oakridgensis* (Tab. 2 or Fig. 5B-D). The T2SS seems to be functional, since we were able to detect protease activity in the supernatant of *L. oakridgensis* cultures. In *L. pneumophila*, the T2SS is essential for *L. pneumophila* virulence (Rossier *et al.*, 2001; Cianciotto, 2005; De Buck *et al.*, 2007).

As reported for *L. pneumophila* Paris (Cazalet *et al.*, 2004), *L. oakridgensis* seems to exhibit a T5SS, a putative outer membrane autotransporter protein with no significant identity to any known *Legionella* protein (Fig. 5E). Gene *loa2004* encodes for a protein of 995 amino acids homolog to an autotransporter of *Polynucleobacter necessarius* and a hemagglutinin related protein of *Janthinobacterium sp*. Marseille. The autotransporter protein exhibits a putative N-terminal signal peptide, seven parallel beta-helix repeats, often found in enzymes with polysaccharide substrates, and a C-terminal autotransporter beta domain. Yet, we do not know, if this gene encodes a functional autotransporter.

We also identified a putative type 4 pilus (Tfp) and a mannose-sensitive hemagglutinin-like pilus (Msh) system (Fig. 5F, G). *L. pneumophila* Tfp proteins promote the attachment to host cells, are required for natural competence, for twitching motility and are involved in biofilm formation (Stone *et al.*, 1998; Stone *et al.*, 1999; Coil *et al.*, 2009; Stewart *et al.*, 2009; Coil *et al.*, 2010). The core components necessary for a functional Tfp pilus are present within the genome sequence of *L. oakridgensis*. The second Tfp system encodes a putative mannose-sensitive hemagglutinin (MSHA)-like pilus. These flexible peritrichious pili were shown to be involved in the attachment of *Pseudoalteromonas tunicata* to surfaces of

green alga or other eukaryotes (Dalisay *et al.*, 2006). For *Vibrio cholerae*, the MSHA-pilus was shown to be involved in the adherence to zooplankton (Watnick *et al.*, 1999; Chiavelli *et al.*, 2001; Meibom *et al.*, 2004). Yet, we do not know if the MSHA-like pilus of *L. oakridgensis* is expressed and if it is involved in the attachment to biotic or abiotic surfaces.

As mentioned above, *L. oakridgensis* ATCC 33761 exhibits the T4ASS Lvh on a genomic island integrated into the tmRNA containing three different direct repeats (Fig. 6A, B). The Lvh island of *L. oakridgensis* exhibits an additional region 2/III (Fig. 6A) containing genes for a DNA-restriction/modification system. The DNA of the island is 96-99% identical to the Lvh-encoding region of *L. pneumophila* strain Lens. As mentioned above, the DNA identity of the other secretion systems was only ~70%. This corroborates that horizontal transfer of the island between different *Legionella* species may exist. Surprisingly, the genome of *L. oakridgensis* RV-2-2007 does not exhibit this Lvh-island, but instead it contains the T4ASS Trb-1 island integrated into the tRNA-Pro gene (Fig. 6C, D). The DNA of this island is 91-98% identical to the *trb/tra* region of the Trb-1-encoding island of *L. pneumophila* Corby, but region 1/II of Trb-1 of *L. oakridgensis* differs from that *of L. pneumophila* Corby (Fig. 6C). This result again indicates that Trb-1 is able to be transferred horizontally between *Legionella* strains, as it was shown recently by an *in vitro* experiment (Glöckner *et al.*, 2008). Using inverse PCR, we demonstrated that region 2/IV is part of the Lvh island and that both islands (Trb-1 and Lvh) can also exist in their excised episomal form (data not shown), as it is described for *L. pneumophila* (Glöckner *et al.*, 2008; Doleans-Jordheim *et al.*, 2006).

Strain RV-2-2007 exhibit an additional genomic island (LorGI-1, ~70,608 bp, ~80 ORFs), encoding a putative genomic island-associated T4SS with homology to LpcGI-1 of *L. pneumophila* Corby (*lpc2190* to *lpc2314*, Lautner *et al.*, 2013). The island seems to be integrated into the $tRNA^{Met}$ gene and we yet identified three integrases, several cation efflux systems (*helABC, copA, cepABC, and ctpA*), the putative T4SS encoding region and the

regulatory operon *lvrRABC*. The overall DNA identity is 82-85% to the corresponding regions of *L. pneumophila* Corby, Paris, Lens or 130b (data not shown). However, yet we were not able to identify the repeat (attR), defining the right site of the integrated LorGI-1 island within the draft genome of strain RV-2-2007. A similar region is also present in the *L. pneumophila* genomes, encoding various genes involved in heavy metal transport or resistance (Schroeder *et al.*, 2010). T4SS are believed to be capable of the transport of proteins and nucleic acids into cells (Nagai *et al.*, 2003; Juhas *et al.*, 2008; Voth *et al.*, 2012). With the exception of the *lvh* genes, all mentioned genes of the different secretion systems of *L. oakridgensis* ATCC 33761 were also present in the same gene order in strain RV-2-2007 (data not shown).

The Dot/Icm secretion system, effector proteins and eukaryote-like proteins

We demonstrated by electron microscopy that after infection with *L. oakridgensis* ribosomes respectively mitochondria were found in close proximity to the LCV-membrane. We also identified all genes of the Dot/Icm secretion system (T4BSS) within the genome sequence of *L. oakridgensis* and our experimental data revealed that this system is functional (see above). The genomic organisation of the Dot/Icm secretion system (T4BSS) of *L. oakridgensis* is similar to *L. pneumophila*, with the exception that *icmV* is localized adjacent to *dotA* (Fig. 5D).

Therefore, we investigated the genome sequence of *L. oakridgensis* for putative effector proteins of the Dot/Icm system. *In silico* analysis revealed the presence of 24 Dot/Icm effector proteins and 12 of them exhibit homologs to known effector proteins of other *L. pneumophila* strains (Tab. 2). The *in silico* analysis of the genome may have overlooked a view further putative effectors with no homology to yet known effectors. However, *L. oakridgensis* homologs of the effectors LidA and SdhA are present which are known to be involved in the recruitment of secretory vesicles to the LCV and in inhibition of host cell death, respectively (Derre *et al.*, 2005; Laguna *et al.*, 2006). In addition, the genomes of *L.*

pneumophila strains encode a large number of eukaryote-like proteins and of proteins with eukaryote-like domains (Albert-Weissenberger *et al.*, 2007, Nora *et al*., 2009). In the genome of *L. oakridgensis* ATCC 33761, we identified 14 genes encoding eukaryote-like proteins without and 43 with a homolog in a *Legionella* strain (Tab. 3). We identified various proteins (35) with domains preferentially found in eukaryotic proteins: putative proteins containing a TPR-domain (11), several with an ankyrin repeat (10), one F-box containing protein, two Ser/Thr kinases, three proteins exhibiting a SNARE domain, three with a Sel1-repeat, three with a SET domain and one Tyr-phosphatase. However, we could not identify any U-box or Sec7 domain containing protein (Tab. 3). With the exception of *loa0052*, encoding a putative UDP-sulfoquinovose synthase, all mentioned genes are also present in the genome sequence of *L. oakridgensis* RV-2-2007 (data not shown). Compared to *L. longbeachae*, there are less proteins with domains preferentially found in eukaryotic proteins (*Loa*/*Llo*; Ankyrin repeat: 10/22-29, F-box: 1/3, Sel-1: 3/3). However, the overall amount of eukaryote-like proteins is comparable to the amount identified in *L. longbeachae* (Cazalet *et al.*, 2010; Kozak *et al.*, 2010). All the mentioned proteins are known to be involved in the pathogen-host interplay during the intracellular replication of *L. pneumophila* (Albert-Weissenberger *et al.*, 2007; Hubber *et al.*, 2010; Lurie-Weinberger *et al.*, 2010; Gomez-Valero *et al.*, 2011).

L. oakridgensis seems to have a very limited set of hosts and replicates significantly slower compared to *L. pneumophila* Corby. This may be in accordance to the low amount of effector proteins and eukaryote-like proteins (total ~80) identified, when compared to what is known for *L. pneumophila* (>300 effector proteins) (Vogel *et al.*, 1998; Vincent *et al.*, 2006; Burstein *et al.*, 2009; Hubber *et al.*, 2010). A recent *in silico* analysis of genome sequences revealed a core set of approximately 107 effector proteins present in *L. pneumophila* strains (Schroeder *et al.*, 2010). However, the available set of effectors and of eukaryote-like proteins seems to be sufficient for intracellular replication of *L. oakridgensis* within a macrophage-like cell line and to cause Legionnaires` disease.

Virulence genes, gene regulation and the flagellar regulon

L. oakridgensis was shown to be pathogenic for humans. Therefore, we also investigated the genome sequences of both strains for known virulence genes and regulators. We could identify various known virulence factors of *L. pneumophila* (e.g. Mip, Msp, LasB, Hsp60, EnhABC, MviN, Tab. 4). In addition, via the above mentioned Scatter Screen we identified three new virulence genes of *L. oakridgensis* (*bipA, gspA, loa2026*) yet not known to be involved in the virulence of *Legionella* species.

Furthermore, we identified the common set of regulatory proteins known from other *Legionella* strains (e.g. CsrA, Hfq, LetAS, LetE, RpoS, RpoD, RpoH, RpoN, FliA, PilSR, PmrAB, CpxAR, RelA and SpoT) (Tab. 4). Therefore, the proposed model for the regulatory network of *L. pneumophila* (Jacobi *et al*., 2004; Molofsky *et al.*, 2004; Heuner and Albert-Weissenberger, 2008; Albert-Weissenberger *et al.*, 2010; Dalebroux *et al.*, 2010) seems to be transferable to *L. oakridgensis*, since the regulatory genes (CsrA, LetA, LetS, LetE, RpoS, RelA, SpoT), known to be involved in the regulation of the switch from exponential to stationary phase (life-cycle), and regulators such as RpoN, PmrA/B and CpxR/A are also present in *L. oakridgensis*. We did not identify a homolog of *fleQ* or *fleR* within both genomes.

