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The Tat pathway in *Listeria monocytogenes*

Henrique Ramalho Machado

Mestrado em Microbiologia Aplicada

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Dissertação orientada por Professora Luísa Brito (ISA/UTL)

e Doutora Sandra Chaves (FCUL-BioFIG)

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Instituto Superior de Agronomia
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IBMC
INSTITUTO DE BIOLOGIA MOLECULAR E CELULAR
INSTITUTE FOR MOLECULAR AND CELL BIOLOGY

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Master Thesis

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This thesis was performed at the Microbiology Lab of the Instituto Superior de Agronomia (CBAA), at the Group of Molecular Microbiology of the Institute for Molecular and Cell Biology (IBMC) and at the Department of Biochemistry and Molecular Microbiology of the Southern Denmark University (BMB-SDU).

Acknowlegments

Ao **Instituto Superior de Agronomia**, por me ter acolhido na realização deste trabalho.

À Professora **Luísa** um enorme OBRIGADO por ter aceite orientar-me, já sabendo que não seria fácil, e por toda a paciência, compreensão e apoio que teve para comigo.

À Doutora **Sandra Chaves** por me co-orientar neste trabalho e pela disponibilidade que demonstrou para me receber ao longo deste ano.

Ao Professor **Rogério Tenreiro** pelo excelente trabalho realizado como coordenador de mestrado e por ser sempre tão prático e disponível.

To Dr. **Didier Cabanes** for having me in his lab, allowing me to learn so many things, and for his words of wisdom when I left.

To Prof. Dr. **Birgitte Kallipolitis** a special thanks, not only for having me in her lab as an Erasmus student, but most of all for everything I learned there and for the great time I had in Denmark, loved every second I spent in the lab, it made me grow both as an individual and as a scientist.

Aos **Micro Team** (Catarina, Elisa, Espiga, Né, Sofia, Sara, Rui, Zé e Mariana) que se encontram por todo o mundo, por estarem sempre presentes, por todo o apoio e carinho, a todos vocês um “Up and down, up and down, up and down, Esgota!”.

A todos os meus **amigos da Benedita e Truquel** (sim, Tru) (em especial, Anita, Rafael, Sarinha, Carol, Briii, Piga e Rasko) por não me abandonarem apesar das longas estadas ausente e por estarem sempre quando mais preciso.

A todo o **grupo de Listeria** (e não só) do ISA (António, Carla, Elsa, Ana [Fifi], Paula, Mara, André, Suse e Rute) por todos os conhecimentos transmitidos, apoio e amizade. To **Mahesh** for all the wisdom sentences he came up with, when I was felling down, and for being such a good hearted person. À D. **Manuela** e à D. **Lena** por tudo o que fazem e pela boa companhia que são. Aos restantes membros do **laboratório de Microbiologia**, e aos membros dos laboratórios de **Fisiologia** e de **Genética** pelo tanto que me ensinaram e por toda a ajuda que me deram.

Aos membros do **grupo de Microbiologia Molecular do IBMC** (Filipe, Ana Filipa, Teresa, Olga, Ana, Elsa e Rita) por me terem recebido e integrado tão bem, e por me terem ensinado tanta coisa.

À **Lúcia** e à **Inês** por me receberem como só elas sabem na Residência das Relvas, no Porto. =)

À **Mariana Ayala** por sempre acreditar que eu tinha uma estrelinha, espero ainda vir a comprová-lo.

To **everyone in Birgitte's Lab** for including me and teaching me so much, I really enjoyed. Thank you for making my stay abroad so enjoyable. A special thanks to **Eva** for being so patient with me and teaching me everything.

To **all the friends I made in Denmark** (Simon, Susana, Debbie or "Deburah", Gonçalo, Annika, Simone, Fabian, Katrien, Nina, Marc, Oscar, Claudia, Pedro, Carolina, Nicola, Loïc, Rougec, and so on…), it was such a great and fun time with you guys! You made my first experience abroad unbelievable! It was, wait for it… AWESOME!

A very special thanks to my schnücki **Katharina** for all the love, support, patience and advice.

Finalmente, e mais importante que tudo, à minha **Família**! Aos meus **pais** por tudo o que são e por tudo o que me proporcionam, principalmente o apoio incondicional. À minha madrinha **Berta** por tudo, tudo tudo, és incrível! Aos **Carochos** pelos bons fins-de-semana passados em casa da avó e por todo o apoio e carinho, demonstrado à sua particular maneira! =P Aos **Ramalhos** por sempre se preocuparem e quererem saber como ia o trabalho.

E a todos os que me esqueci e que de alguma forma contribuiram para este trabalho ou para a minha formação como pessoa, muito obrigado!

And to everyone else I forgot and that somehow contributed to this work or to my formation as a human being.

Abstract

Listeria monocytogenes, a foodborne pathogenic bacterium, remains a serious public health concern due to its frequent occurrence in food products coupled with a high mortality rate, specially among immunocompromised hosts. Bacterial pathogenicity depends greatly on the ability to secrete virulence factors to or beyond the bacterial cell surface. Thus, the study of secreted proteins is of crucial importance to further develop defensive strategies.

The Tat pathway, one of the secretion systems present in *L. monocytogenes*, was until now only investigated *in silico*. Therefore, a better understanding of the Tat pathway was needed. In *L. monocytogenes*, strain EGDe, two proteins constitute the Tat pathway and are encoded in the genes *tatC* (*lmo0361)* and *tatA* (*lmo0362)*.

In the present study, a *L. monocytogenes* mutant strain lacking the genes coding for the Tat pathway was successfully constructed (EGDe Δ*tatAC*). The mutant showed the ability to grow at the same growth rate as the parent strain, proving that the Tat pathway is not essential for *L. monocytogenes* survival. Moreover this study showed that both genes are transcribed in a bicistronic and growth-phase dependent manner.

The deletion mutant for the Tat pathway showed no differences in the *in vitro* virulence potential, but significant differences ($p < 0.05$) were found when *in vivo* virulence potential was assessed, being the *tat* mutant more virulent than the wild-type strain.

Regulation of *tatAC* was also investigated, and a deletion mutant for *lmo0364*, a gene coding for a transcription regulator, localized close to the *tat* genes, was constructed. Our results show that Lmo0364 is not related to the other genes in the *locus*, as it is not involved in the transcription regulation of any of the genes analyzed.

This is, to our knowledge, the first experimental study on the Tat pathway of *L. monocytogenes*. Here we show that this pathway is not essential for the bacterium and that it might be impairing its virulence ability.

Keywords:

Listeria monocytogenes; secretion system; Tat pathway; deletion mutant.

Resumo

Listeria monocytogenes é actualmente considerada uma bactéria patogénica de risco para a Indústria Alimentar, afectando essencialmente grávidas, crianças, idosos e indivíduos imunocomprometidos. Este risco torna-se cada vez mais importante, à medida que aumentam os casos de doentes imunocomprometidos, quer por infecções por VIH ou por tratamentos como quimioterapia e radioterapia, entre outros.

L. monocytogenes é uma bactéria gram-positiva em forma de bastonete com dimensões de 0,4 µm por 1 a 1,5 µm. Esta bactéria caracteriza-se pelo seu baixo teor em G+C, por ser anaeróbia facultativa, catalase positiva e oxidase negativa. A sua principal característica é a sua versatilidade no que diz respeito às condições de crescimento, nomeadamente, temperatura (1 a 45 ˚C), pH (4,4 a 9,6), cloreto de sódio (10 a 20% (m/v)), sais biliares (10 a 40% (m/v)), actividade da água (a_w) (≥0,92) e a tolerância a alguns metais, geralmente tóxicos para outras bactérias, como o lítio, o tálio e o telúrio.

Listeriose é o nome dado à doença provocada por *L. monocytogenes* e encontra-se associada uma elevada taxa de mortalidade (20 a 30%). Na Europa, o número de casos confirmados aumentou 19% em 2009, comparativamente com 2008. *L. monocytogenes* é um parasita intracelular facultativo. Após ingestão de alimentos contaminados, a bactéria tem a capacidade de atravessar o epitélio intestinal e disseminar-se através dos vasos linfáticos ou sanguíneos, para tecidos mais profundos. O fígado e o baço são órgãos-alvo primários para a sucessiva multiplicação bacteriana, com a possível formação de abcessos. Uma das principais características fisiopatológicas de *L. monocytogenes* é a capacidade de atravessar barreiras epiteliais, como a barreira hemato-encefálica e a barreira placentária, levando a meningo-encefalite ou infecção do feto, podendo eventualmente causar aborto e morte ou meningite neonatal.

A patogenicidade bacteriana encontra-se bastante dependente da capacidade das bactérias segregarem factores de virulência, que são expostos à superfície da célula, segregados para o ambiente extracelular ou, até mesmo, injectados directamente nas células hospedeiras. Enquanto que em bactérias gram-negativas, para que haja secreção de proteínas para o exterior da célula têm de ser transpostas duas membranas biológicas, em bactérias gram-positivas, os mecanismos de transposição da membrana citoplasmática permitem a segregação eficaz de proteínas para o exterior da célula.

Em bactérias gram-positivas, são, actualmente, reconhecidos sete sistemas de secreção: Sec (*Secretion*), Tat (*Twin-arginine translocation*), FPE (*Fimbrilin – Protein Exporter*), transportadores ABC (*ATP-binding cassette*), FEA (*Flagella Export Apparatus*), Holinas e Sistema Wss.

Relativamente a *L. monocytogenes*, embora a secreção de proteínas seja de extrema importância, quer no processo de colonização de ambientes bióticos e abióticos, quer na sua virulência, ainda muito pouco é conhecido. Assim, a partir dos genomas sequenciados e com recurso à bioinformática, foram indentificados, em *L. monocytogenes*, todos os sistemas descritos para gram-positivos.

O sistema Tat é responsável pela secreção de proteínas na sua conformação final, tendo as proteínas secretadas por este sistema um motivo N-terminal [(S/T)TRRXFLK] de consenso. Este sistema de secreção foi primeiramente identificado em bactérias gram-negativas, sendo essencial para a sua funcionalidade três proteínas membranares: TatA, TatB e TatC. A maioria das bactérias gram-positivas possui um sistema Tat "minimalista" pois apenas as proteínas TatA e TatC estão presentes, sendo TatA bifuncional, colmatando a inexistência de TatB.

Este sistema de secreção está presente em vários microrganismos patogénicos, para os quais foi demonstrada a sua importância para os estilos de vida, saprófito ou patogénico. A secreção de proteínas por este sistema demonstrou ser importante para a mobilidade em *Escherichia coli* O157:H7, para a infecção e para a aquisição de ferro em *Pseudomonas aeruginosa*, para o crescimento, divisão e formação de biofilme em *Legionella pneumophila* e para a capacidade infecciosa *in vivo* de *Staphylococcus aureus*.

Em *L. monocytogenes* apenas uma cópia dos genes *tatA* e *tatC* foi identificada, sendo que a proteína considerada como secretada por este sistema se encontra codificada num operão muito próximo deste *locus*.

O trabalho que aqui se apresenta teve como objectivo o estudo do sistema Tat em *L. monocytogenes*.

Para avaliar o papel deste sistema de secreção, construiu-se um mutante de delecção da estirpe EGDe nos genes codificantes para o sistema Tat. O mutante foi construído usando um sistema de dois passos: um primeiro passo de integração e segundo passo de desintegração do plasmídeo no genoma, através de regiões homólogas às regiões anterior e posterior aos genes. Tanto quanto se sabe, trata-se do primeiro mutante de delecção descrito para o sistema Tat em *L. monocytogenes*. O sistema Tat não se mostrou essencial ao crescimento de *L. monocytogenes* em meio completo e em meio mínimo, apresentando o mutante e a estirpe selvagem taxas específicas de crescimento semelhantes.

