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# An oxaloacetate decarboxylase homologue protein influences the intracellular survival of *Legionella pneumophila*

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**Abstract**

*Legionella pneumophila* is a facultative intracellular parasite which is able to survive in various eukaryotic cells. We characterised a Tn*5*-mutant of the *L. pneumophila* Corby strain and were able to identify the insertion site of the transposon. It is localised within an open reading frame which shows high homology to the α-subunit of the oxaloacetate decarboxylase (OadA) of *Klebsiella pneumoniae*. The OadA homologous protein of *L. pneumophila* was detected in the wild-type strain by Western blotting. Since the intracellular multiplication of the *oad*A$^-$ mutant strain is reduced in guinea pig alveolar macrophages and human monocytes, it is concluded that the *oad*A gene product has an effect on the intracellular survival of *L. pneumophila*.

*Keywords:* *Legionella pneumophila*; Oxaloacetate decarboxylase; Intracellular survival

## 1. Introduction

*Legionella pneumophila*, the causative agent of Legionnaires' disease, is a Gram-negative, rod-shaped, facultative intracellular bacterium [1]. In its natural aquatic habitat as well as in man-made water systems, *L. pneumophila* is able to parasitise and replicate within free-living amoebae [2,3]. From this environment *L. pneumophila* can be transmitted by inhalation of bacteria containing aerosols to humans where it colonises the respiratory tract and multiplies intracellularly in the alveolar macrophages [4]. Only a few factors have been identified and characterised that contribute to the survival of the bacteria in eukaryotic cells [5], such as the major outer membrane porin OmpS [6,7], the Mip protein (macrophage infectivity potentiator) [8] and the two genetic loci designated *icm* (intracellular multiplication) and *dot* (defect in organelle trafficking) [9,10]. To date, little is known about the physiology and metabolism of *Legionella* which differs from other bacterial species in using solely amino acids as main energy source and not glucose [11], and being very fastidious if cultivated under laboratory conditions. Nothing is known about the physiological parameters of *Legionella* strains, which may be essential to survive intracellularly in the different host cells.

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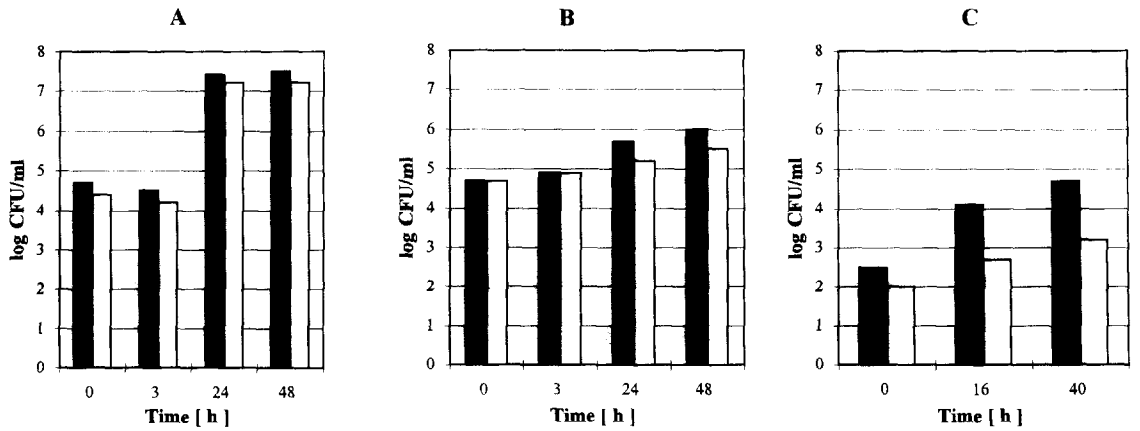


Fig. 1. Intracellular survival of *L. pneumophila* strains in *A. castellanii* (A), human monocytes (B) and guinea pig alveolar macrophages (C). After an incubation time of 0, 3, 24 and 48 h, the formation of CFU of bacteria was determined in doublicates taken from three independent experiments. The absence of error bars indicated that the standard deviation of the mean was smaller than the capacity of the graphic program. Shown are the wildtype *L. pneumophila* Corby (black bars) and the Tn*5*-mutant *L. pneumophila* Corby E 25 (white bars).