Using electron microscopy, we could corroborate that strain ATCC 33761 is non-flagellated and the flagellin could not be detected by Western blot analysis with a flagellin-specific antiserum in any of the three strains investigated (Fig. S2B and C). This is in agreement with the observation of the first report about *L. oakridgensis* (Orrison *et al.*, 1983). Although the three *L. oakridgensis* strains investigated were flagellin negative, we screened both genome sequences for the presence of genes of the flagellar regulon. We detected the alternative sigma factor-28 FliA, its negative regulator FlgM (Anti-sigma-28 factor) and the alternative sigma factor-54 RpoN. However, all other genes of a complete flagellar regulon

(Heuner and Albert-Weissenberger, 2008; Albert-Weissenberger *et al.*, 2010) were absent (Fig. 7). In contrast to *L. longbeachae*, another aflagellated strain (Heuner *et al.*, 1995; Cazalet *et al.*, 2010; Kozak *et al.*, 2010), also the master regulatory protein FleQ and the regulators FleR, FleS and FleN are missing in the *L. oakridgensis* genomes (Fig. 7). This indicates that these regulators are not necessary for *L. oakridgensis* survival and virulence, although it was shown for *L. pneumophila* that FleQ and FleR are involved in the regulation of virulence genes (Heuner *et al.*, 2002a; Brüggemann *et al.*, 2006; Albert-Weissenberger *et al.*, 2010; Schulz *et al.*, 2012). The presence of the direct regulator of the flagellin gene expression FliA (Heuner *et al.*, 1997) indicates the importance of this alternative sigma factor for *Legionella*. However, generally the anti-sigma-28 factor FlgM is secreted via a functional basal body of the flagellum to induce FliA activity and therefore the expression of FlaA. Since there is no basal body present in *L. oakridgensis*, the mechanism of the interaction between FliA and FlgM must be different in this strain. For *L. pneumophila* it is known that FliA is involved in the establishment of infection, the cytotoxicity to BMMs and for the *in vivo* fitness of *L. pneumophila* (Hammer *et al.*, 1999; Hammer *et al.*, 2002; Heuner *et al.*, 2002a; Molofsky *et al.*, 2005; Schulz *et al.*, 2012). Furthermore, transcriptome studies of *L. pneumophila* Paris identified several genes belonging to the FliA regulon that do not play a role in flagellar gene regulation or flagellum assembly (Albert-Weissenberger *et al.*, 2010). We started to knock out the *fliA* gene of *L. oakridgensis* to analyse the function of FliA without the disruptive influence of any other gene involved in flagellar gene regulation or flagellum assembly. Yet, *L. pneumophila* was shown to be flagellated but exhibits no chemotaxis genes and *L. longbeachae* to be non-flagellated but to exhibit a putative chemotaxis gene cluster. *L. oakridgensis* is now the first *Legionella* species shown to be non-flagellated but also negative for chemotaxis genes.

Major pathways of the metabolism, carbohydrate utilization and investigated phenotypes

We also screened the genome sequence of *L. oakridgensis* for genes involved in the degradation of carbohydrates and for general metabolic pathways. We identified the genes of a complete glycolysis pathway (EMP), the Entner-Doudoroff (ED) and the Pentose Phosphate (PP) pathway as well as of a complete citrate (TCA) cycle (Tab. 5). Similar to *L. pneumophila*, no homologs of the gluconate-6-phosphate dehydrogenase (Gnd) and transaldolase could be identified and the glyoxylate cycle seems also to be absent. In contrast to *L. pneumophila*, *L. oakridgensis* exhibits a fructose-2,6 bisphosphatase (*loa2092*), putatively involved in glycolysis or gluconeogenesis (Tab. 5). In addition, within the *zwf* operon of *L. oakridgensis*, a homolog of the glucose transporter protein (Lpp0488) is lacking and the glucoamylase gene (*gamA*) and its putative regulator *yozG* are also missing. This may explain the inability of *L. oakridgensis* to degrade starch and glycogen. GamA was shown to be responsible for glycogen-degrading activity in *L. pneumophila* (Herrmann *et al.*, 2011). In addition, our isotopologue profiling experiments demonstrated that *L. oakridgensis*, in contrast to *L. pneumophila*, is not able to use supplied glucose as a carbon source for *de novo* synthesis of amino acids or PHB. This is in good compliance with the absence of the glucose transport protein Lpp0488 in *L. oakridgensis*, present in the *zwf* operon of *L. pneumophila* (Eylert *et al.*, 2010; Harada *et al.*, 2010). However, we could identify genes encoding a chitinase (*loa2513*) and two endoglucanases (*loa1304* and *loa1565*) explaining the observed ability of *L. oakridgensis* to degrade cellulose (Tab. 4). In addition, whereas a homolog of the glucose transporter of Lpp0488 is missing, several putative sugar phosphate permeases are present (*loa0545, loa1948, loa2006, loa2190, loa2450, loa2451, loa2474, loa2475* and *loa2618*).

Using serine as a ^{13}C-labelled carbon source for *in vitro* growth, we found a similar pattern of ^{13}C-labelled amino acids and PHB as for *L. pneumophila* (Eylert *et al.*, 2010 and Fig. 4), demonstrating that *L. oakridgensis* uses serine as a carbon source for the *de novo* synthesis of amino acids and PHB. However, in contrast to *L. pneumophila*, a higher amount

of added serine was incorporated directly into proteins and the mol% of ^{13}C of PHB in *L. oakridgensis* was higher than in *L. pneumophila* (Eylert *et al.*, 2010).

So in summary, *L. oakridgensis* is able to degrade proteins (Fig. S2F) and to use serine (amino acids) as a carbon source, but it seems to be unable to metabolize extragenously supplied glucose. However, the main pathways to degrade glucose (EMP, ED, PP) are present, probably involved in gluconeogenesis or in the catabolism of carbohydrates. It is conceivable that *L. oakridgensis* is able to import glucose-phosphate (glucose-1-phosphate or glucose-6-phosphate), since nine sugar-phosphate permeases (Tab. 5) are present within the genome of *L. oakridgensis.* Subsequently the sugar-phosphates could be metabolized through the above mentioned pathways.

Conclusion

L. oakridgensis is a human pathogen, but is a less virulent strain which seems to have a reduced range of hosts. Our hypothesis is, that this may be explained by the presence of a limited set of effector proteins of a functional Dot/Icm system. However, further investigations are necessary to prove this hypothesis. Since *L. oakridgensis* is non-flagellated but FliA positive, seems to contain a different pattern of glucose catabolism, has a limited set of genomic islands and putative new virulence factors, it is an interesting species for further research.

Nucleotide sequence accession number

The *L. oakridgensis* draft genome sequence of strain RV-2-2007 has been submitted to NCBI (BioProject number PRJNA184190). The genome sequence of *L. oakridgensis* ATCC 33761 has been entered in GenBank accession numbers CP004006 (chromosome) and CP004007 (plasmid). The genome sequence of *L. pneumophila* strain Corby is accessible at CP000675.2.

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Figure Legends

Fig. 1. *In vitro* (A) and *in vivo* (B-D) growth of *L. oakridgensis* strains ATCC 33761 (*Loa*, black), RV-2-2007 (*Lor*, brown), W09-391-2 (*Low*, green) and *L. pneumophila* Corby (*Lpc*, red). (A) All strains were grown in AYE broth at different temperatures for 24 h. Optical density was measured after centrifugation and resuspension of bacteria to remove the brown pigment produced by the bactria. (B-D) Monolayers of *A. lenticulata* 45 (B+C) and U937 cells (D) were infected with bacteria at a MOI of 1, washed after 2 h and incubated for 4 days at the respective temperature. The number of CFU per well was determined by plating on BCYE agar plates. Results are mean standard deviations of three (two for A) independent experiments of duplicate samples. Symbols: open circle, 30 °C; open square, 37 °C; open up-pointing triangle, 40 °C; open down-pointing triangle, 42 °C; filled circle, *Loa*; filled square, *Lor*; up-pointing filled triangle, *Low*; down-pointing filled triangle, *Lpc*.

Fig. 2. Thin section EM of *A. lenticulata* 45 (A-C) and U937 cells (D-F) infected with *L. oakridgensis* ATCC 33761 at a MOI of 10 (respective growth curve, see Fig. S1A), washed after 2 h and incubated for 48 h at 37 °C. (A+D) Host cells with large LCV containing many bacteria. (B+E) Host cells with small LCV and few bacteria. (C+F) Details of LCV of the respective cells shown in B and E which show either ribosomes (C) or mitochondria (F) in vicinity of the LCV membrane. Bars: 1 μm.

Fig. 3. Intracellular replication of *L. oakridgensis* ATCC 33761 wild-type (WT) and various Tn5 mutant strains within *A. lenticulata* 45 (A) and U937 cells (B). Monolayers of host cells were infected with bacteria at a MOI of 1, washed after 2 h and incubated for 3-4 days at 37 °C. The number of CFU per well was determined in duplicate by plating on BCYE agar plates. Results are mean standard deviations of duplicate samples and are representative of at least three (A) or two (B) experiments (* two experiments; # only once).

Fig. 4. Overall ^{13}C excess (mol%) of labeled isotopologues in PHB and protein derived amino acids after feeding of *L. oakridgensis* (Loa) and *L. pneumophila* Paris (Lpp) with 11 mM [U-

$^{13}C_6$]glucose (left) or 3 mM [U-$^{13}C_3$]serine (right). The values represent means from three technical replicates and the error bars indicate standard eviations. 1 and 2, biological replicates 1 and 2.

Fig. 5. Genetic organization of secretion systems and type IV pili of *L. oakridgensis* ATCC 33761. Respective genome regions of *L. oakridgensis* (*Loa*) are compared to DNA regions of *L. pneumophila* Corby (*Lpc*). The gene numbers (*loa*) are given below and gene names above the genes which are indicated by arrows. Genes belonging to the respective secretion or pilus systems are given in black, adjacent conserved genes in grey and all others in white.