Foram efectuados estudos transcriptionais relativos ao *locus* do sistema, nomeadamente análises Northern blot e estudos da actividade promotora, fazendo uso do gene codificante para a β-galactosidase, como gene repórter. A transcrição dos genes *tatA* e *tatC* revelou-se dependente da fase de crescimento, sendo a transcrição mais evidente na fase exponencial,

comparativamente com a fase estacionária. Demonstrou-se ainda que a transcrição dos genes codificantes para o sistema Tat é bicistrónica, apresentando o operão correspondente uma forte actividade promotora.

A existência de um regulador de transcrição (Lmo0364) codificado no locus estudado, levou à hipótese de este se encontrar relacionado com a regulação da transcrição do sistema Tat. Esta hipótese foi investigada através da construção de um mutante de delecção neste regulador de transcrição. No entanto, não foram registadas alterações, quer na transcrição, quer na actividade promotora dos genes estudados.

Estudos prévios realizados *in silico*, por outros autores, identificaram apenas uma proteína secretada através deste sistema. Esta proteína está codificada no operão *lmo0365-67*, sendo a última do operão (*lmo0367*). De forma a identificar esta e/ou outras proteínas secretadas por este sistema, procedeu-se a uma análise das proteínas secretadas por SDS-PAGE, não tendo sido no entanto possível detectar alterações entre o secretoma do mutante e o secretoma da estirpe selvagem, possivelmente devido às limitações desta metodologia (limites de resolução e detecção).

Os potenciais de virulência dos dois mutantes (EGDe Δ*tatAC* e EGDe Δ*lmo0364*) foram também avaliados *in vitro,* através da infecção de células epiteliais humanas, e *in vivo*, através da contagem de bactérias no baço, após três dias de inoculação subcutânea em ratinhos. Apesar de não terem sido identificadas diferenças no potencial virulento *in vitro*, foram verificadas diferenças significativas ($p < 0,05$) *in vivo*, sendo que o mutante *tatAC* demonstrou um potencial virulento mais elevado do que a estirpe selvagem, demonstrando que a presença deste sistema de secreção nesta estirpe poderá dificultar o processo de infecção.

Ao contrário do que se verificou noutros microrganismos patogénicos, o sistema Tat não é necessário à sobrevivência de *L. monocytogenes* e muito menos à sua capacidade infecciosa. Tanto quanto se sabe, este trabalho constitui o primeiro trabalho experimental realizado em *L. monocytogenes*, com o objectivo de estudar o sistema de secreção Tat.

Palavras-chave:

Listeria monocytogenes; sistema de secreção; sistema Tat; mutante de delecção.

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1. Introduction

1.1. Listeria monocytogenes

1.1.1. Historical facts

In 1924, after an outbreak in laboratory animals in Cambridge, Murray, Webb and Swann have isolated a gram-positive bacterium which they named *Bacterium monocytogenes* (1), due to the monocytosis disease observed in the infected animals. This was considered the official discovery of *Listeria*, which after the name of *Bacterium monocytogenes* had other names as *Erysipelothrix*, *Listerella* and finally, *Listeria* (2), in honor of Lord Lister (3).

Despite its official discovery date in 1924, there is evidence that this organism had probably been observed in histological sections many years before its official discovery and it had been cultivated and described by Hülphers in 1911 (4).

Although the clinical descriptions of infection by *L. monocytogenes* in animals and humans date from the twenties of the last century, only in 1952, in Germany, listeriosis was recognized as an important cause of neonatal meningitis and septicemia (5). In 1981, *L. monocytogenes* was for the first time associated with the Food Industry and considered as a foodborne pathogen, following an outbreak of listeriosis in Nova Scotia, Canada, with 41 cases and 18 deaths reported, mostly children and pregnant women, epidemiologically linked by the consumption of coleslaw contaminated with sheep feces containing *L. monocytogenes* (6).

1.1.2. Characteristics

The presence of *L. monocytogenes* in food products is currently considered a risk in the Food Industry. This pathogenic microorganism affects primarily pregnant women, children, the elderly and immunocompromised individuals. This risk becomes more important as the cases of immunocompromised patients, either by HIV infection or by treatments such as chemotherapy and radiotherapy, among others increases.

Taxonomically, *L. monocytogenes* belongs to the *Bacteria* domain, phylum *Firmicutes*, class *Bacilli*, order *Bacillales*, family *Listeriaceae* and gender *Listeria*. Eight species have been described for the genus *Listeria*: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, *L. grayi*, *L. marthii* (7) and *L. rocourtiae* (8). *L. monocytogenes* infects ruminants and it has been considered the only one pathogenic for humans (9) (10), *L. ivanovii* was thought to infect only ruminants but recently a case of infection by this species in a man was

reported, suggesting that the rarity of human listeriosis due to this species can be explained not only by the host tropism factors but also by the rare occurrence of this species in the environment, compared with *L. monocytogenes* (11).

Thirteen serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7), from three different lineages (lineages I, II and III) have been described for *L. monocytogenes* (12).

L. monocytogenes is a gram-positive rod-shaped bacterium with dimensions of 0.4 µm for 1 to 1.5 µm. This bacterium has a low G+C content, is a facultative anaerobe, catalase positive and oxidase negative. It has peritrich flagella, which presence is very limited when grown at 37 ˚C (13). *L. monocytogenes* can grow in a range of temperatures from 1 to 45 ˚C (14) (15), although its optimal growth temperature ranges from 30 to 37 ˚C. The growth versatility of this bacterium is also observed with respect to pH, as it has the ability to grow at pH values between 4.4 and 9.6 (14), although the optimal pH for its growth is neutral or slightly alkaline.

L. monocytogenes is quite tolerant to sodium chloride (10 to 20% (w/v)) (16) (13) (17), bile salts (10 to 40% (w/v)), and to some metals, which are usually toxic for other bacteria, such as lithium, thallium and tellurium. It has also the ability to grow in low water activity (a_w) values, equal or greater than 0.92 (18), but its optimal growth occurs at 0.97.

1.1.3. Infectious cycle

L. monocytogenes is responsible for listeriosis, a disease with a high mortality rate (20% to 30%). In the EU, the number of confirmed cases increased 19% in 2009 compared to 2008 (19). *L. monocytogenes* is a facultative intracellular bacterium. After ingestion of contaminated food it is able to cross the intestinal epithelium and disseminate via the lymph and blood-streams to deeper tissues. The liver and the spleen are primary target organs for further bacterial multiplication, resulting in possible abscess formation (20). One of the main characteristics of *L. monocytogenes* physiopathology is its capacity of crossing major epithelial barriers, the blood-brain barrier and the placental barrier, leading to meningo-encephalitis or fetus infection, ultimately it can eventually cause abortion, stillbirth or neonatal meningitis.

Briefly, after being ingested this bacterium has the ability of entering the intestine epithelial cells and escape from the phagosome, becoming free in the cell cytoplasm. By actin polymerization it propels through the cell cytoplasm and into the adjacent cell's outer membrane which encapsulates this protuberance and acquires the bacteria that will become once again free in the cell cytoplasm (Figure 1).

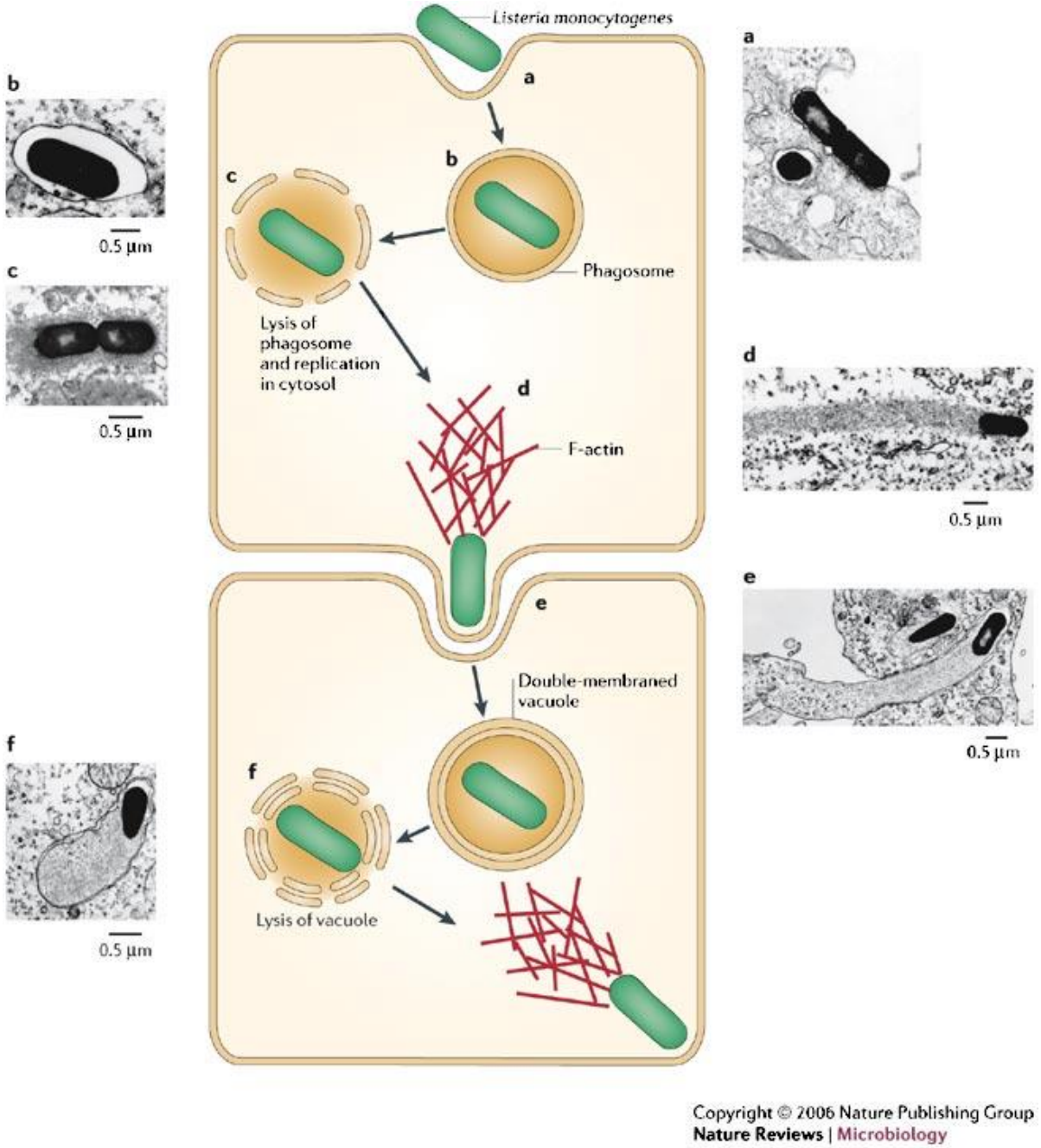

Figure 1 – Schematic representation and electron micrographs of the *Listeria monocytogenes* life cycle.

a - *L. monocytogenes* induces its entry into a non-professional phagocyte. b - Bacteria are internalized in a vacuole (also known as a phagosome). c,d - The membrane of the vacuole is disrupted by the secretion of two phospholipases, PlcA and PlcB, and the pore-forming toxin listeriolysin O. Bacteria are released into the cytoplasm, where they multiply and start to polymerize actin, as observed by the presence of the characteristic actin tails. e - Actin polymerization allows bacteria to pass into a neighbouring cell by forming protrusions in the plasma membrane. f - On entry into the neighbouring cell, bacteria are present in a double-membraned vacuole, from which they can escape to perpetuate the cycle. F-actin, filamentous actin (21).

1.2. Bacterial Secretion Systems

Bacterial protein secretion is extremely important because secreted proteins are the main tool used by bacteria to interact with their environment. Bacterial pathogenicity depends greatly on the ability of bacteria to secrete virulence factors which are displayed on the bacterial cell surface, secreted into the extracellular milieu or even injected directly into the host cell (22). Many nonpathogenic organisms also secrete proteins that are important for its growth, for example degradative enzymes such as cellulases, secreted by saprophytic bacteria.