In this report, we describe the identification of a gene in *L. pneumophila* with homology to the α-subunit of the oxaloacetate decarboxylase of *Klebsiella pneumoniae*. This gene, designated *oad*A, seems to modulate the ability of *L. pneumophila* to grow within alveolar macrophages of guinea pigs and human monocytes.

## 2. Material and methods

### 2.1. Bacterial strains

The strain *L. pneumophila* Corby and the Tn*5*-derivative *L. pneumophila* Corby E 25 were described elsewhere [12,13]. The *Legionella* strains were cultured on buffered charcoal yeast extract (BCYE)-agar at 37°C with a 5% $CO_2$ atmosphere for 2–3 days [14].

The *Escherichia coli* strain DH5α [15] was used for DNA amplification and was grown on Luria-Bertani (LB)-agar or in LB-broth. For cloning purposes the vector pUC18 and the vector pBC KS+ were used.

### 2.2. Infection of eukaryotic host cells with *L. pneumophila*

For invasion experiments human blood monocytes and alveolar macrophages of guinea pigs were used. In addition, the protozoa *Acanthamoeba castellanii* was infected as an example for a natural host of *L. pneumophila*. The assays were carried out as described previously [13,16,17].

### 2.3. DNA manipulations and sequence analysis

Isolation of plasmid DNA and recombinant DNA techniques were performed as described by Sambrook et al. [18]. DNA amplification by polymerase chain reaction (PCR) was performed according to the method of Saiki et al. [19] using a Thermocycler 60 apparatus from Biomed (Theres, Germany). DNA sequence determination was carried out as described by the manufacturer with the T7 polymerase sequencing kit from Pharmacia (Freiburg, Germany) by using specific oligonucleotides as primers. Sequence data were analysed and displayed using the University of Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package [20]. Homology searches were conducted against the Genbank, EMBL, and Swiss-Prot data-bases using the FASTA program [21].

### 2.4. SDS-PAGE and Western blot analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (10%) as described previously [22]. Western blotting was performed by semidry