Fig. 6. Genetic organization of the genomic islands Lvh (A) and Trb-1 (C) of *L. oakridgensis* ATCC 33761 (*Loa*) and *L. oakridgensis* RV-2-2007 (*Lor*), respectively. The nucleotide sequence of the repeats of Lvh (B) and Trb-1 (D) are given. Lvh is integrated within the tmRNA and Trb-1 within the $tRNA^{Pro}$ gene (black arrows). Genes of a gene cluster are shaded in the same pattern. The gene numbers (*loa*, *lor*, *lpl* or *lpc* for *L. oakridgensis* strains ATCC 33761, RV-2-2007, *L. pneumophila* Lens or Corby, respectively) are given below and gene names above the genes which are indicated by arrows.

Fig. 7. Structure of the genes belonging to the flagellar regulon of *L. pneumophila* Corby (*Lpc*), *L. longbeachae* NSW150 (*Llo*) and *L. oakridgensis* ATCC 33761 (*Loa*). Gene names are given above the genes which are indicated by arrows. Black arrows represent genes of the flagellar regulon and putative chemotaxis genes are indicated by grey arrows. Hence, in *L. oakridgensis* only *fliA, flgM* and *rpoN* are present.

Supporting information

Fig. S1. Intracellular replication of *L. oakridgensis* within different amoebal strains. (A) Replication of *L. oakridgensis* ATCC 33761 within *A. lenticulata* 45 and U937 cells infected with an MOI of 10 for electron microscopy analysis shown in Fig. 2. (B-D) Co-culture studies of *L. pneumophila* Corby (*Lpc*, red) and *L. oakridgensis* strains ATCC 33761 (*Loa*, black), RV-2-2007 (*Lor*, brown) and W09-391-2 (*Low*, green) with monolayers of *A. castellanii* (B), *Naegleria gruberi* (C) and *Hartmannella vermivormis* (D). Amoebae were infected with bacteria at a MOI of 1, washed after 2 h and incubated for 4 days at 37 °C. The number of CFU per well was determined by plating on BCYE agar plates. Results are mean standard deviations of duplicate samples and are representative of at least three independent

experiments. Symbols: filled circle, *Loa*; filled square, *Lor*; up-pointing filled triangle, *Low*; down-pointing filled triangle, *Lpc*.

Fig. S2. Phenotypic analysis of *L. oakridgensis* strains ATCC 33761 (*Loa*), RV-2-2007 (*Lor*), W09-391-2 (*Low*), *L. pneumophila* strain Corby (*Lpc*) and its isogenic *icmX* mutant strain (Δ*icmX*). (A) Growth on BCYE agar plates with (+) and without (-) additional cysteine (Cys). (B) Western blot analysis of cell lysates of BCYE agar grown bacteria with a polyclonal specific anti-flagellin (FlaA, 48 kDa) antiserum. (C) Negative staining EM of *L. oakridgensis* ATCC 33761 grown in AYE medium for one day at 30 °C. Bar represents 1 µm. (D-F) Cellulose (D), starch (E) and casein (F) degradation by *Legionella* strains on agar plates. Supplemented agar plates with 0.1% cellulose (Soluble derivative CMC), 0.1% starch or 0.9% casein inoculated with cell-free culture supernatants or whole cells (pellet). Halos around the inoculation site revealed the presence of degrading activity after 3 days of incubation at 37 °C. The results represented are representative of at least 3 independent experiments.

Fig. S3. Mauve alignment of the genomes of (A) *L. oakridgensis* strains ATCC 33761 and RV-2-2007 (draft genome) and (B) *L. oakridgensis* ATCC 33761 and *L. pneumophila* Corby. The colored boxes represent homologous segments free of genomic rearrangements. Blocks below the center line indicate regions with inverse orientation. Homologous regions are connected by lines between genomes. Non-boxed regions lack homology between genomes. Within each block there is a similarity profile of the DNA sequences. White areas indicate that the sequences are specific to a genome.

Fig. S4. Organization of the IncF plasmids of *L. oakridgensis* strains ATCC 33761 (A, *Loa*) and RV-2-2007 (B, *Lor*). Genes (ORFs) are indicated by arrows. Gene names are given above and the gene number below the genes. Genes of the transfer region (*tra*) are given in black (region I). Plasmid of *Loa* exhibits an additional region (II) putatively involved in metal ion resistance and *Lor* a region (II) encoding a putative type I modification/restriction (MR) system.

Table 1. Comparison of *L. oakridgensis* and other *Legionella* genomes

Species	**Size (bp)**	**Plasmids**	**Protein coding genes**	**G+C content (%)**	**Reference**
L. oakridgensis ATCC 33761	2773209	1	2932	40.86	This study
L. oakridgensis RV-2-2007	~2861000[a]	1	nd[b]	40.8	This study
Lp[c] Philadelphia	3397754	0	2943	38.3	Chien *et al*., 2004
Lp Paris	3503610	1	3027	38.4	Cazalet *et al*., 2004
Lp Lens	3345687	1	2878	38.4	Cazalet *et al*., 2004
Lp Corby	3576469	0	3204	38.5	Glöckner *et al*., 2008; Steinert *et al*., 2007
L. longbeachae NSW150/ D-4968	4077332/ 4050119	1	3512/ 3821	37	Cazalet *et al*., 2010; Kozak *et al*., 2010
L. drancourtii LLAP12	4062386	0	3965	39	Moliner *et al*., 2009
L. dumoffii TEX-KL/NY-23	nd[a]	2	~3562/~3637	39.7	Qin *et al*., 2013

[a] draft genome; [b] nd= not determined; [c] *L. pneumophila*

Table 2. General secretory (Sec) genes and Dot/Icm effector proteins

Sec genes

Gene name	***loa*-number**	**Gene product**
secA	*1871*	General secretory protein A
secB	*2256*	General secretory protein B
secD	*1060*	General secretory protein D
secE	*0375*	General secretory protein E
secF	*1061*	General secretory protein F
secG	*2731*	General secretory protein G
secY	*0410*	General secretory protein Y
yaiC	*1059*	General secretory protein
yidC	*2947*	General secretory protein

Dot/Icm effector proteins

Protein name	***loa*-number**	**Description/proposed function**
AnkH (LegA3)	*0901*	Ankyrin, NFkappaB inhibitor
AnkI (LegAS4)	*2188*	Ankyrin, SET domain
AnkK (LegA5)	*2268*	Ankyrin
AnkY (LegA9)	*1776*	Ankyrin
Astacin	*1581*	Astacin protease
Ceg25-like	*2231*	Coiled-coil
CegC2-like	*0163*	Ninein

LegC7-like	*1402*	Putative TATA-binding
LidA-like	*1171*	Rab1 binding and sequestration
Lpg1717	*2053*	Unknown
Lpg2527	*1821*	Coiled-coil domain
SdhA	*0799*	Coiled-coil, anti apoptosis
in silico identified, putative:	*0317, 0517, 1390, 1430, 1592, 2000, 2243, 2761, 2768, 2869, 2878, 2879*	Unknown

Table 3. Eukaryotic-like proteins and proteins with domains preferentially found in eukaryotic proteins

Eukaryotic-like proteins

Gene name or product or domain	***loa***	**Best BLAST Hit**
with no homolog in *Legionella*:		
Actacin family protein	*1581*	Q7Q209, *Anopheles gambiae*
Acyl-CoA synthetase	*2517*	D8FE94, delta proteobacterium Naph S2
CelA (Endoglucanase)	*1565*	B2STW0, *Xanthomonas oryzae*
CelB (Endoglucanase)	*1304*	B2STW0, *Xanthomonas oryzae*
Chitinase class I	*2513*	A4CA61, *Pseudoalteromonas tunicata* D2
Glycosyl transferase family 2	*0283*	Q4P697, *Ustilago maydis*
Multimeric flavodoxin	*1387*	B8BRD8, *Thalassiosira pseudodonana*
Putative UDP-sulfoquinovose synthase	*0052*	D3RMC8, *Allochromatium vinosum*
putative uncharacterized protein	*2024*	A8PLV0, *Ricketsiella grylli*
Short chain dehydrogenase	*1547*	A3Z051, *Synechococcus* sp WH 5701
Troponin-like, Tyr sulfation site	*2763*	Q7SOC9, *Neurospora crassa*
Troponin-like, Tyr sulfation site	*2549*	DON7S0, *Phytophtora infestans* T30-4
UbiB	*0943*	AOEA14, *Paramecium tetraurelia*
Uncharacterized protein	*2427*	C7PTG1, *Chitinophaga pinensis*
with a homolog in *Legionella*:		
3-Methyladenine DNA glycosylase	*1905*	Lpl0656[a]
5' nucleotidas family	*2493*	Lpp0109[a]
6-pyruvoyl tetrahydropterin synthase	*2933*	Llb_1699[a]
AhpC/TSA family	*0019*	Lpw_00101[a]
Ankyrin repeat protein	*2879*	Llb0528
Carbamoyl phosphate synthase, subunit	*2299*	Ldg_0664[a]
Carnitine racemase	*1261*	Ldg_2046
CoA-binding domain	*1464*	Lpw06901
Collagen triple helix repeat	*1840*	Lpp2697
Collagen triple helix repeat	*1084*	Lpp2697
Cysteine synthase	*0120*	Ldg_1125
Cytochrome p450	*0739*	Lpc_2075
DegP protease (HtrA)	*1881*	Lpl0935
Deoxyribodipyridine photolyase	*2810*	Lpl0266