Protein secretion systems have been extensively investigated in a wide range of gram-negative bacterial species and six secretion systems were identified (numbered from I to V, plus the chaperone pathway), although they are not all systematically present in a single bacterium and its presence and activity varies from one bacterium to another (23). In contrast, information about secretion systems in gram-positive bacteria is still essentially restricted to *Bacillus subtilis*, although other studies concerning gram-positive bacteria such as *L. monocytogenes* (24) (25) and *Staphylococcus aureus* (26) (27) are starting to be reported.

In gram-positive bacteria seven protein secretion systems are currently recognized: the Sec (Secretion) pathway; the Tat (Twin-arginine translocation) pathway; the FPE (Fimbrilin-Protein Exporter); some ABC (ATP-binding cassette) protein exporters; the FEA (Flagellum Export Apparatus); the holins (hole-forming) and the Wss (WXG100 secretion system) (25). As occurs in gram-negative bacteria, not all of the secretion pathways are systematically present in a single organism, and their respective contribution in protein transportation varies from one organism to another.

All the secretion pathways described for gram-positive bacteria were identified in *Listeria monocytogenes* as schematically shown in Figure 2.

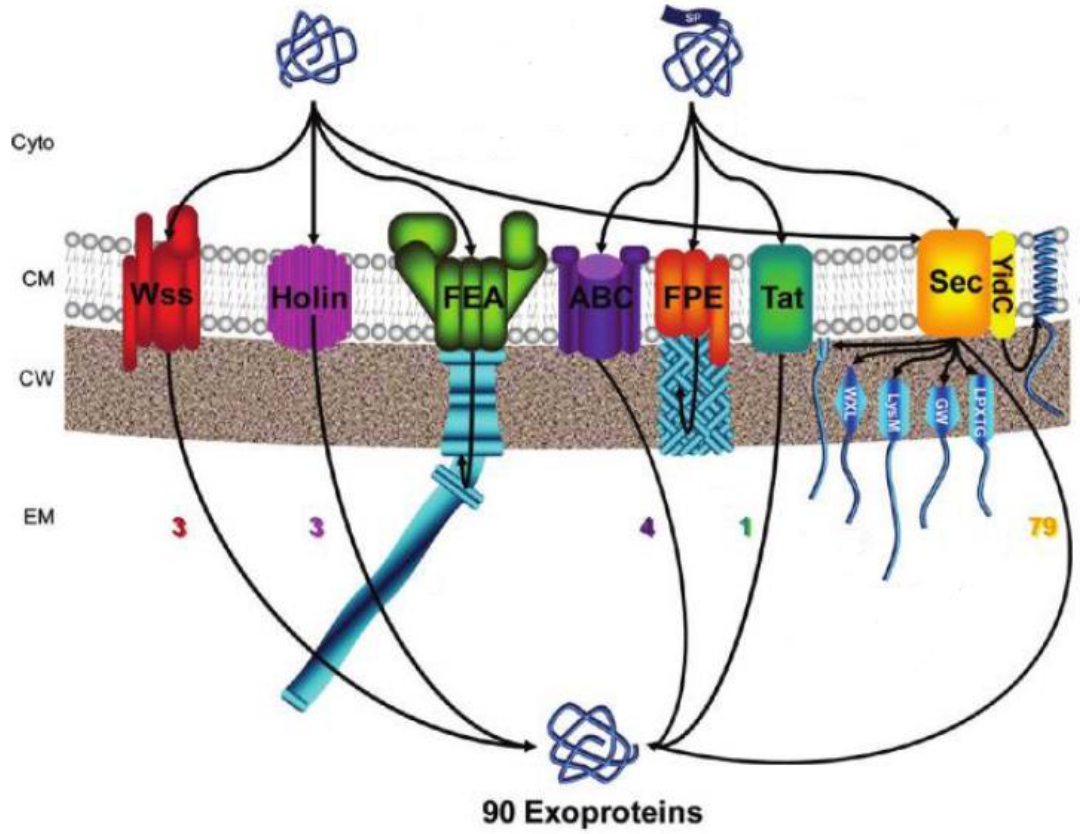

Figure 2 – Schematic representation of the secretion systems and the number of proteins secreted by each system in *Listeria monocytogenes*.

SP, signal peptide; Sec, secretion; Tat, twin-arginine translocation; FPE, fimbrilin-protein exporter; ABC, ATP-binding cassette exporter; FEA, flagella export apparatus; holin, hole forming; Wss, WXG100 secretion system; Cyto, cytoplasm; CM, cytoplasmic membrane; CW, cell wall; EM, extracellular milieu. Adapted from Desvaux et al, 2010 (25).

The **Sec pathway** is the major secretion system in prokaryotes and eukaryotes, not only because of the number of proteins considered as exported by this pathway, but also because of their importance. Although the presence of the Sec pathway in *L. monocytogenes* has not been experimentally proved, its existence was inferred due to the high number of proteins carrying the putative translocation signal peptide and the presence of these proteins in the extracellular milieu or in the cell surface (28) (29). This system consists of a heterotrimeric SecYEG complex, which is the central component as it forms a channel in the cytoplasmic membrane. A cytosolic ATPase SecA is also essential for protein transport. It acts through cycles of adenosine triphosphate (ATP) binding and hydrolysis, thereby facilitating the binding of proteins to the system, leading to stepwise export of the proteins (24). In *L. monocytogenes* a SecA paralogue named SecA2 was identified (30) and unlike SecA this is not essential for cell viability but it is involved in the secretion of proteins that contribute to virulence (31), such as p60 hydrolase (30), NamA (N-acetilmuramidase A) (32) and FbpA (Fribonectin-binding protein A) (33).

The proteins secreted by the **Tat pathway** have a N-terminal signal peptide and an essential twin arginine motif, (S/T)TRRXFLK, which straddles the N-domain and the hydrophobic H-domain (34). Unlike the Sec system, the Tat pathway is considered to secrete proteins in their final conformation (35) and somehow rejects the ones that are unfolded. In *L. monocytogenes* EGDe (serovar 1/2a) only one copy of the genes coding for *tatA* and *tatC* was identified while in the sequenced *L. monocytogenes* 4b serovars no genes coding for the Tat pathway were identified.

The **Fimbrilin-Protein Exporter (FPE) system** is responsible for the secretion and agglomeration of proteins similar to pilin, and its components are encoded in *comG* locus. Although the existence of this system has never been experimentally demonstrated in *L. monocytogenes*, it is referred because of its similarity with the genetic *locus comG* of *Bacillus subtilis*.

ATP-binding cassette (ABC) protein exporters are transmembrane proteins that utilize the energy of ATP hydrolysis to carry out various biological processes, including translocation of substrates across membranes. Only recently this type of system was considered as responsible for the secretion of four bacteriocins in *L. monocytogenes* (25).

In gram-negative bacteria, the **Flagellum Export Apparatus (FEA)** is related with the type III secretion system, together with the Hrp (Hypersensitive response and pathogenicity) pilus export apparatus and the injectisome export apparatus (24). From the nine FEA components identified in gram-negative bacteria, seven were identified in *Listeria*, FlhA, FlhB, FliP, FliQ, FliR, FliI and FliH.

Holins are small membrane proteins that allow the transport across the cytoplasmic membrane of proteins lacking N-terminal signal sequences. These proteins, which derived from phages, are involved in the secretion and activation of proteins with murein hydrolyzing activity in early stages of cell lysis, which is very important for apoptosis. Holins are homo-oligomeric complexes that form pores in the cytoplasmic membrane, allowing an energy independent translocation of proteins (24).

The **Wss system** is formed by a protein with 100 amino acids, which has a coil-coil domain and a conserved WXG motif, called WXG100 and a membrane-bound ATPase, homologous to *B. subtilis* YukAB. In *Listeria* only one copy of the gene coding for the protein YukAB was identified, which is also present in the non-pathogenic species *L. innocua* (36).

1.3. The Tat Pathway

The twin-arginine translocation (Tat) pathway is responsible for the secretion of proteins in their folded conformation and, as referred before, targeted proteins to be secreted via Tat pathway have a N-terminal consensus motif [(S/T)TRRXFLK] (34). The energy required for protein translocation is exclusively acquired from the transmembrane proton electrochemical gradient (Δp) (37).

This secretion system was first characterized in plant chloroplasts and in 1998 a homologous bacterial Tat pathway was identified in *Escherichia coli*, encoded by the *tat* genes (*tatA, tatB, tatC* and *tatE*) (38).

In gram-negative bacteria, where this pathway was first identified in prokaryotes, the Tat translocation requires three integral membrane proteins belonging to TatA, TatB and TatC families, and all of them have been demonstrated to be essential for protein translocation (Figure 3A) (34).

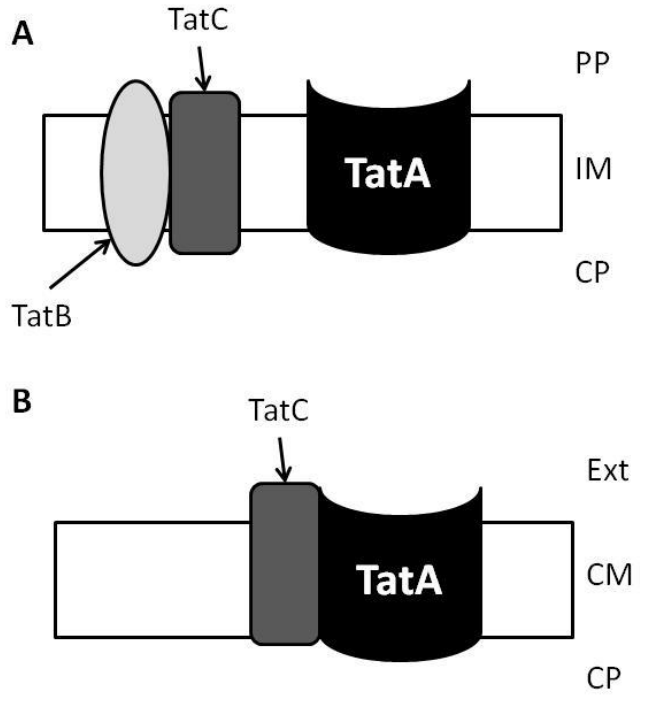

Figure 3 – Schematic representation of Tat pathways (A) in gram-negative and (B) gram-positive bacteria.

PP, periplasm; IM, inner membrane; CP, cytoplasm; Ext, extracellular milieu; CM, cytoplasmic membrane.

Most gram-positive bacteria and *Archaea* possess a simpler and minimalist Tat pathway as only *tatA* and *tatC* genes are present, lacking the gene for TatB component, which function is performed by a bifunctional TatA component (Figure 3B) (39). Although the TatB is missing in gram-positive bacteria, it has been proved its functionality in *B.subtilis*. This operon (*tatAdCd,* named d due to the existence of more than one copy of these genes in *B. subtilis* genome) was expressed in an *E. coli tat* null mutant and its ability to export several Tat substrates was proved (39).

Despite the differences between gram-positive and gram-negative bacteria, homologues of *E. coli tat* genes have been identified in the genomes of many bacterial pathogens, including *E. coli* O:157, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Vibrio cholerae*,

Helicobacter pylori, *Mycobacterium tuberculosis*, *L. monocytogenes*, *Salmonella enterica*, *Legionella pneumophila*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Pasteurella multocida*, *Yersinia pestis*, *Xanthomonas campestris*, *X. axonopodis, Xyllela fastidiosa*, *S. aureus* and *Ralstonia solanacearum* (38). For some of these pathogens the importance of the Tat pathway was assessed and studied in detail. Those studies proved the involvement of the Tat pathway in different lifestyles of bacteria, from saprophytic to pathogenic. Translocation of proteins by this system showed to be important for *E. coli* O157:H7 motility, for infection and iron acquisition in *P. aeruginosa* and for cell growth, division and biofilm formation in *L. pneumophila* (38). In *S. aureus,* a bacterium taxonomically closer to *L. monocytogenes*, there are evidences of the Tat pathway function in transporting an iron-dependent peroxidase (FepB) and its need to successful *in vivo* infection was shown (26).