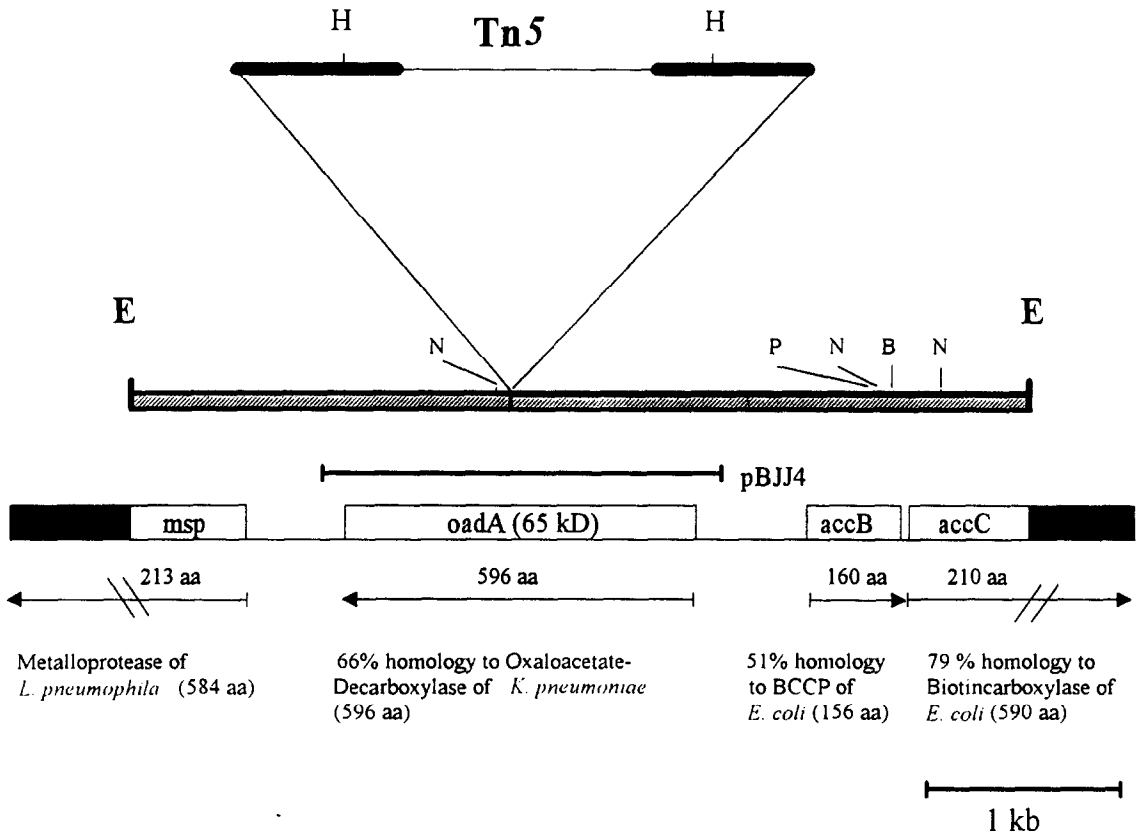


Fig. 2. Map of the subcloned Tn*5* insertion region of the mutant *L. pneumophila* E 25. Coding regions corresponding to *msp*, *oad*A, *acc*B and *acc*C are indicated by arrows. The sequenced region is shown as shaded bars and the unkwown sequence as black bars. BCCP is the biotin carboxyl carrier protein. Restriction endonuclease sites: B, *Bam*HI; H, *Hind*III; E, *Eco*RI; N, *Nsi*I; P, *Pvu*I.

electroblotting in a graphite chamber according to the method of Kyhse-Anderson [23]. Polyclonal rabbit antibodies against the OadA of *K. pneumoniae* were applied to the Western blot in a 1 : 500 dilution. For the detection of bound antibodies, peroxidase conjugated swine anti-rabbit IgG antibodies (DAKO, Hamburg, Germany) were used. The colour reaction was developed using 4,1-chloronaphtol.

## 3. Results and discussion

### 3.1. Intracellular survival of the L. pneumophila Corby Tn5-mutant E 25 in several host cells

In order to identify new virulence-associated genes, a mutagenesis using the transposon Tn*5* was recently performed with the *L. pneumophila* strain Corby [13], and the Tn*5*-mutant E 25 was identified. The mutant E 25 showed no difference compared to the wild-type strain when tested for the expression of the genes *ppl*A, *mip*, *lly*, *htp* and *fla*A in Western Blot analysis with specific antibodies (data not shown). To determine the capacity of mutant strain E 25 to multiply within host cells, we infected a variety of potential host cells with E 25 and its parental strain. The results are shown in Fig. 1. The wild-type strain *L. pneumophila* Corby and the mutant strain *L. pneumophila* E 25 were tested in cells such as human blood monocytes, cultured alveolar macrophages of guinea pigs and their natural aquatic host *Acanthamoeba castellanii*. In *A. castellanii* the Tn*5*-mutant E 25 showed only a 2-fold unspecific difference in its ability to multiply intracellularly compared to the wild-type strain *L. pneumophila* Corby (Fig. 1A). In contrast to this protozoa model a significant im-

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          51                                                  100
K.pn.  FDACIRFLGE DPWVRLRELK KAMPKTPLQM LLRGQNLLGY RHYADDVVER
S.ty.  FDACIRFLGE DPWLRLRELK KAMPKTPLQM LLRGQNLLGY RHYADDVVER
L.pn.  FDACLRFLKE DPWSRLRQLR QALPNTQLSM LLRGQNLLGY RNYADDVVRA