Endonuclease	*1567*	Lpp1144
Exinuclease ABC A subunit	*1195*	Lpp1144
Exonuclease III, endonuclease	*2457*	Lpp0702
FAD-dependent oxidoreductase	*2056*	Llb_0031
Glutamine amidotransferase	*0341*	Lpg2606
Glutamine amidotransferase	*1599*	Lpl2649
Golgi nucleoside diphosphatase	*1277*	Lpc_1359
HAD superfamily hydrolase	*0908*	Llo_0589[a]
Histone methylation protein	*0113*	Lpa_04331[a]
NADH flavin oxidoreduktase	*0806*	Lpc_1816
Peptide chain relase factor	*2606*	Ldg_2678
Polysaccharide deacetylase (chitin)	*0512*	Lpp1607
Pteridine reductase	*2936*	Ldg_1060
Putative chloroplast amino acid transporter	*2283*	Lpp0653
Putative lipoprotein	*1020*	Lpl0761
Putative mitochondrial carrier protein	*0737*	Llo_1358
Putative uncharacterized protein	*0212*	Lpl0173
Putative uncharacterized protein	*0095*	Lpg0087
Putative uncharacterized protein	*1265*	Ldg_1024
Putative uncharacterized protein	*1292*	Ldg_3253
Pyridoxal phosphate enzyme	*0857*	Lpg2014
Pyridoxamine 5-phosphate oxidase	*2065*	Lpg2139
S1/P1 nuclease	*1540*	Lpw_15931
S-Adenosyl-L-homocysteine hydrolase	*1921*	Llo_1088
SAM-dependent methyl transferase	*1453*	Ldg_1248
Serine carboxy peptidase	*0318*	Llo_0042
Short chain reductase	*1009*	Lpc_2583
Stress responsive A/B barrel domain	*1568*	Llo_1612
Thaumatin/PR5-like protein	*0625*	Lpp1283

Proteins with domains preferentially found in eukaryotic proteins

Protein name	***loa*-number**
Ankyrin repeat1	*0901, 1390, 1592, 1776, 2000 2188, 2243, 2268, 2869, 2879*
F-box	*0656*
Sec7 domain	--
Sel1-repeat	*0831, 1623, 1891*
Ser/Thr kinase	*2019, 2042*
SET domain	*0317, 2188, 2243*
SNARE	*1976, 2371, 2579*
TPR	*0706, 0831, 1004, 1327, 1558 1560, 1561, 1623, 1789, 2262, 2268*
Tyr-Ptase	*0748*
U-box	--

[a] Ldg, *L. drancourtii*; Llo, *L. longbeachae* NSW159; Lpa, *L. pneumophila* Alcoy; Lpc, *L. pneumophila* Corby; Lpg, *L. pneumophila* Phil-1; Lpl, *L. pneumophila* Lens; Lpp, *L. pneumophila* Paris; Lpw, *L. pneumophila* 130b

Table 4. Regulatory proteins and genes encoding virulence factors

Regulatory genes	***loa*-number**	**function**
cpxA	*1775*	Two component sensor protein CpxA
cpxR	*1774*	Transcriptional regulator protein CpxR
csrA (chromosomal)	*1097, 1513*	Carbon storage regulator A
csrA2 (*lvrC*, genomic island)	*0186*	Carbon storage regulator A
fis1, fis2, fis3	*0547, 1608, 2131*	DNA-binding protein Fis
fleQ	--	Transcriptional regulator FleQ
fleR	--	Transcriptional regulator FleR
flgM	*1456*	Anti-sigma-28 factor FlgM
fliA	*2161*	RNA polymerase sigma-28 factor FliA
fur	*0437*	Ferric-uptake regulation protein Fur
hfq	*0017*	Translational regulator Hfq
letA	*0826*	*Legionella* transmission regulator LetA
letE	*0542*	Transmission trait enhancer LetE
letS	*0926*	*Legionella* transmission sensor LetA
ompR	*2529*	Transcriptional regulator protein OmpR
pilR	*1434*	Type IV pilus regulator PilR
pilS	*1433*	Type IV pilus sensor PilS
pmrA	*1966*	DNA-binding response regulator PmrA
pmrB	*1968*	Two componen sensor protein PmrB
rpoD	*0764*	RNA polymerase sigma-70 factor RpoD
rpoH	*2655*	RNA polymerase sigma-32 factor RpoH
rpoN	*0795*	RNA polymerase sigma-54 factor RpoN
rpoS	*1974*	RNA polymerase sigma factor RpoS

Selection of virulence genes

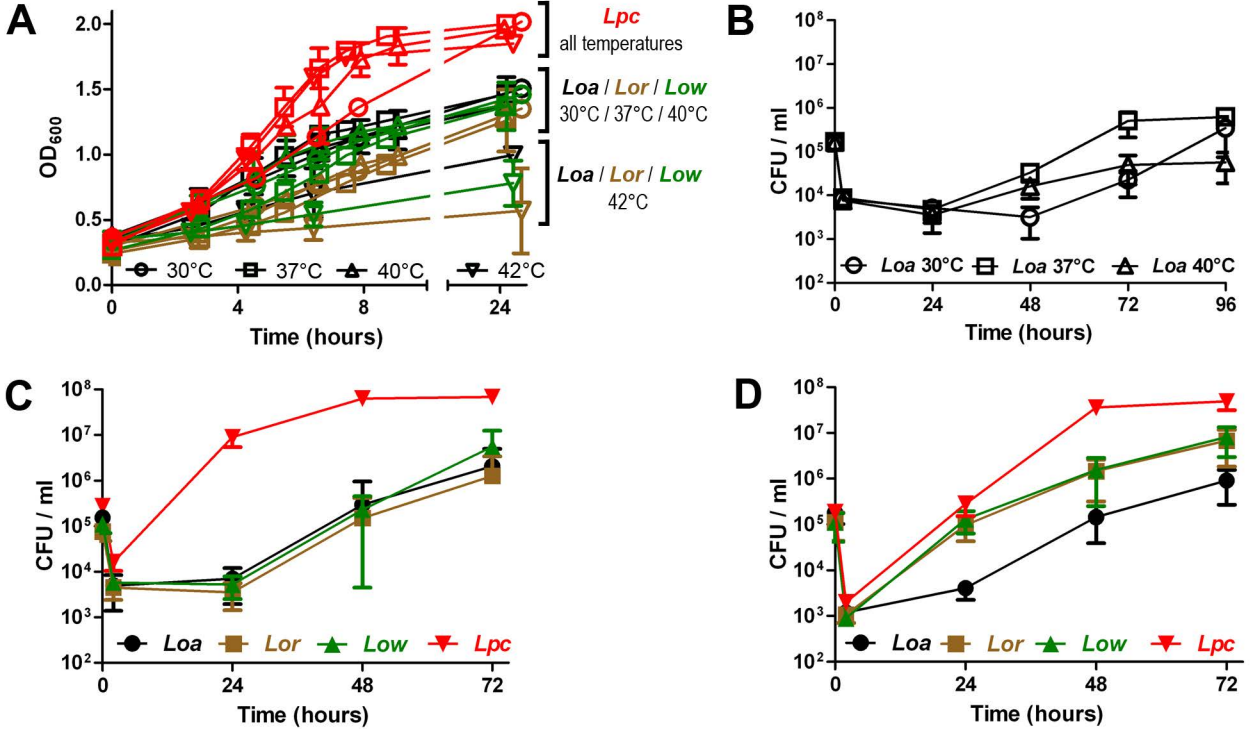
Gene or protein name	***loa*-number**	**Description/proposed function**
Beta lactamase	*0260, 0666, 1588*	Beta lactamase
bipA	*2771*	GTP-binding protein A
celA, celB	*1565, 1304*	Endoglucanase, putative cellulase
Chitinase	*2513*	Chitinase
enhA, enhA2	*0829, 1647*	Enhanced entry protein A
enhB	*0830*	Enhanced entry protein B
enhC	*0831*	Enhanced entry protein C
ereA	*2407*	Erythromycin esterase
gpsA	*2257*	Glycerol-3-phosphate dehydrogenase (GpsA)
htpB/A (Hsp60)	*1036/1035*	Heat shock protein 60
lasB	*2804*	Metallo proteinase
lbtABC	*0324-26*	Legiobactin
lvrE, lvrE2	*199, 1572*	*Legionella* virulence region protein E
mip	*1195*	Macrophage infectivity potentiator

momp	*0105,0106, 2888*	Major outer membrane protein
mviN	*0843*	Integral membrani protein MviN
Patatin	*0152, 2277, 2750*	Phospholipase (Patatin)
Phospholipases	*0809, 1463, 1487*	Phospholipase/lecithinase
proA	*0779*	Zinc metallo proteinase Msp
relA	*1761*	(p)ppGpp synthetase I
sodB, sodC	*0100, 0804*	Superoxide dismutase
spoT	*0864*	Guanosine-3,5-bis 3-pyrophosphohydrolase
Sulfotransferase	*2026*	Putative sulfotransferase
Sulfotransferase	*0043*	Putative sulfotransferase

Table 5 Main metabolic pathways

Gene or protein name	***loa*-number**	**Gene product**
Glycolysis		
Glk	*1753*	Glucose kinase
Pgi	*1094*	Phosphoglucose isomerase
PfkA	*0925*	Phosphofructokinase
Fba	*0782*	Fructose-1,6-bisphosphate aldolase
TpiA	*2732*	Triosephosphate isomerase
Gap	*0166*	Glycerole 3-phosphate dehydrogenase
Pgk	*0165*	Phosphoglycerate kinase
Pgm	*2344*	Phosphoglycerate mutase
Eno	*2648*	Enolase
PykA	*0164*	Pyruvate kinase
PpsA (gluconeogenesis)	*1209*	PEP synthase
Loa2092	*2094*	Fructose-2,6 bisphosphatase
Loa2135	*2135*	Archael fructose-1,6 bisphosphase/Inositol monophosphatase
Entner-Doudoroff pathway		
Glk	*1532*	Glucose kinase
Zwf	*1756*	Glucose-6-phosphate dehydrogenase
Pgl	*1755*	6-phosphogluconolactonase
Edd	*1754*	6-phosphogluconate dehydratase
Eda-1, Eda-2	*1752, 1170*	2-keto-3-deoxy-6-phosphogluconate aldolase
TktA	*0167*	Transketolase
Pentose-Phosphate pathway		
Gnd	--	6-phosphogluconate dehydrogenase
Rpe	*1938*	Ribulose-5-phosphate epimerase
RpiA	*0121*	Ribose-5-phosphate isomerase
TktA	*0167*	Transketolase
Tal	--	Transaldolase
TCA		
Pyruvate dehydrogenase	*1811-1813*	Pyruvate dehydrogenase complex
	1542-1544	Pyruvate dehydrogenase complex