In *L. monocytogenes* EGDe, the two proteins which constitute the Tat pathway are encoded in the genes *lmo0361* and *lmo0362*. The gene located four genes away of the *tat* genes corresponds to the gene coding for an iron-dependent Dyp-peoxidase (*lmo0367*), with 40 % similarity to FepB of *S. aureus* considered secreted by the Tat pathway. This indicates that probably Lmo0367 is also secreted by the Tat system. This protein is part of an operon formed by genes *lmo0365*, *lmo0366* and *lmo0367* (40), all of them related with iron. Between the two *locus* mentioned there is a gene coding for a peptidase and a transcriptional regulator, *lmo0363* and *lmo0364*, respectively. A schematic representation of the *locus* coding for the Tat Pathway and the protein putatively secreted by this system is shown in Figure 4.

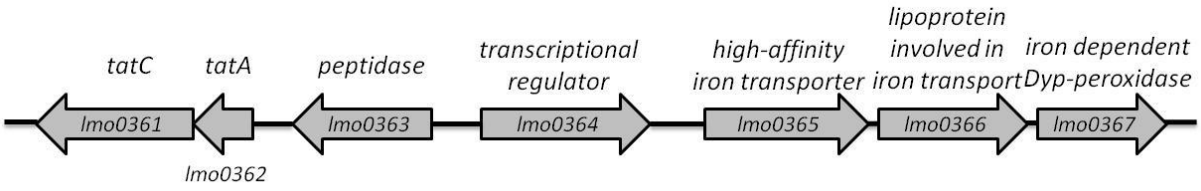

Figure 4 – Organization of the genes coding for the Tat pathway and the *lmo0365-7* operon of *Listeria monocytogenes* EGDe.

2. Materials and Methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study and its characteristics are described in Tables 1 and 2, respectively.

Table 1 – Bacterial strains used in this study.

Strains	Relevant genotype or characteristics	Reference
***E. coli* TOP10**	*E. coli* strain used for cloning	Invitrogen
L. monocytogenes		
EGDe	Wild-type serovar 1/2a strain	(41)
Δ*lmo0361* Δ*lmo0362*	EGDe Δ*lmo0361* Δ*lmo0362*	This study
Δ*lmo0364*	EGDe Δ*lmo0364*	This study
3993	Low virulent	(42)
EGDe pTCV	EGDe containing plasmid pTCV-lac	This study
EGDe pTCV-lmo0361	EGDe containing plasmid pTCV-lmo0361	This study
EGDe pTCV-lmo0362	EGDe containing plasmid pTCV-lmo0362	This study
EGDe pTCV-lmo0363	EGDe containing plasmid pTCV-lmo0363	This study
EGDe pTCV-lmo0364	EGDe containing plasmid pTCV-lmo0364	This study
EGDe pTCV-lmo0365	EGDe containing plasmid pTCV-lmo0365	This study
Δ*lmo0361* Δ*lmo0362* pTCV	EGDe Δ*lmo0361* Δ*lmo0362* containing plasmid pTCV-lac	This study
Δ*lmo0361* Δ*lmo0362* pTCV-lmo0361	EGDe Δ*lmo0361* Δ*lmo0362* containing plasmid pTCV-lmo0361	This study
Δ*lmo0361* Δ*lmo0362* pTCV-lmo0362	EGDe Δ*lmo0361* Δ*lmo0362* containing plasmid pTCV-lmo0362	This study
Δ*lmo0361* Δ*lmo0362* pTCV-lmo0363	EGDe Δ*lmo0361* Δ*lmo0362* containing plasmid pTCV-lmo0363	This study
Δ*lmo0361* Δ*lmo0362* pTCV-lmo0364	EGDe Δ*lmo0361* Δ*lmo0362* containing plasmid pTCV-lmo0364	This study
Δ*lmo0361* Δ*lmo0362* pTCV-lmo0365	EGDe Δ*lmo0361* Δ*lmo0362* containing plasmid pTCV-lmo0365	This study
Δ*lmo0364* pTCV	EGDe Δ*lmo0364* containing plasmid pTCV-lac	This study
Δ*lmo0364* pTCV-lmo0361	EGDe Δ*lmo0364* containing plasmid pTCV-lmo0361	This study
Δ*lmo0364* pTCV-lmo0362	EGDe Δ*lmo0364* containing plasmid pTCV-lmo0362	This study
Δ*lmo0364* pTCV-lmo0363	EGDe Δ*lmo0364* containing plasmid pTCV-lmo0363	This study
Δ*lmo0364* pTCV-lmo0364	EGDe Δ*lmo0364* containing plasmid pTCV-lmo0364	This study
Δ*lmo0364* pTCV-lmo0365	EGDe Δ*lmo0364* containing plasmid pTCV-lmo0365	This study

Table 2 – Plasmids used in this study.

Plasmids	Relevant genotype or characteristics	Reference
pAULA	Erm^r; Cloning plasmid for gene replacements in gram-positive bacteria	(43)
pMAD	Amp^r; Erm^r; Cloning plasmid for gene replacements in gram-positive bacteria	(44)
pMAD – Δ*lmo0361* Δ*lmo0362*	Amp^r; Erm^r; pMAD derivative containing homologous regions upstream EGDe *lmo0362* and downstream of EGDe *lmo0361*	This study
pAULA – Δ*lmo0364*	Erm^r; pAULa derivative containing homologous regions up- and downstream of EGDe *lmo0364*	This study
pTCV-lac	Kan^r; Transcriptional lacZ fusion vector; Low copy-number	(45)
pTCV-lmo0361	Kan^r; Truncated *lmo0361* region (-143 to +36) inserted in pTCV-lac upstream of *lacZ*	This study
pTCV-lmo0362	Kan^r; Truncated *lmo0362* region (-152 to +48) inserted in pTCV-lac upstream of *lacZ*	This study
pTCV-lmo0363	Kan^r; Truncated *lmo0363* region (-208 to +42) inserted in pTCV-lac upstream of *lacZ*	This study
pTCV-lmo0364	Kan^r; Truncated *lmo0364* region (-178 to +36) inserted in pTCV-lac upstream of *lacZ*	This study
pTCV-lmo0365	Kan^r; Truncated *lmo0365* region (-219 to +30) inserted in pTCV-lac upstream of *lacZ*	This study

2.2. Growth conditions

Escherichia coli strains were grown at 37 ˚C with shaking in Luria-Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, pH 7.2) or on LA plates (LB supplemented with 1.5 % (w/v) agar). When required, ampicillin (Amp), erythromycin (Erm) or kanamycin (Kan) was added to a final concentration of 100 µg/mL, 150 µg/mL and 50 µg/mL, respectively.

L. monocytogenes strains were grown at 37 ˚C with shaking in brain heart infusion (BHI) broth or on BHI plates (BHI supplemented with 1.5% (w/v) agar) and on minimal medium Modified Welshimer Broth (MWB) (46) or on Tryptone Soya Yeast Extract Agar (TSA-YE, 1.5% (w/v) agar). When required, erythromycin (Erm) or kanamycin (Kan) was added to a final concentration of 5 µg/mL and 50 µg/mL, respectively.

2.3. Genomic DNA extraction

Overnight cultures of *L. monocytogenes* strains were centrifuged at 3500 g for 5 min. The pellets were resuspended in 100 µL 1xPBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, adjusted to a final pH of 7.4) and 5 µL of lysozyme (10 mg/mL) was

added, following 15 min incubation at 37 ˚C, with shaking. The DNA was extracted and purified using FastDNA kit and protocol from Bio101 (Bio101, USA).

The DNA quality and concentration was verified by agarose gel electrophoresis (1% agarose).

2.4. Construction of mutants

*2.4.1. Construction of double deletion mutant strain (*L. monocytogenes *EGDe Δ*lmo0361 *Δ*lmo0362*)*

In order to generate the *lmo0361 lmo0362* double deletion mutant, an approximately 1000 basepair (bp) large region upstream of *lmo0362* and downstream of *lmo0361* was generated from *L. monocytogenes* EGDe genomic DNA. To generate these regions, a standard polymerase chain reaction (PCR) was performed using DNA polymerase high-fidelity (Fermentas, Canada), primers Tat_A together with Tat_B, and primers Tat_C together with Tat_D, as listed in Appendix 1 (for schematic representation see Figure 5). The first fragment (AB) was digested with restriction enzymes SalI and MluI and cloned in the temperature sensitive plasmid pMAD (Appendix 2), resulting the pMAD-TatAB plasmid. The second fragment (CD) was digested with restriction enzymes MluI and BglII and cloned in the pMAD-TatAB plasmid, resulting the pMAD-TatAD plasmid which had two approximately 1000 bp region, each homologous to the up and down-stream of the genes of interest.

The pMAD-TatAD plasmid was electroporated into *L. monocytogenes* EGDe, according to Park *et al*. (47). To induce chromosomal integration of the pMAD-TatAD, the cells were grown at a non permissive temperature (42 ˚C) on BHI plates containing erythromycin (5 µg/mL). Colonies containing the integrated plasmid were grown for eleven days at a permissive temperature (30 ˚C) without antibiotics, allowing the plasmid excision from the chromosome and its loss from the cell, thus allowing excising the genes *lmo0361* and *lmo0362* from the chromosome (schematic representation of gene deletion in Appendix 3). Finally, the presence of *lmo0361* and *lmo0362* regions was investigated through amplification from the resulting mutants using the internal primers for both genes, TatA_fw with TatA_rv and TatC_fw with TatC_rv, respectively (for schematic representation see Figure 5).

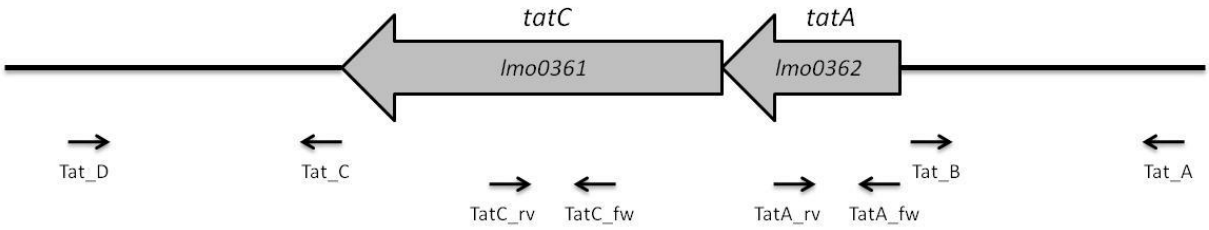

Figure 5 – Schematic representation of primer annealing positions for primers used in the construction of the *tatAC* deletion mutant.

2.4.2. Construction of deletion mutant strain (L. monocytogenes EGDe Δlmo0364)

In order to generate the *lmo0364* (transcription regulator) deletion mutant, an approximately 400 bp large region upstream and downstream of *lmo0364* was generated from *L. monocytogenes* EGDe genomic DNA. To generate these regions, a standard polymerase chain reaction (PCR) was done using Phusion polymerase (Finnzymes, Finland), primers P1-lmo0364 and P2-lmo0364, and primers P3-lmo0364 together with P4-lmo0364, as listed in Appendix 1. The resulting products were used to produce an approximately 800 bp fragment by splicing by overlap extension (SOE-ing) (48). After digestion of this fragment with EcoRI and BamHI it was cloned in the temperature sensitive plasmid pAULA (Appendix 2) resulting the pAULA–Δlmo0364, which construction was confirmed by sequencing with primers pAUL-1 and pAUL-2 (Appendix 1).

The pAULA–Δlmo0364 plasmid was electroporated into *L. monocytogenes* EGDe (47). To induce chromosomal integration of the pAULA–Δlmo0364, the cells were grown at a non permissive temperature (42 ˚C) on BHI plates containing erythromycin (5 µg/mL). Colonies containing the integrated plasmid were grown for eight days at a permissive temperature (30 ˚C) without antibiotics, allowing the plasmid excision from the chromosome and its loss from the cell, thus allowing the excision of gene *lmo0364* from the chromosome (schematic representation of gene deletion in Appendix 3). Finally, to confirm that the gene was deleted from the chromosome, the *lmo0364* region was amplified in wild-type and mutant strain DNA following sequencing using primers PC1_lmo0364 and PC2_lmo0364 (Appendix 1).