         101                                                  150
K.pn.  FVERAVKNGM DVFRVFDAMN DPRNMQAALQ AVRRHGAHAQ GTLSYTTSPA
S.ty.  FVERAVKNGM DVFRVFDAMN DPRNMKAALQ AVRSHGAHAQ GTLSYTTSPA
L.pn.  FVKLAVNNGV DVFRVFDALN DARNLKVAID AIKSHKKHAQ GAICYTTSPV

         151                                                  200
K.pn.  HTLQTWLDLT EQLLETGVDS VAIKDMSGIL TPHAAFELVS EIKKRYDVTL
S.ty.  HTLQTWLDLT EQLLETGVDS IAIKDMSGIL TPMAAYELVS EIKKRFEVRL
L.pn.  HTLDNFLELG KKLAEMGCDS IAIKDMAGLL TPTVTVELYA GLKQATGLPV

         201                                                  250
K.pn.  HLHCHATTGM AEMALLKAIE AGVDGVDTAI SSMSATYGHP ATEALVATLA
S.ty.  HLHCHATTGM AEMALLKAIE AGVDGVDTAI SSMSATYGHP ATEALVATLA
L.pn.  HLHSHSTSGL ASICHYEAVL AGCNHIDTAI SSFSGGASHP PTEALVAALT

         251                                                  300
K.pn.  GTPYDTGLDI HKLESIAAYF REVRKKYHAF EGQLKGTDSR ILVAQVPGGM
S.ty.  GTEHDTGLDI LKLENIAAYF REVRKKYHAF EGQLKGYDSR ILVAQVPGGM
L.pn.  DTPYDTELDL NILLEIDDYF KAVRKKYSQF ESEAQNIDPR VQLYQVPGGM

         301                                                  350
K.pn.  LTNLEGQLKQ QSAAHRLDEV LAEIPRVRED LGFIPLVTPT SQIVGTQAVL
S.ty.  LTNLESQLKQ QNAADKLDQV LAEIPRVRED LGFIPLVTPT SQIVGTQAVL
L.pn.  ISNLYNQLKE QNALDKMDAV HKEIPRVRKD LGYPPLVTPT SQVVGTQAVI

         351                                                  400
K.pn.  NVLTGERYKT IAKETAGILK GEYGRTPAPV NAALQARVLD GADPVTCRPA
S.ty.  NVLTGERYKT IAKETAGILK GEYGHTPVPV NAALQARVLE GGAPVTCRPA
L.pn.  NVLTGERYKT ITNEVKLYCQ GKYGTPPGKI SSALRKKAIG RTEVIEVRPG

         401                                                  450
K.pn.  DLLKPELAQL EADVRRQAQE KGITLAENAI DDVLTVALFP QPGLKFLENR
S.ty.  DLLKPELAEL EADVRRQAQE KGITLAGNAI DDVLTVALFP QIGLKFLENR
L.pn.  DLLPNELDQL QNEISDLALS D......... EDVLLYAMFP EIGRQFLEQR

         451                                                  500
K.pn.  HNPAAFEPVP QAEAAQPVAK AEKPAASGVY TVEVEGKAFV VKVSDGGDVS
S.ty.  NNPAAFEPLP QAEAAQPVAK AEKPAASGIY TVEVEGKAFV VKVSDGGDIS
L.pn.  KNNQLI.... .PEPLLTQSS APDNSVMSEF DIILHGENYH VKVAGYGMIE

         501                                                  550
K.pn.  Q......... ..LTAAAPAP APAPAPASAP AAAAPAGAGT PVTAPLAGTI
S.ty.  Q......... ..LTAAVPAA SSAPVQ.... .AAAPAGAGT PVTAPLAGNI
L.pn.  HGQQSCFLWV DGVPEEVVVQ HSELHDKIER SSVNNKIGPG DITVAIPGSI