GltA (CIT)	*1799*	Citrate synthase
AcnA (ACN)	*0585, 1683*	Aconitase
IcdA (IDH)	*1220*	Isocitrate dehydrogenase
SucA	*0536, 0537*	Succinyl-CoA synthase subunit A
SucB	*0538*	Succinyl-CoA synthase subunit B
SucC	*0539*	Succinyl-CoA synthase subunit C
SucD	*0540*	Succinyl-CoA synthase subunit D
SdhA	*0534*	Succinate dehydrogenase subunit A
SdhB	*0535*	Succinate dehydrogenase subunit B
SdhC	*0532*	Succinate dehydrogenase subunit C
SdhD	*0534*	Succinate dehydrogenase subunit D
FumC	*0080, 2809*	Fumarase
Mdh	*0771*	Malate dehydrogenase
KorA	*1176*	2-oxoacid:acceptor oxidoreductase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OD600
2.0
1.5
1.0
0.5
0.0
0
4
8
24
Time (hours)
30°C
37°C
40°C
42°C
Lpc
all temperatures
Loa / Lor / Low
30°C / 37°C / 40°C
Loa / Lor / Low
42°C
B
CFU / ml
Loa 30°C
Loa 37°C
Loa 40°C
0
24
48
72
96
Time (hours)
C
CFU / ml
Loa
Lor
Low
Lpc
0
24
48
72
Time (hours)
D
CFU / ml
Loa
Lor
Low
Lpc
0
24
48
72
Time (hours)

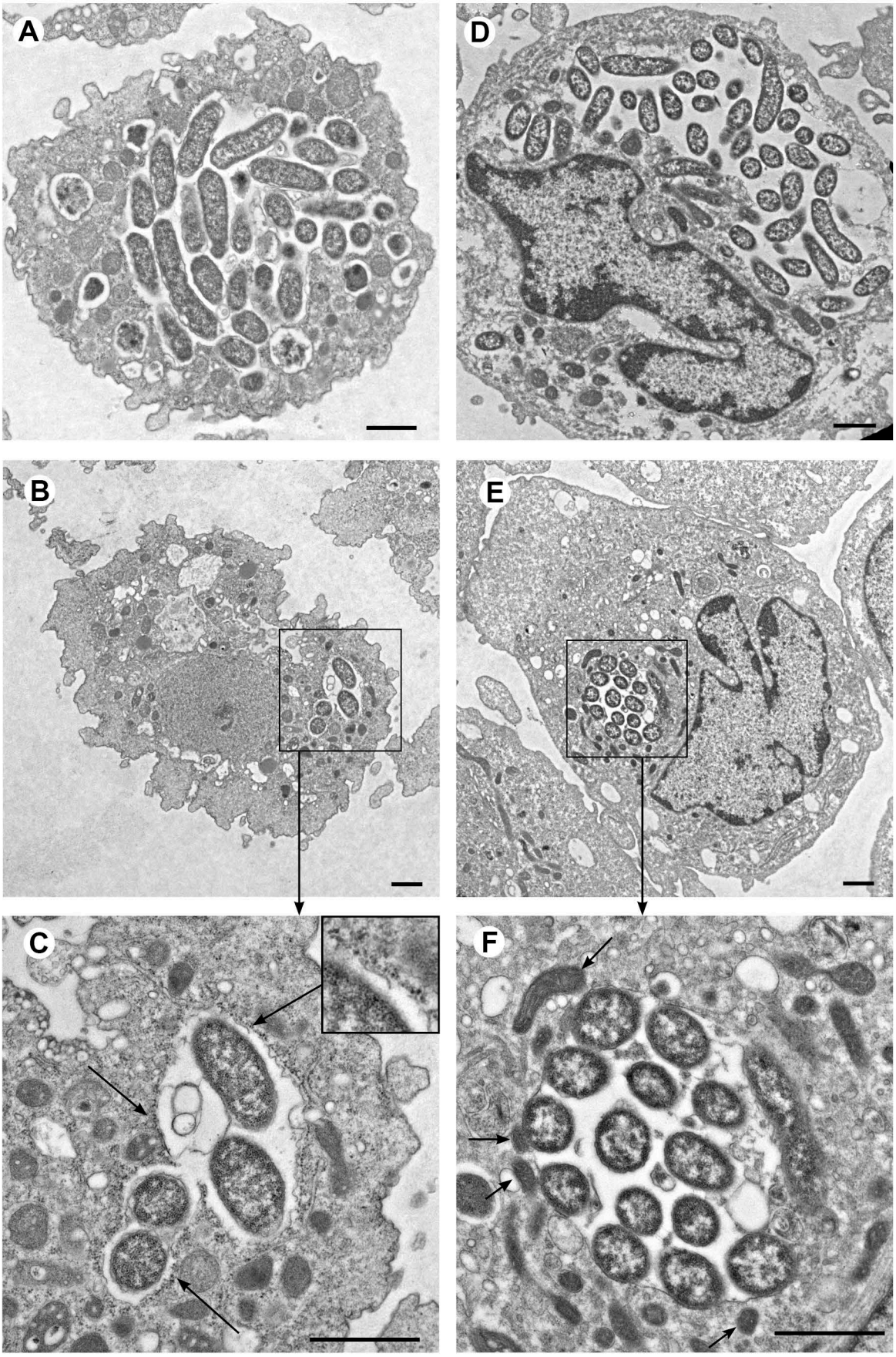
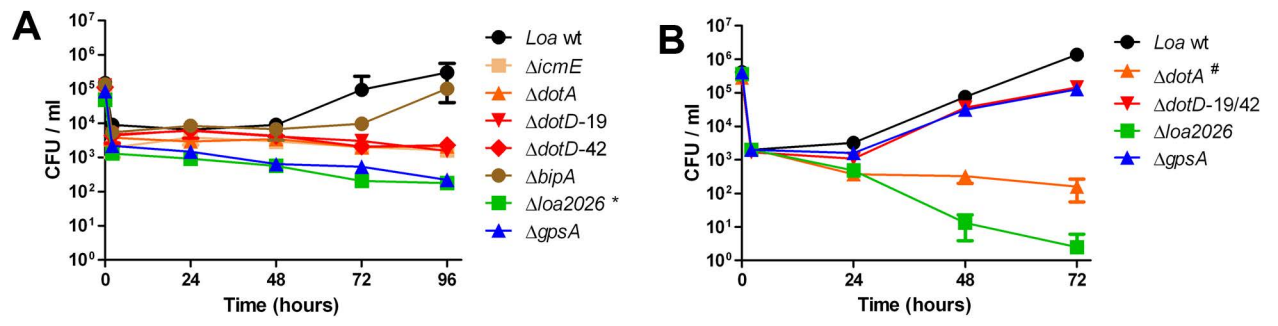
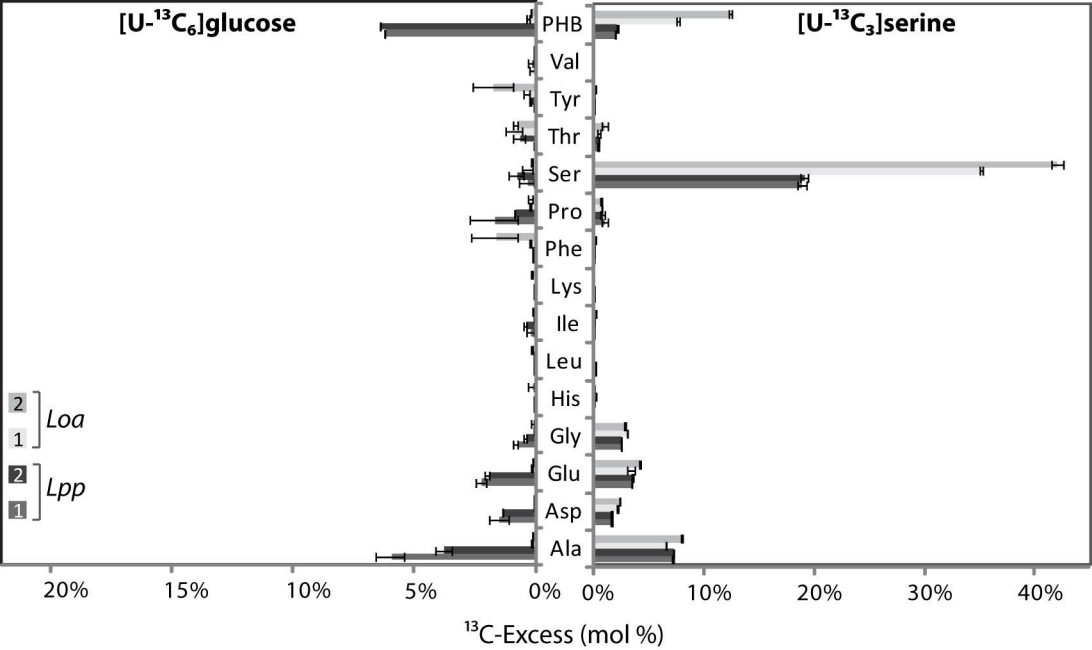
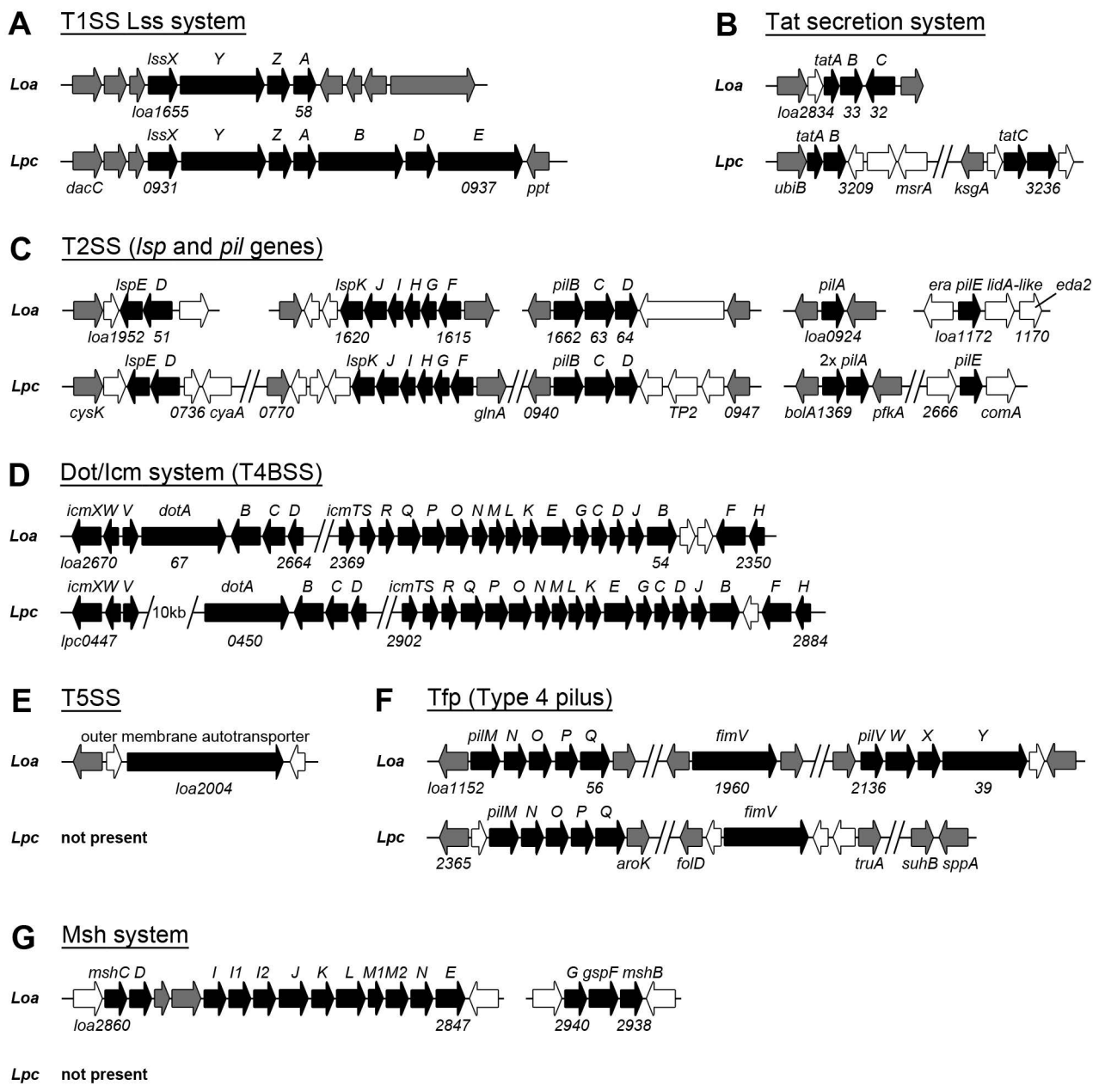