2.5. Preparation of total RNA

Overnight cultures of *L. monocytogenes* strains were diluted to an A_{600} = 0.02 in BHI (corresponding to time 0) and grown at 37 ˚C. At various time points (3, 4.5, 6 and 8 hours) 20 mL samples of the growing cultures were shortly cooled in liquid nitrogen (N_2) and the cells were harvested by centrifugation at 8000 g for 3 min at 4 ˚C, snap frozen in liquid

nitrogen and stored at -80 ˚C. The cells were resuspended in 900 µL TRI reagent (MRCGENE, USA) and lysed using a FastPrep instrument. RNA was subsequently extracted using chloroform and precipitated with ethanol and sodium acetate, overnight at -80 ˚C. The samples were centrifuged at 20000 g for 30 min at 4 ˚C, washed with 70% ethanol (-20 ˚C) and resuspended in 50 µL Milli-Q water.

The RNA quality and quantification was assessed on a NanoDrop 2000. The integrity and the predicted concentration of the RNA was verified by agarose gel electrophoresis (1% agarose).

2.6. Northern blot analysis

For Northern blotting, loading buffer (95% formamide and 20 mM EDTA, 0.05% BPB and 0.05% xylene cyanol) was added to 10 µg of total RNA and samples were boiled at 95 ˚C for 3 min (to denaturate the RNA) and immediately cooled on ice. The total RNA was separated on a 4% polyacrylamide-7M urea gel and transferred to a Zeta probe nylon membrane (Bio-Rad, USA) by semi-dry electroblotting at 400 mA for 2 hours.

For detection of RNA, the membranes were pre-incubated for 30 min at 42 ˚C in PerfectHyb hybridization buffer (Sigma-Aldrich, USA) and hybridized with a specific ^{32}P-labelled DNA probe, prepared using poly nucleotide kinase (PNK) (Fermentas, Canada) and γ-[^{32}P]-ATP (PerkinElmer, USA). The membranes were then washed with a 2X saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) solution for 5 min and a 0.5X SSC, 0.1% SDS solution for 20 min, both at 42 ˚C, and finally visualized by phosphor imaging using a Typhoon scanner.

2.7. Transcriptional fusions

The promoter regions of *lmo0361*, *lmo0362*, *lmo0363*, *lmo0364* and *lmo0365-lmo0367*, respectively (see Figure 4 in the Introdution, section 1.3.) were amplified from *L. monocytogenes* EGDe chromosomal DNA under standard PCR conditions using Phusion polymerase (Finnzymes, Finland) with primers listed in Appendix 2. The resulting products of approximately 200 bp were digested with EcoRI and BamHI and cloned in the low copy-number plasmid pTCV-*lac* (Appendix 2). The construction was confirmed by sequencing with primers vLac1 and vLac2 (Appendix 1).

The constructed plamids, pTCV-lmo0361, pTCV-lmo0362, pTCV-lmo0363, pTCV-lmo0364 and pTCV-lmo0365 were each one electroporated into *L. monocytogenes* EGDe, Δ*lmo0361*

Δ*lmo0362* and Δ*lmo0364* (47) strains. Transformants were selected by kanamycin resistance.

2.8. Beta-galactosidase assay

Strains for testing β-galactosidase activity were diluted from an overnight culture to an A_{600}=0.02 in BHI. At different growth times (3, 4, 5, 7 and 24 hours) 1 mL of each sample were collected by centrifugation at 14000 g for 4 min, thoroughly aspirated, frozen and stored at -80 ˚C until use. For the assay, the pelleted cells were thawn on ice and resuspended in 1 mL Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0). Cells were permeabilized by adding 0.5 % toluene and 4.5 % ethanol, vortexing after each addition. From the permeabilized cell suspension, 100 µL was mixed with 400 µL of Ortho-nitrophenyl-β-galactosidase (ONPG) solution (1 mg/mL ONPG, 0.075 % SDS dissolved in Z-buffer) and incubated at 37 ˚C until the samples present a yellow colour ($A_{420} \approx$ 0.6 - 0.9). When this colour was reached, the reaction was stopped by addition of 500 µL of 1 M Na_2CO_3.

The β-galactosidase activity was determined as described by Miller (49), using the equation:

$$Specific\ Activity\ \ (\beta - galactosidase\ Units) = \frac{A_{420} - 1.75\,A_{550}}{t \times v \times A_{600}},$$

where t is the time of reaction in minutes, v is the volume of culture used in the assay (mL), A_{420} and A_{550} is the absorbance of the β –galactosidase sample at 420 nm and 550 nm, respectively, and A_{600} is the absorbance of the cell culture when harvested.

Strains carrying an empty vector were also subjected to β-galactosidase measurements and this background activity was subtracted of the obtained data.

2.9. Secreted protein analysis

2.9.1. Bacterial cultures

L. monocytogenes strains were cultured overnight at 37 ˚C on TSA-YE. A single isolated colonie was suspended in 25 mL of minimal medium MWB (46) and incubated overnight at 37 ˚C with shaking. Overnight cultures were adjusted to an A_{600} = 0.05 using fresh MWB and grown at the same conditions used before (37 ˚C with shaking) for about 20 hours. The cells were harvested at exponential phase ($A_{600} \approx$ 0.8) by centrifugation (3 000 g, 10 min, 4 ˚C). The obtained supernatants were filtered with 0.22 µm Millipore Express Membranes and phenylmethylsulphonyl fluoride (PMSF) was added to a final concentration of 0.2 mM (29).

2.9.2. Protein precipitation

The proteins present in the supernatants were precipitated with 0.2 mg/mL sodium deoxycholate (DOC) for 30 min at 4 ˚C, followed by addition of 6 % (w/v) trichloroacetic acid (TCA) and overnight incubation at 4 ˚C. The precipitate was centrifuged (17 900 g, 15 min, 4 ˚C), washed with ice-cold acetone and resuspended in Milli-Q water.

2.9.3. Protein quantification

Protein concentration was determined by a modified Lowry method (50) using the bovine serum albumin (BSA) as standard.

2.9.4. SDS-PAGE

Samples containing 50 µg of total protein were dissolved in sample buffer: 80 mM Tris-HCl, pH 6.8, containing 2 % (w/v) SDS, 0.1 mM β-mercaptoethanol, 15 % glycerol and 0.01 % (w/v) *m*-cresol purple. Samples were then heated at 100 ˚C for 4 min and submitted to SDS-PAGE 15 % (w/v) in gels with 8 cm x 7.3 cm x 0.75 mm, on a Mini-PROTEAN 3 Cell (Bio-Rad, USA).

Separated proteins were visualized by colloidal Coomassie Brilliant Blue G-250 staining (51). Apparent masses were calculated based on the molecular masses of standard LPM (14 to 66 kDa) (Sigma-Aldrich, USA).

2.10. *In vitro* test of virulence

2.10.1. Cell line and culture conditions

The human adenocarcinoma cell line HT-29 (ECACC n.º 850611109) was used between passages 62 and 64. Cells were routinely grown in 75 cm^2 flasks in high glucose Dulbecco's modified Eagle's medium (D-MEM) (Gibco/Invitrogen, UK), supplemented with sodium bicarbonate (3.7 g/L), fetal calf serum (10% (v/v)) and L-glutamine (2mM) (complete medium). Penicillin (100 IU/mL) and streptomycin (100 µg/mL) were always added to the culture medium except for the medium used 24 hours prior to the virulence assays. Cells were maintained in a humidified atmosphere using an incubator at 37 ˚C under 5% (v/v) CO_2 in air.

2.10.2. Plaque-forming assay (PFA)

HT-29 cells were trypsinized from the 75 cm^2 flasks and 3 x 10^4 cells were deposited per well in a 96-well tissue culture plate. To obtain confluent monolayers, the plates were incubated for 3 days with antibiotics followed by incubation for 24 h without antibiotics.

The overnight grown *Listeria* strains (TSA-YE, 37 ˚C) were suspended in buffered saline to a concentration of 4 x 10^8 cell mL^{-1} and serial dilutions were made in complete medium. HT-29 cell monolayers were infected with dilution series of 10^2 to 10^7 *L. monocytogenes* cells per well, and incubated for 2 h at 37 ˚C. The inocula concentration was assessed by duplicate plating of the appropriate dilutions onto TSA-YE and incubation at 37 ˚C for 24 h, before counting colonies.

After 2 h incubation with the bacterial cells, the suspensions were removed and cell monolayers were incubated for 1.5 h with complete D-MEM medium containing 100 µg gentamicin mL^{-1}, and lacking penicillin and streptomycin. The wells were then covered with complete D-MEM medium supplemented with 10 µg gentamicin mL^{-1} and 2.5 % (m/v) agarose. Once the agar media was solidified, complete medium was added to the top of the agar media to prevent cell starvation. Culture plates were incubated for 24 to 48 h at 37 ˚C under 5 % (v/v) CO_2 in air. Formed plaques were counted 24 h after bacteria deposition on the HT-29 cell monolayer, and confirmed after 48 h of incubation. Enumeration of the plaques was done using an inverted microscope (52) (53).

2.11. *In vivo* test of virulence

To test the *in vivo* pathogenicity by subcutaneous injection (50 µL) of the wild-type and the mutants, seven-week-old immunocompetent Swiss female mice were used (54). They were maintained on sterilized wood shavings with free access to water and sterilized food. For each isolate, groups of five mice were inoculated subcutaneously into the left hind footpad.

Bacterial strains were grown on BHI plates for 17 hours at 37 ˚C. The culture was standardized turbidimetrically and diluted appropriately in phosphate buffered saline (PBS, pH 7.3) to obtain 4 log CFU (colony forming unit) in 50 µL for subcutaneous infection. The inocula used were verified by determining viable counts on TSA (Trypic Soy Agar) plates.

Mice were sacrificed by cervical dislocation three days after subcutaneous injection and spleens were aseptically removed. Homogenate samples were appropriately diluted in PBS and plated onto TSA plates. Viable numbers of bacteria were assessed after incubation at 37

˚C for 48 hours. Animal handling and care conditions were consistent for all experimental runs.

2.12. Expression of results and data analysis

For SDS-PAGE gels, the images were analyzed and band quantification was done using the densitometric curves obtained in the Gel Compare II version 5.1 (Applied Maths, Belgium).

For the β-galatosidase assay, the promoter activities were expressed as the mean of β-galactosidase units, from experiments done in duplicate, from three independent trials. The standard deviation was also assessed and it is shown in the graphic representations of promoter activities.

For the *in vitro* test, the pathogenic potential of the isolates was expressed as the mean log of the number of plaques formed (for 10^7 *Listeria* per well) (log PFA), in duplicate, from at least two independent trials. ANOVA of the log PFA values from the plaque-forming isolates was carried out using least significant differences (LSD) *post hoc* multiple comparison tests by running the program Statistica, version 6 (Statsoft).

For the mouse virulence assay, the virulence potential of the isolates was expressed as the mean log of the number of colony forming units (log CFU) per spleen, multiplied by the ratio of number of positive cultures in the spleen to inoculated mice in the subcutaneous infection test. The significant differences between strains were assessed using the log CFU mean and the standard deviations by applying a t-test.

Those values whose probability of occurrence was greater than 95 % ($p < 0.05$) were considered as significant values.

3. Results

3.1. Deletion mutant for the Tat pathway

To investigate the importance of the Tat pathway in *L. monocytogenes*, a deletion mutant for the genes coding for this system was constructed as described before in section 2.3.1. The confirmation of the deletion of the genes was done by polymerase chain reaction (PCR), using internal primers for the genes *lmo0361* (*tatA*) and *lmo0362* (*tatC*) as well as the primers Tat_A and Tat_D, used in the production of the homologous fragment for gene replacement (for primers sequence see Appendix 1). As a PCR control, internal primers for the *L. monocytogenes iap* gene were used.