         551                                                  600
K.pn.  WKVLASEGQT VAAGEVLLIL EAMKMETEIR AAQAGTVRGI AVKAGDAVAV
S.ty.  WKVIATEGQT VAEGDVLLIL EAMKMETEIR AAQAGTVRGI AVKSGDAVSV
L.pn.  IAIHVSAGDE VKAGQAVLVI EAMKMETEIK APANGVVAEI LCQKGDKVTP

         601
K.pn.  GDTLMTLA..
S.ty.  GDTLMTLA..
L.pn.  GQVLIRVEVS
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Fig. 3. Amino acid sequence comparison of the *L. pneumophila* OadA and the oxaloacetate decarboxylases of *K. pneumoniae* and *S. typhimurium*. Conserved amino acids are printed in bold letters.

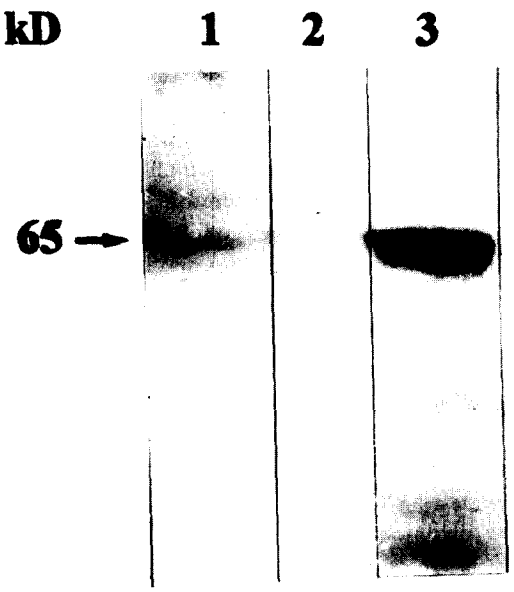


Fig. 4. Western blot analysis of total bacterial cell extracts using a polyclonal antibody against the OadA of *K. pneumoniae*. *L. pneumophila* Corby and *L. pneumophila* E 25 were grown on BCYE-agar plates for 2–3 days. Equal amounts of extracts of *L. pneumophila* Corby (lane 1), *L. pneumophila* E 25 (lane 2) and *E. coli* DH5α(pBJJ4) (lane 3) were applied to each lane.

paired intracellular growth within freshly isolated blood monocytes and cultured guinea pig alveolar macrophages could be detected (Fig. 1B,C). In comparison to the *L. pneumophila* Corby about 5-fold less bacteria were recovered from monocytes and about 10-fold less bacteria from alveolar macrophages of guinea pigs infected with the *L. pneumophila* Tn*5*-mutant E 25. These results implicate that the genetic defect of mutant strain E 25 may be of important significance for the survival of *L. pneumophila* in an environment offered by human blood monocytes and guinea pig alveolar macrophages, but not in *A. castellanii*. In spite of numerous similarities in the host–parasite relationship and the intracellular pathway of *L. pneumophila* in its different host cells like protozoa, human monocytes and alveolar macrophages [24–26], we can conclude from our data that there occurs a host specificity in certain mutants of *L. pneumophila*. Obviously some factors seem to have an influence on the intracellular survival in monocytes and macrophages, which are not important in protozoa like *A. castellanii*.

### 3.2. Identification of the Tn*5*-insertion in the *L. pneumophila* chromosome

The chromosomal DNA of the mutant *L. pneumophila* E 25 was digested with the restriction enzyme *Eco*RI to subclone the DNA fragment containing the transposon insertion into the vector pUC18. The desired fragment was identified by selection for the kanamycin resistance encoded by the Tn*5*. The resulting plasmid pBJE25 was subjected to sequence analysis. The DNA region upstream and downstream of the Tn*5* insertion was sequenced on both strands with specific oligonucleotides as primers. Sequence analysis revealed the presence of four open reading frames (ORF) as indicated in Fig. 2. We were able to identify the 5′-end of the *L. pneumophila* metalloprotease encoding gene *msp* and two additional ORFs located on the opposite strand which displayed high homology to the biotin carboxyl carrier protein (*acc*B) and the biotin carboxylase (*acc*C) of *E. coli*. While we could detect the entire ORF of *acc*B, the 3′-end of *acc*C was not present on pBJE25. The fourth ORF contains 1788 bp and is interrupted by the transposon insertion. The ORF is preceded by a potential ribosome-binding site at an appropriate distance from the putative initiation codon. This sequence will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number X99678.