Fig.2


A
CFU / ml
Time (hours)
Loa wt
ΔicmE
ΔdotA
ΔdotD-19
ΔdotD-42
ΔbipA
Δloa2026 *
ΔgpsA
B
CFU / ml
Time (hours)
Loa wt
ΔdotA #
ΔdotD-19/42
Δloa2026
ΔgpsA


[U-13C6]glucose
[U-13C3]serine
PHB
Val
Tyr
Thr
Ser
Pro
Phe
Lys
Ile
Leu
His
Gly
Glu
Asp
Ala
2
1
Loa
2
1
Lpp
20%
15%
10%
5%
0%
0%
10%
20%
30%
40%
13C-Excess (mol %)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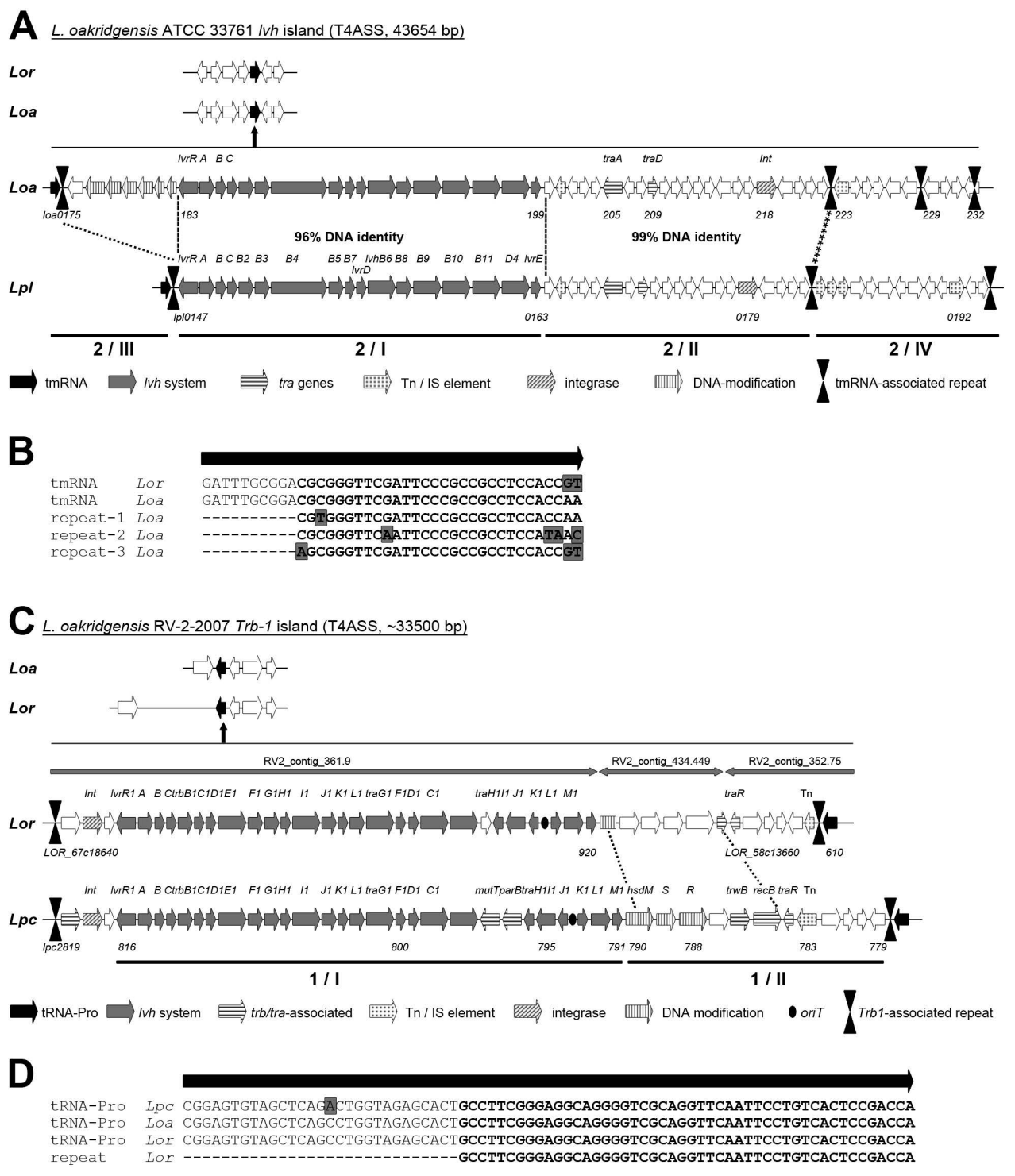
T1SS Lss system
Loa
lssX Y Z A
loa1655 58
Lpc
lssX Y Z A B D E
dacC 0931 0937 ppt
B
Tat secretion system
Loa
tatA B C
loa2834 33 32
Lpc
tatA B tatC
ubiB 3209 msrA ksgA 3236
C
T2SS (lsp and pil genes)
Loa
lspE D
loa1952 51
lspK J I H G F
1620 1615
pilB C D
1662 63 64
pilA
loa0924
era pilE lidA-like eda2
loa1172 1170
Lpc
lspE D
cysK 0736 cyaA
lspK J I H G F
0770 glnA
pilB C D
0940 TP2 0947
2x pilA
bolA1369 pfkA
pilE
2666 comA
D
Dot/Icm system (T4BSS)
Loa
icmXW V
dotA
B C D
icmTS R Q P O N M L K E G C D J B
F H
loa2670 67 2664 2369 54 2350
Lpc
icmXW V
10kb
dotA
B C D
icmTS R Q P O N M L K E G C D J B
F H
lpc0447 0450 2902 2884
E
T5SS
Loa
outer membrane autotransporter
loa2004
Lpc
not present
F
Tfp (Type 4 pilus)
Loa
pilM N O P Q
fimV
pilV W X Y
loa1152 56 1960 2136 39
Lpc
pilM N O P Q
fimV
2365 aroK folD truA suhB sppA
G
Msh system
Loa
mshC D
I I1 I2 J K L M1M2 N E
G gspF mshB
loa2860 2847 2940 2938
Lpc
not present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L. oakridgensis ATCC 33761 lvh island (T4ASS, 43654 bp)
Lor
Loa
lvrR A B C
traA traD Int
loa0175 183 199 205 209 218 223 229 232
96% DNA identity
99% DNA identity
lvrR A B C B2 B3 B4 B5 B7 lvhB6 B8 B9 B10 B11 D4 lvrE
lvrD
Lpl
lpl0147 0163 0179 0192
2 / III
2 / I
2 / II
2 / IV
tmRNA
lvh system
tra genes
Tn / IS element
integrase
DNA-modification