The mutant strain showed a ~2000 bp band, the same size as the constructed plasmid (Figure 6), which represents ~1000 bp upstream *lmo0362* and ~1000 bp downstream *lmo0361.* The wild-type strain EGDe presented a ~3000 bp band. The difference between the mutant and the wild-type was explained by the deletion of *tatA* and *tatC* (911 bp) (Figure 6). The amplification done using the internal primers for the genes *lmo0361* (*tatC*) and *lmo0362* (*tatA*) resulted in no bands for the mutant and a band of ~200 bp for the wild-type strain, as expected.

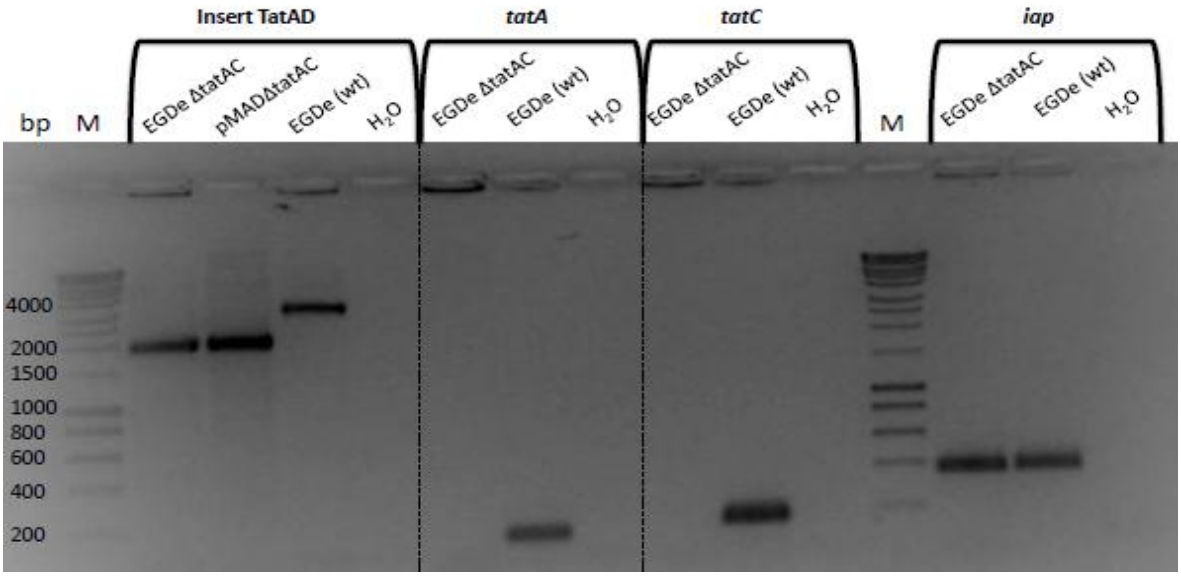

Figure 6 – Polymerase chain reaction results confirming the construction of a *tatAC* deletion mutant from the parent strain *L. monocytogenes* EGDe.

1% agarose gel. M, Ladder; bp, basepair.

In order to investigate if the deletion of *tatAC* genes was impairing for mutant growth, the mutant strain and the wild-type were grown in BHI at 37 ˚C (Figure 7) and growth rates (μ_{max}) were calculated. The wild-type showed a μ_{max} = 1.26 h^{-1} and the mutant showed a μ_{max} = 1.31 h^{-1}, showing that Tat pathway is not essential for *L. monocytogenes* survival.

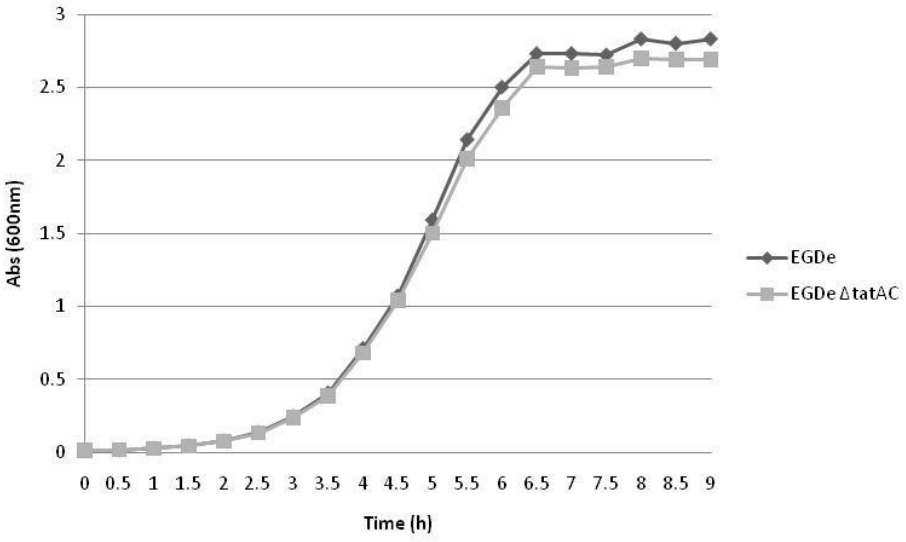

Figure 7 – Growth curves of *L. monocytogenes* EGDe and EGDe Δ*tatAC* mutant grown in complete medium (BHI) at 37 ˚C.

3.2. Transcriptional analysis

3.2.1. Northern blot

The transcription of the genes *lmo0361* (*tatC*) and *lmo0362* (*tatA*) was analyzed during the exponential and stationary phase. The total RNA was analyzed by Northern blot, by using probes for genes *lmo0361*, *lmo0362*, *lmo0363* and a control probe for 16S rRNA gene (Appendix 1). The genes *lmo0361* and *lmo0362* presented the same pattern of expression (Figure 8). The same RNA was observed with both probes indicating that genes *lmo0361* and *lmo0362* are transcribed in a bicistronic manner. The transcription of *tatAC* genes was growth-phase dependent, since it seemed to be less expressed in the stationary phase, in comparison to the exponential phase, as shown in Figure 8. As expected, no transcripts were detected in the deletion mutant.

Proteins exported by the Tat pathway have a conserved N-terminus motif which is cleaved when the protein is secreted. The gene *lmo0363* upstream the genes coding for the Tat pathway codes for a peptidase and it is located just 96 bp from *lmo0362* (see Figure 4 in section 1.3.). To investigate if gene *lmo0363* is transcribed together with genes *lmo0362* and

lmo0361 a Northern blot analysis for this gene was also performed (Figure 8). Transcripts of the *lmo0363* were practically undetectable and showed to be transcribed independently from *lmo0362* and *lmo0361*.

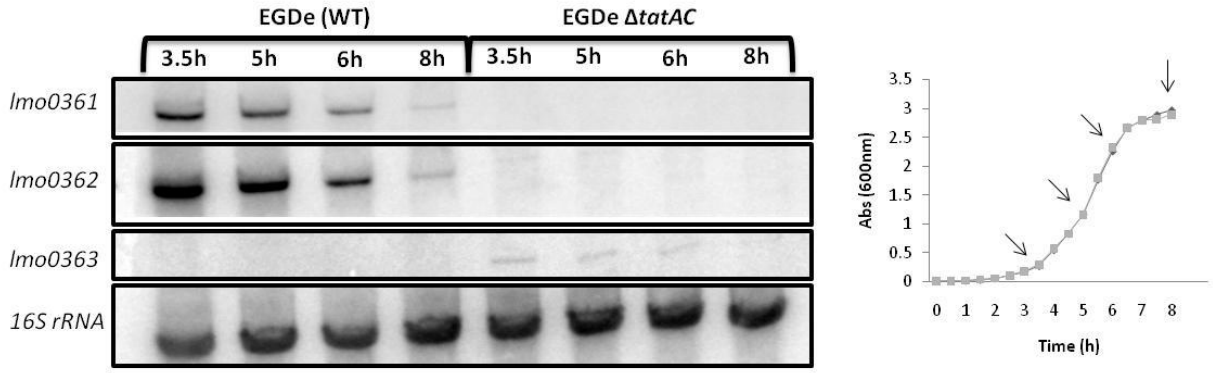

Figure 8 – Northern blot analysis of *lmo0361*, *lmo0362* and *lmo0363* transcripts in wild-type and *tatAC* mutant strain. RNA was isolated from both strains at different times of growth (3.5, 5, 6 and 8 hours, signed with an arrow in growth curve), corresponding to the exponential and stationary phases.

The *locus* for *lmo0361* and *lmo0362* in *L. monocytogenes* EGDe (serovar 1/2a) is similar to a *locus* present in the nonpathogenic *L. innocua*, but it is absent in the genome of the *L. monocytogenes* F2365 (serovar 4b) (55).

Between the genes coding for the secretion system and the operon containing the gene for the secreted protein, there is a gene coding for a transcription regulator (*lmo0364*) (see Figure 4 in section 1.3.).

To address the possible involvement of this transcription regulator in transcription regulation of genes *tatA* and *tatC*, a new transcription analysis was performed. For that a deletion mutant in this gene was also constructed. In the deletion mutant, the resulting protein lacks in the N-terminal, including the DNA-binding domain, 247 amino acids, out of 313 that constitute Lmo0364.

The results in Figure 9 show that the *lmo0364* gene product has no influence in the transcription of *tatA* and *tatC*, since no difference between the mutant and the wild-type strain was detected.

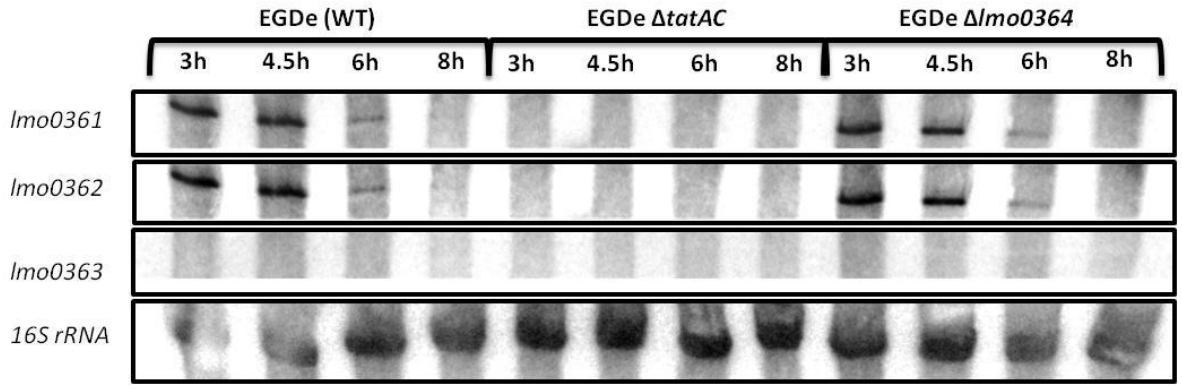

Figure 9 - Northern blot analysis of *lmo0361*, *lmo0362* and *lmo0363* transcripts in wild-type, *tatAC* and *lmo0364* mutant strains. RNA was isolated from both strains at different times of growth (3, 4.5, 6 and 8 hours), corresponding to the exponential and stationary phases.

3.2.2. Promoter activity

The promoter activity of the genes *lmo0361*, *lmo0362*, *lmo0363*, *lmo0364* and of the operon *lmo0365-67* was analyzed for the wild-type strain using a β-galactosidase assay.

Interestingly, the promoters showed different levels of activity, with *lmo0362* being the most active promoter, followed by the operon *lmo0365-67* promoter (Figure 10). Although transcripts of the gene *lmo0363* were undetectable in the Northern blot, promoter activity was low but recorded. Even much lower than the activity of the *lmo0362* and *lmo0365* promoters, the transcription regulator *lmo0364* also showed promoter activity (Figure 10). The promoter of *lmo0361* gene showed no promoter activity when compared to the empty plasmid demonstrating once more that this gene is not transcribed on its own but in a bicistronic transcript with *lmo0362*.