### 3.3. Identification of an OadA homologue protein

The gene product deduced from the interrupted ORF consisted of 596 amino acids with a calculated molecular mass of 65 666 Dalton. Data-bank searches revealed a high degree of identity of the putative protein with the α-subunit of oxaloacetate decarboxylase of *Klebsiella pneumonia* [27] and *Salmonella typhimurium* [28] with a similarity of 66% and 48% (Fig. 3). This enzyme consists of three different subunits, α, β and γ encoded by the genes *oad*GAB which are clustered on the genome of *K. pneumoniae* and *S. typhimurium* in that order. The oxaloacetate decarboxylase is an essential enzyme of the citrate fermentation pathway of these organisms. Under anaerobic conditions citrate is catabolised via oxaloacetate to acetate and formate. The biotin enzyme oxaloacetate decarboxylase catalyses the decar-

boxylation of oxaloacetate to pyruvate and carbon dioxide, and has the additional function as a primary sodium pump. $Na^+$ bioenergetics are important as a link between exergonic and endergonic reactions in the membrane [29]. The oxaloacetate decarboxylase conserves the free energy of decarboxylation by conversion into an $Na^+$ gradient.

As indicated in Fig. 4 Western blot analysis of the wild-type and mutant strain using a polyclonal antibody against the OadA protein of *Klebsiella pneumoniae* showed that the antibody reacted with an approximately 65 kDa protein in the *L. pneumophila* Corby. This protein is not expressed in the Tn*5*-mutant strain E 25. As a positive control we applied the strain DH5α(pBJJ4) to lane 3 (Fig. 4) where the OadA homologous protein is expressed. A 2.1 kb DNA fragment containing the wild-type *oad*A gene was amplified from the chromosome of *L. pneumophila* Corby by PCR, and subcloned in the vector pBC KS+, resulting in the recombinant plasmid pBJJ4 (Fig. 2). These data suggest that *L. pneumophila* expresses a protein homologous to the α-subunit of oxaloacetate decarboxylase of *K. pneumoniae* [27], and that the absence of this protein in the mutant *L. pneumophila* E 25 is due to the inactivation of the corresponding gene by the insertion of the Tn*5* in this specific gene locus.

There is a possible explanation for the role of the oxaloacetate decarboxylase as a determinant of virulence in *L. pneumophila*. The intracellular microenvironment in which *Legionella* strains reside is not well understood. Although *Legionella* requires a complex medium for growth in vitro, the intracellular nutritional requirements are probably provided by the phagosome of human phagocytes and freshwater protozoa. It is known, that besides amino acids also α-ketoglutarate and pyruvate can stimulate growth of *Legionella*. Whether the protein homologous to the oxaloacetate decarboxylase α-subunit is part of an oxaloacetate decarboxylase complex or participates as a partial enzyme in another complex enzymatic reaction, e.g., the carboxylation of pyruvate, is unknown. In any case, the enzymatic reaction catalysed by this enzyme may be important to support growth of *Legionella* in its natural environment.

This is not the first reported case of a housekeeping protein being important for the virulence of an organism. Recently, a pyruvate oxidase mutant of *Streptococcus pneumoniae* was found to be less virulent in the animal model, because the altered carbohydrate metabolism of these bacteria has an influence on the adherence to host cells [30]. The virulence of *L. pneumophila* is a highly complex, multifactorial phenomenon. Therefore it is likely that under certain intracellular conditions, enzymes like the oxaloacetate decarboxylase may play an essential role in the physiology of the bacteria and subsequently exerting a global effect on the intracellular survival of *L. pneumophila*.

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