tmRNA-associated repeat
B
tmRNA Lor GATTTGCGGACGCGGGTTCGATTCCCGCCGCCTCCACCGT
tmRNA Loa GATTTGCGGACGCGGGTTCGATTCCCGCCGCCTCCACCAA
repeat-1 Loa ----------CGTGGGTTCGATTCCCGCCGCCTCCACCAA
repeat-2 Loa ----------CGCGGGTTCAATTCCCGCCGCCTCCATAAC
repeat-3 Loa ----------AGCGGGTTCGATTCCCGCCGCCTCCACCGT
C
L. oakridgensis RV-2-2007 Trb-1 island (T4ASS, ~33500 bp)
Loa
Lor
RV2_contig_361.9
RV2_contig_434.449
RV2_contig_352.75
Int lvrR1 A B CtrbB1C1D1E1 F1 G1H1 I1 J1 K1 L1 traG1 F1D1 C1
traH1I1 J1 K1 L1 M1
traR
Tn
LOR_67c18640 920 LOR_58c13660 610
Lpc
Int lvrR1 A B CtrbB1C1D1E1 F1 G1H1 I1 J1 K1 L1 traG1 F1D1 C1
mutTparBtraH1I1 J1 K1 L1 M1 hsdM S R trwB recB traR Tn
lpc2819 816 800 795 791 790 788 783 779
1 / I
1 / II
tRNA-Pro
lvh system
trb/tra-associated
Tn / IS element
integrase
DNA modification
oriT
Trb1-associated repeat
D
tRNA-Pro Lpc CGGAGTGTAGCTCAGACTGGTAGAGCACTGCCTTCGGGAGGCAGGGGTCGCAGGTTCAATTCCTGTCACTCCGACCA
tRNA-Pro Loa CGGAGTGTAGCTCAGCCTGGTAGAGCACTGCCTTCGGGAGGCAGGGGTCGCAGGTTCAATTCCTGTCACTCCGACCA
tRNA-Pro Lor CGGAGTGTAGCTCAGCCTGGTAGAGCACTGCCTTCGGGAGGCAGGGGTCGCAGGTTCAATTCCTGTCACTCCGACCA
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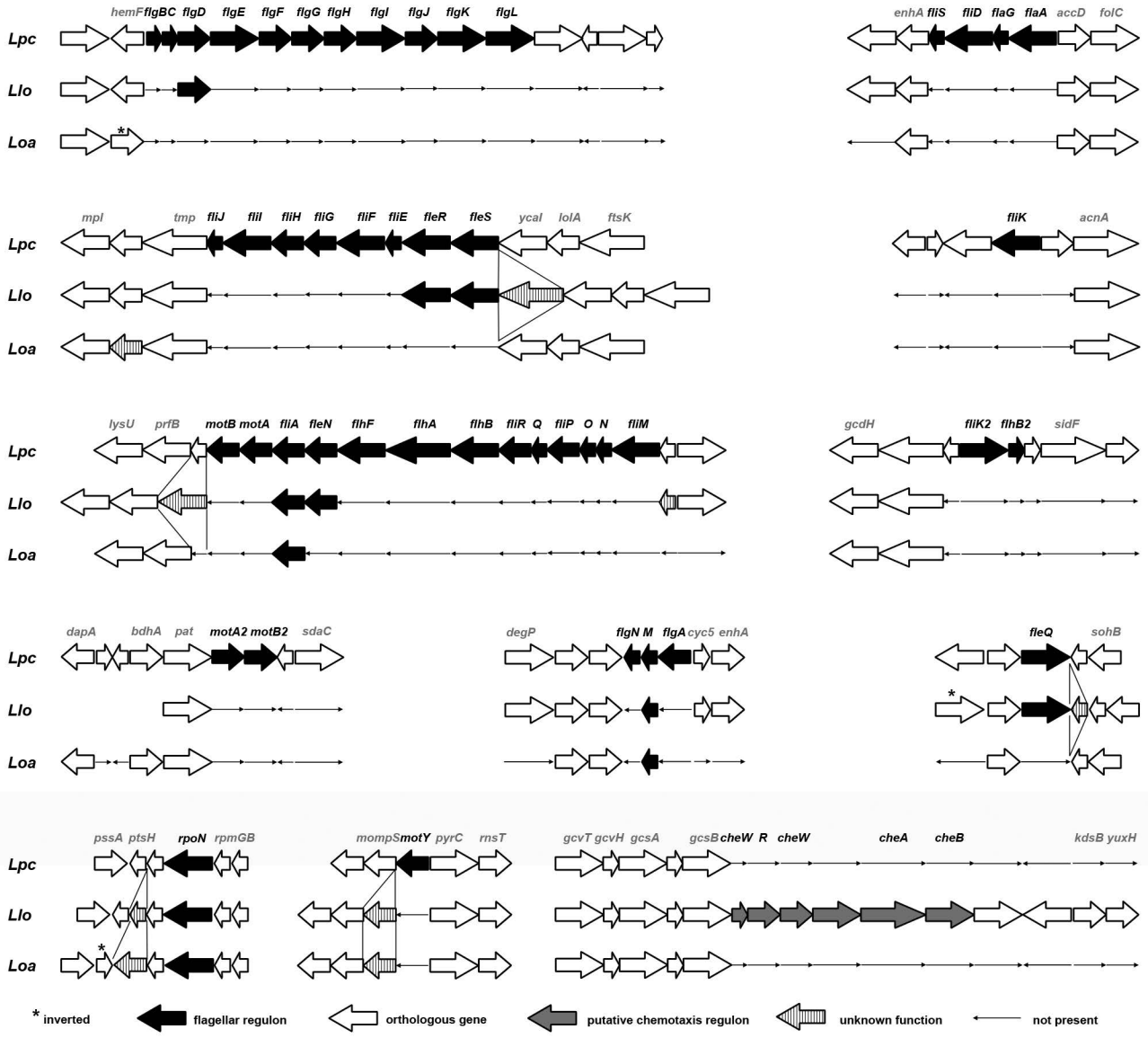
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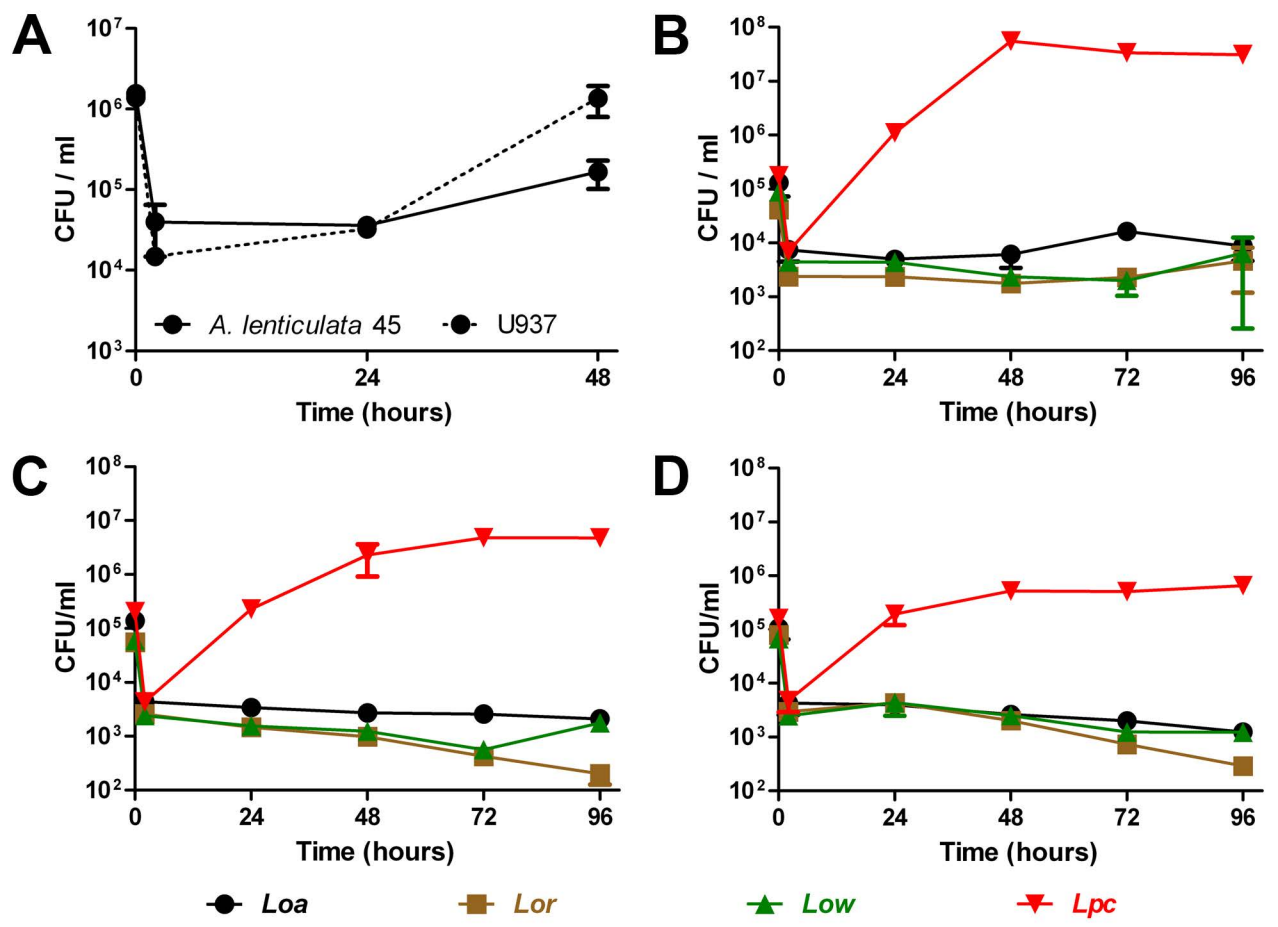
enhA fliS fliD flaG flaA accD folC
Lpc
Llo
Loa
mpl tmp fliJ fliI fliH fliG fliF fliE fleR fleS ycal lolA ftsK
fliK acnA
lysU prfB motB motA fliA fleN flhF flhA flhB fliR Q fliP O N fliM
gcdH fliK2 flhB2 sidF
dapA bdhA pat motA2 motB2 sdaC
degP flgN M flgA cyc5 enhA
fleQ sohB
pssA ptsH rpoN rpmGB
mompS motY pyrC rnsT
gcvT gcvH gcsA gcsB cheW R cheW cheA cheB kdsB yuxH
* inverted
flagellar regulon
orthologous gene
putative chemotaxis regulon
unknown function
not present