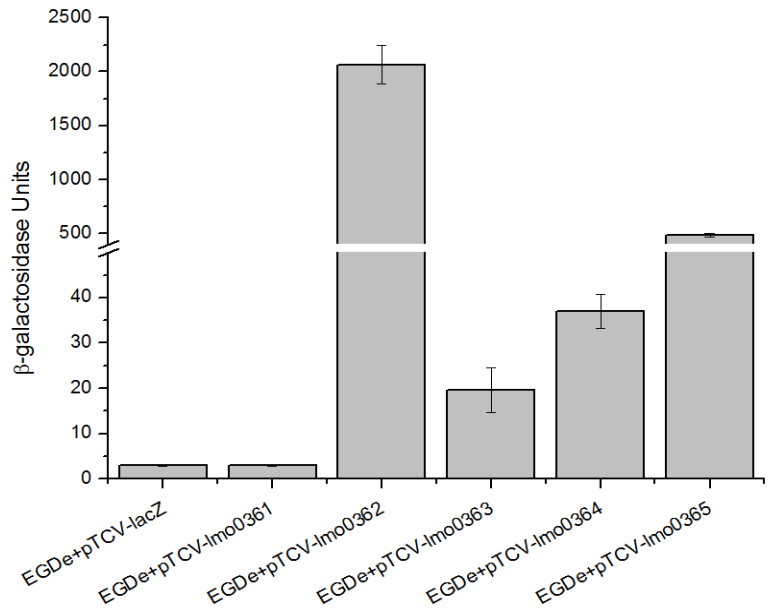

Figure 10 – Determination of promoter activity by using *lacZ* fusion.

Promoter activity was measured for strains containing the empty plasmid and plasmids with the promoter regions of the genes *lmo0361*, *lmo0362*, *lmo0363*, *lmo0364* and of the operon *lmo0365-67*, determined using the β-galactosidase assay method. All samples were collected after five hours of culture growth in BHI, at 37 ˚C. The presented activities are the averages of three independent experiments each conducted in duplicate. Standard deviation bars are shown.

The *lmo0362* transcript level showed to be growth-phase dependent in the Northern blot analysis, being the gene less expressed during the stationary phase. The promoter activity was investigated at different time points during culture growth (Figure 11). The promoter activity of the *lmo0362* gene showed an increased activity during exponential phase, followed by a decrease during the stationary phase.

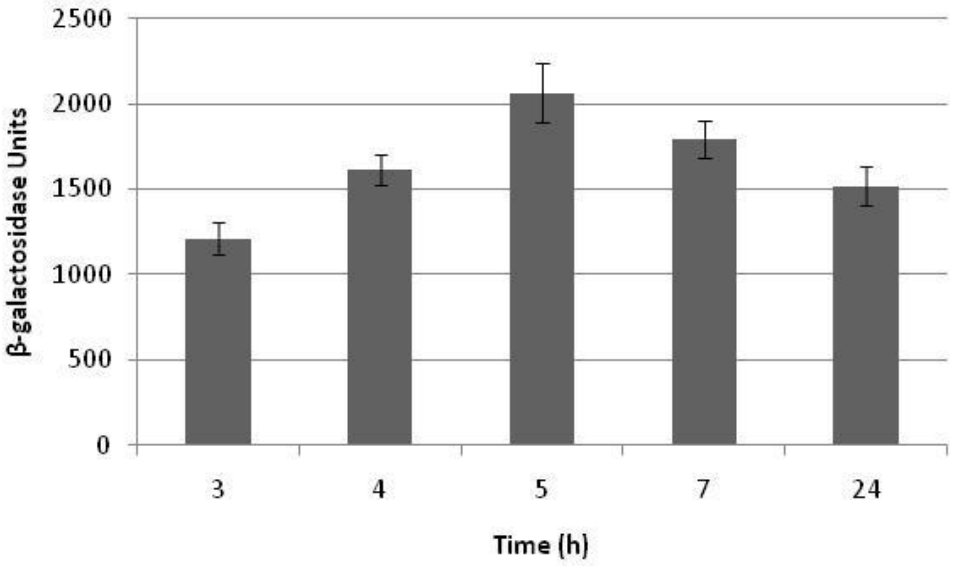

Figure 11 – Comparison of *lmo0362* promoter activity at different time points during growth (3, 4, 5, 7 and 24 hours in BHI, at 37 ˚C).

The presented activities are the averages of three independent experiments each conducted in duplicate. Standard deviation bars are shown.

In order to investigate if the transcription regulator (Lmo0364) was related to the transcription of the operon *lmo0365-67,* where the last gene codes for the protein hypothetically secreted by the Tat pathway (25), and to confirm the result from the Northern blot for *lmo0362*, the promoter activity of *lmo0362* and *lmo0365* (Figure 12 A and B, respectively) was assessed in the wild-type and the deletion mutants Δ*tatAC* and Δ*lmo0364*.

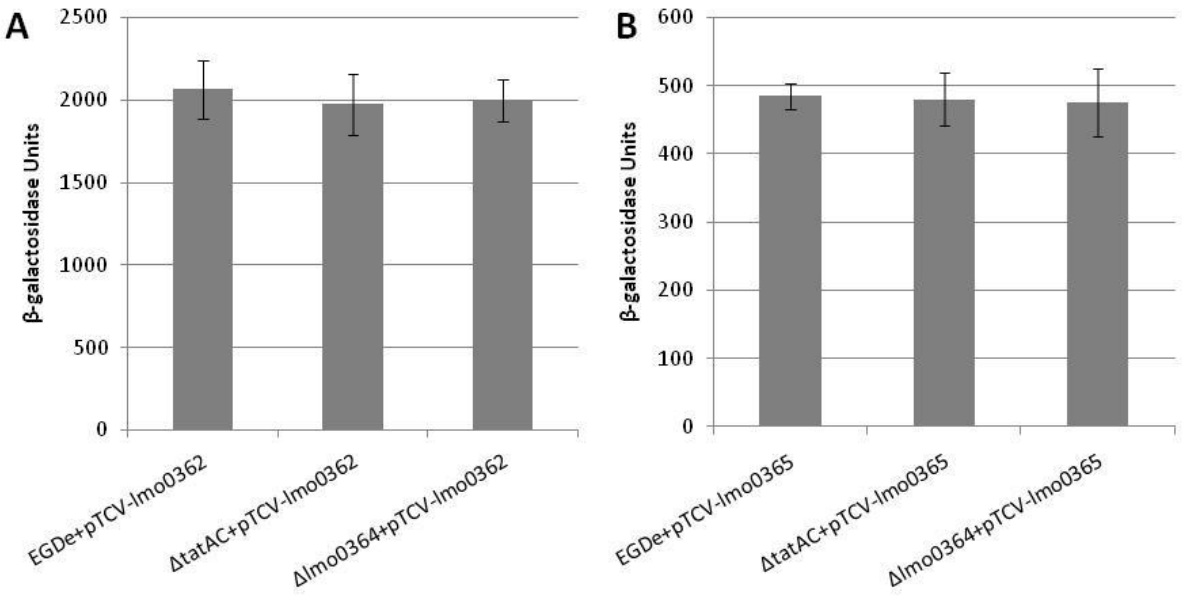

Figure 12 – Determination of promoter activities in the EGDe (wt), Δ*tatAC* and Δ*lmo0364* mutant strais. Cells were harvested in the exponential growth phase. (A) Promoter activity measured for the gene *lmo0362*. (B) Promoter activity measured for the operon *lmo0365-67*.

The presented activities are the averages of three independent experiments each conducted in duplicate. Standard deviation bars are shown.

No differences were found among the wild-type and the mutant strains both for the activities of *lmo0362* and *lmo0365-67* promoters, indicating that there is not a direct relationship between the transcription regulator Lmo0364 and the promoter activities of the genes *lmo0362* and *lmo0365-67*.

The results obtained for the activity of *lmo0362* promoter (Figure 12A) also indicate that the presence of this secretion system and the promoter activity of the corresponding genes are not related. The same could be verified for the *lmo0365-67* operon. This operon hosts the gene coding for the protein putatively secreted by the Tat pathway. Nevertheless, the promoter activity of the operon was not different in the Tat pathway mutant.

To confirm that the transcriptional regulator was not associated to the transcription of the genes studied and that the results were not disguised by a down regulation of its transcription in the growing conditions used, the promoter activity of the *lmo0364* was also assessed. No differences were found among strains (Figure 13) or time points during growth (data not shown). Finally, the influence of Lmo0364 in the promoter activity of *lmo0363* gene was also investigated. The obtained results (not shown) also indicated the absence of differences between the wild-type and Δ*lmo0364* mutant strains.

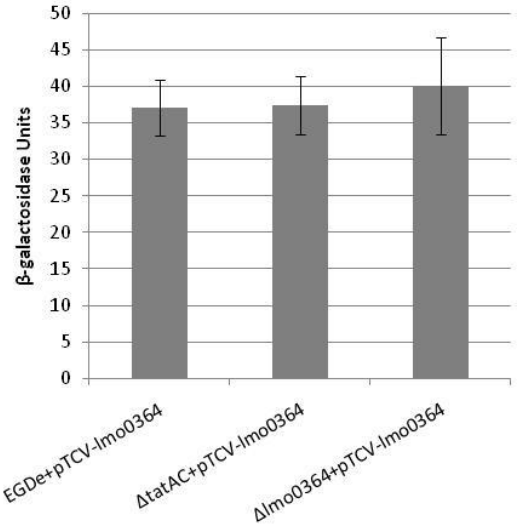

Figure 13 – Determination of promoter activity measured for the gene *lmo0364*.

The presented activities are the averages of three independent experiments each conducted in duplicate. Standard deviation bars are shown.

3.3. Proteomic analysis

3.3.1. SDS-PAGE analysis of secreted proteins

The hypothesis that *L. monocytogenes* has a secretion system (Tat pathway) that secretes only a single protein was predicted *in silico* (25).

In order to investigate this hypothesis, the secretome of *L. monocytogenes*, grown in minimal medium at both 20 and 37 ˚C (the saprophytic and infectious temperatures, respectively) was analyzed for differences in the presence or absence of proteins in SDS-PAGE gels (Figure 14). This preliminary analysis showed no relevant differences between the wild-type strain and the *tatAC* mutant. The protein predicted to be secreted by the Tat pathway (Lmo0364) should present approximately 40 kDa. No identifiable change between the mutant and the wild-type strain protein patterns was observed at this range size or at any other range sizes (Figure 14).

At 20 ˚C, the most intense band (between 29 and 36 kDa, marked with an arrow) is thicker in the mutant compared to the wild-type. Its relative amount was assessed using Gel Compare II version 5.1 (Applied Maths, Belgium), showing that there was a relatively higher amount (1.3 times) in the Δ*tatAC* mutant when compared with EGDe wild-type. By comparison with previously identified bands from other strains of the same serovar 1/2a (data not published), this could be flagellin, the main constituent of the flagellar filament.

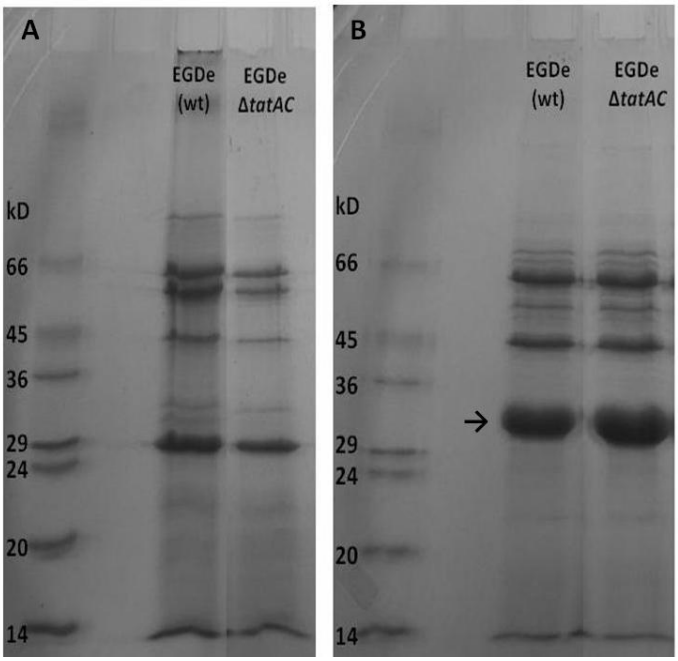

Figure 14 - The secretome of *L. monocytogenes* strains grown in minimal medium and harvested at late exponential phase (A_{600} = 0.8).