A
B
C
D
CFU / ml
CFU/ml
Time (hours)
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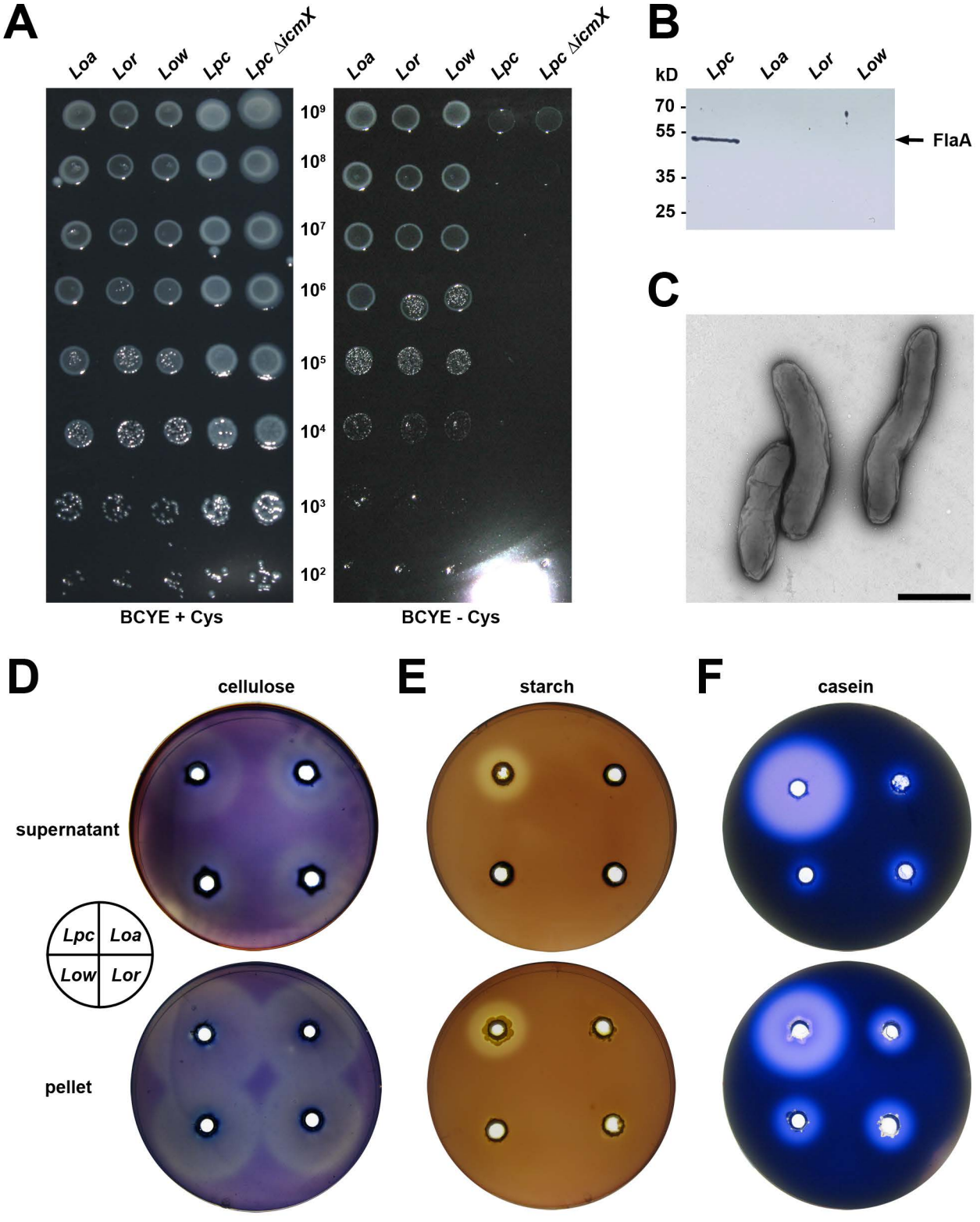
U937
Loa
Lor
Low
Lpc


A
Loa
Lor
Low
Lpc
Lpc ΔicmX
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
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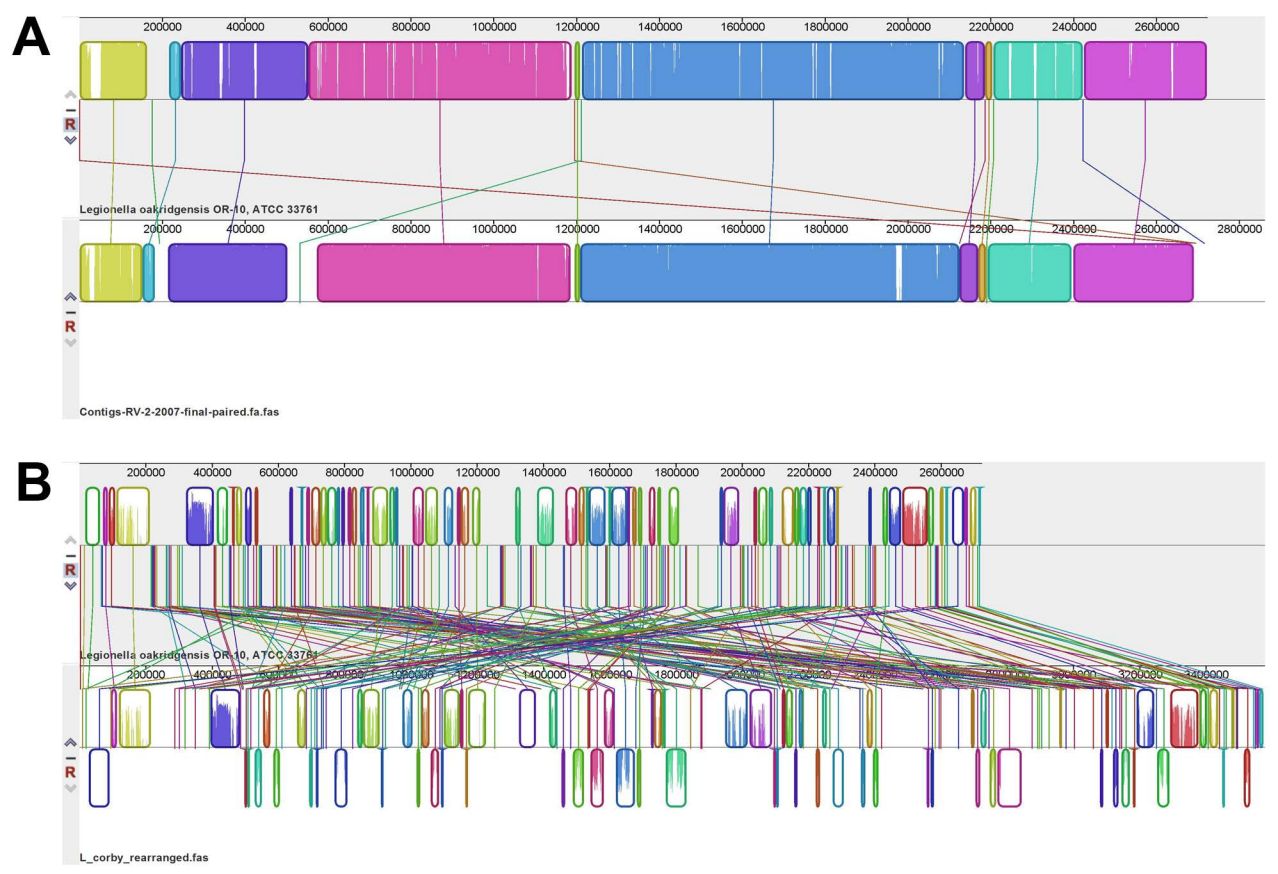
10²
BCYE + Cys
BCYE - Cys
B
kD
70
55
35
25
FlaA
C
D
cellulose
supernatant
Lpc
Loa
Low
Lor
pellet
E
starch
F
casein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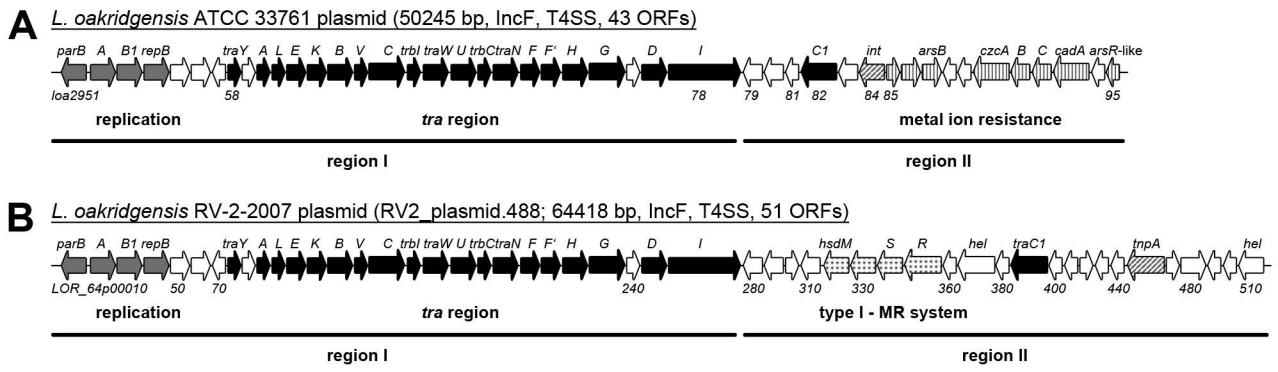
200000
400000
600000
800000
1000000
1200000
1400000
1600000
1800000
2000000
2200000
2400000
2600000
Legionella oakridgensis OR-10, ATCC 33761
2800000
Contigs-RV-2-2007-final-paired.fa.fas
B
Legionella oakridgensis OR-10, ATCC 33761
L_corby_rearranged.fas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L. oakridgensis ATCC 33761 plasmid (50245 bp, IncF, T4SS, 43 ORFs)
parB A B1 repB traY A L E K B V C trbI traW U trbCtraN F F' H G D I C1 int arsB czcA B C cadA arsR-like
loa2951 58 78 79 81 82 84 85 95
replication
tra region
metal ion resistance
region I
region II
B
L. oakridgensis RV-2-2007 plasmid (RV2_plasmid.488; 64418 bp, IncF, T4SS, 51 ORFs)
parB A B1 repB traY A L E K B V C trbI traW U trbCtraN F F' H G D I hsdM S R hel traC1 tnpA hel
LOR_64p00010 50 70 240 280 310 330 360 380 400 440 480 510
replication
tra region
type I - MR system
region I
region II