(A) Cells grown at 37 ˚C. (B) Cells grown at 20 ˚C.

3.4. Phenotypical analysis

3.4.1. In vitro *virulence*

The importance of the secretion systems in virulence is related with the fact that the bacterial virulence depends on the ability to secrete virulence factors. To assess if the absence of the Tat pathway influenced the infectious ability of the *tatAC* mutant, plaque forming assays (PFAs) were performed (Table 3). These assays allow the evaluation, not only of the adhesion of the bacteria to the epithelial cells, but also its ability to internalize, multiply inside the host cell and migrate from one cell to another.

Table 3 – *In vitro* virulence potential of EGDe wild-type strain and Δ*tatAC* mutant.

Strain	Plaque forming assay Mean (log ± SD)[a]
EGDe	6.24 ± 0.46
EGDe Δ*tatAC*	6.46 ± 0.36
442[b]	3.69 ± 0.13

[a] **Number of plaques formed for 10^7 *Listeria* per well. The result corresponds to the mean ± standard deviation (SD) of logarithmic values from two independent experiments done in duplicate.**

[b] **Low-virulence 442 strain used as reference for *in vitro* tests** (42).

Strain EGDe (wild-type) is generally used as a reference for virulent strain, whereas strain 442 is used as reference for low virulence (42).

The deletion mutant for the Tat pathway did not show alteration in the PFA assay results when compared to the wild type strain, demonstrating that this secretion system is not essential for the infectious steps analyzed by the PFA, specifically adhesion, internalization, multiplication and cell to cell spread.

3.4.2. In vivo virulence

Although *in vitro* results are a good methodology for the evaluation of some steps of the infectious cycle, when using animal models the whole process of infection is much more complex and more challenging for the bacteria.

The Tat pathway has been described as responsible for the secretion of an iron dependent dyp-peroxidase (product of gene *lmo0367*), and iron is one of the key factors for pathogenesis. The pathogens need to keep the iron under a strict regulation and, therefore, an *in vivo* study of virulence was performed for the wild type and the deletion mutants Δ*tatAC* and Δ*lmo0364* (Table 4).

Table 4 – *In vivo* virulence potential of EGDe wild-type and mutant strains.

Strain	Subcutaneous test (spleens) Mean (log ± SD)[a]	Number of positive cultures (spleen)
EGDe	4.01 ± 0.24	5/5
EGDe Δ*tatAC*	4.99 ± 0.48	5/5
EGDe Δ*lmo0364*	4.26 ± 0.28	5/5

[a] **Number of bacteria per spleen homogenate. The result corresponds to the mean ± standard deviation (SD) of logarithmic values from contaminated mice.**

The result showed that the *tatAC* mutant was significantly more virulent than the wild-type strain (t-test, p-value < 0.05), while no significant difference was observed for the *lmo0364* mutant.

4. Discussion

4.1. The Tat pathway in *Listeria monocytogenes*

In the present study, a *L. monocytogenes* mutant strain lacking the genes coding for the Tat pathway was successfully constructed (EGDe Δ*tatAC*). Like the parent strain, the mutant showed the ability to grow, in both complete and minimal medium, comfirming that the Tat pathway is not essential for *L. monocytogenes* survival.

For other species, it has been reported that the *tat* genes are organized in an operon, but, to our knowledge, this is the first time that it is experimentally demonstrated that *lmo0361* (*tatC*) and *lmo0362* (*tatA*) genes in *L. monocytogenes* are co-transcribed in a bicistronic and growth-phase dependent manner. Transcription of these genes occurs rather in the early exponential phase, being down regulated in the stationary phase. This was confirmed by Northern blot analysis and by the promoter activity determination of *lmo0362* and the null activity found for *lmo0361* supposed promoter region. Even though transcription of the genes was shown, the Tat pathway functionality still remains to be elucidated.

Previous studies showed that the genes coding for this secretion system were under the negative regulation of CtsR, a transcriptional repressor of class III stress response genes, and σ^B, an alternative sigma factor that positively regulates the transcription of class II stress response genes (56). It has also been shown that Fur, a regulator of iron acquisition systems, was responsible for down regulation of the *tatAC* genes (40).

The growth-phase dependent transcription observed for *tatA* and *tatC* is similar to the one described for the *fri* gene, which codes for a ferritin-like protein and is under the direct regulation of Fur (57). Even though the *tat* genes may be under direct or indirect regulation by different proteins, its transcription pattern, similar to the *fri* gene, suggests a participation of Fur in the observed down regulation.

Fur has been studied and some direct interactions between Fur and several promoter regions were identified (*fur* boxes). For some repression activities, Fur binding to *fur* boxes has been shown to be iron dependent, but iron independent when binding different *fur* boxes promoters (40). The pathways involved in this mechanism are still unclear.

While transcription of *tatAC* showed to be growth-phase dependent by Northern blot analysis, this was not evident when promoter activity was assessed, even though a slight decrease of activity was observed in the stationary phase. This can be explained by the absence of the CtsR binding site previously identified for *tatA* promoter region in *L. monocytogenes* (56). CtsR binding site is located around 270 bp upstream the starting codon

of *tatA* (56), in the coding region of *lmo0363,* and was absent in the construction used for promoter activity determination. Furthermore, its distance from the starting site of *tatAC*, as well as the verification of promoter activity 152 bp upstream of the coding region and its down regulation in the stationary phase, seem to be indicative of the independence of direct regulation by CtsR at the locus previously identified. The down regulation verified in the promoter activity in the stationary phase can then be attributed to other regulators of these genes. The lower level of transcripts in the stationary phase can also be explained by a pos-transcriptional regulation, such as mRNA degradation.

The results obtained in this work with the wild-type and the Δ*tatAC* mutant transformed with the *lmo0362* promoter region, combined with the Northern blot analysis for *tatAC* genes, suggest that *tatAC* expression is not regulated by the level of their product, showing the same promoter activity and level of transcripts both in the wild-type and in the mutant strain.

Previously *in silico* analysis (25) pointed to a 40 kDa protein secreted by the Tat pathway. The SDS-PAGE analysis of *L. monocytogenes* secretome did not show differences between the EGDe wild-type and the Δ*tatAC* mutant, although it does not mean that the protein was not present. It could be present in undetectable levels by the method used. FLAG-tag methodology (58) is an alternative methodology that could be used to assess the presence of the protein both in the supernatant and in the cytoplasm, with a higher detection capability.

For the observed intensity difference of bands possibly corresponding to flagellin (Figure 14B), no plausible explanation was found, although a CtsR mutant was described in *L. monocytogenes* as having reduced *flaA* transcription and FlaA expression (59), showing that the repressor CtsR appears somehow connected to both *flaA* and *tatAC*. Further experimental studies will be needed in order to prove the involvement of the Tat pathway in the secretion of Lmo0367 and to identify other proteins possibly secreted by this system.

Considering strains virulence ability, the presence of Tat pathway showed to be irrelevant for *in vitro* virulence, but significant differences were found when *in vivo* virulence was assessed. Surprisingly, the differences verified *in vivo* showed that the *tat* mutant was significantly more virulent ($p < 0.05$) than the wild-type strain. This result was unexpected as the only protein considered as secreted by the Tat pathway, Lmo0367, is a peroxidase, pointed by some authors as a possible new major virulence factor (25). This protein should be important in the host environment, where oxidative stress due to release of reactive oxygen intermediates takes place (60). It is also interesting to notice an increase of *in vivo* virulence of *tat* mutants, as serovar 4b strains, which are usually more involved in clinical cases, appear to be lacking the genes coding either for the Tat pathway and the *lmo0365-67* operon (55). This may indicate that, instead of being useful and necessary for virulence, the Tat pathway and

possibly the iron-dependent peroxidase Lmo0367 might be, on the other hand, impairing virulence.

4.2. The *lmo0365-67* operon

A first reported *in silico* analysis identified two proteins secreted via Tat pathway: Lmo0367 and Lmo2201 (24). Recently, another study reported that Lmo2201 was most certainly a 50S ribosomal protein L35 and, therefore, it could not be secreted. Thus, only the protein Lmo0367 is considered to be secreted by this secretion system (25). The gene coding for this protein is part of an operon where *lmo0365* and *lmo0366* are also present (40). These three genes are related to iron, as *lmo0365* and *lmo0366* code for a transporter and a lipoprotein, respectively, both involved in iron transport, while lmo0367 codes, as already mentioned, for an iron-dependent peroxidase.

A similar operon was identified in *Staphylococcus* spp., where a peroxidase (FepB), similar to Lmo0367, was demonstrated to be secreted by the Tat pathway. In *S. aureus*, *tatAC* genes are right downstream the iron-operon, similar to *lmo0365-67* (26). Although the proteins encoded in the operon are similar to those found in *Listeria*, their organization is different, raising once again the question of its origin, since it has been shown that this secretion system is also present only in some *Staphylococcus* species. Biswas *et al.* (26) support the possibility that, in *Staphylococcus*, the cluster holding the genes *tat* and the iron-related operon have phage origin and therefore the phage would probably be the transmission vector among different species (26). This hypothesis was based on the fact that it was identified a gene coding for a protein similar to a phage envelop protein, immediately upstream of *tatA*.

Here we demonstrated that the promoter of this operon has a strong activity, although less strong than the one observed for the *tat* genes. Moreover, our results show that there is no influence in promoter activity when the *tat* genes or the *lmo0364* were deleted from the genome. It might be possible that there is no relation between the presence of the secretion system and the transcription of the operon where the putative secreted protein is coded, raising the question about what might happen to the transcript of *lmo0367* in the *tatAC* mutant. Is the mRNA or the protein degraded? Is it possible that the Lmo0367 is secreted through another system or does it accumulates inside the cell? These questions can only be answered by performing a more detailed study on the protein Lmo0367.

Similarly to what has been shown to *tatAC* genes by Ledala et al. (40), the *lmo0365-67* operon is also under the negative regulation of Fur. The *fur* gene has been shown to be expressed in the presence of iron and significantly repressed in the absence of the same

metal (40). However the transcriptional *in vivo* study developed by Camejo *et al.* (61) showed that both *fur* and *lmo0366* were highly upregulated *in vivo* (61), presenting some inconsistency in the predicted mechanisms of regulation. It is also interesting to notice that if free iron is present in almost negligible concentrations *in vivo* (57), the *fur* gene should then be repressed or, at least, not upregulated, as it will repress some genes responsible for iron acquisition (40) (57). This reinforces the hypothesis that the regulation by Fur is far from being understood and that it might be a complex sensor/regulator pathway instead of a negative regulation by binding of Fur to a *fur* box in the promoter region.

4.3. Transcriptional Regulator Lmo0364

The *locus* from gene *lmo0361* to *lmo0367* is a conserved *locus* also present in *L. innocua*. Genes *lmo0361* and *lmo0362* seem to be related to *lmo0367*, since the first two code for the translocation system responsible for the secretion of the product of the last one. In the same *locus*, gene *lmo0364,* coding for a transcriptional regulator required further study. Besides its particular location, the *lmo0364* proved not to be related to the other genes in the *locus*, as it is not involved in the transcription regulation of any of the genes studied. Interestingly, *L. monocytogenes* serovar 4b appears to have genes homologous to *lmo0363* and *lmo0364*, lacking the two operons surrounding these genes.

Considering the loss or acquisition of operons *lmo0361-62* and *lmo0365-67*, depending on the evolutionary moments of serovar divergence, it is interesting to notice that it happened in two different places, although close to each other. The G+C content in these two *loci* is between 36 and 41%, similar to the content determined for *Listeria* species (38%). This may suggest their lost at some point along the species divergence. According to Doumith *et al.* (62), serovar 1/2a diverged from serovar 4b and *L. innocua*, being these last two evolutionary closer than to the serovar 1/2a. This may indicates that, serovar 4b has lost the genes *lmo0361-62* and *lmo0365-67*, present in both serovar 1/2a and in *L. innocua*.

Our results show that the transcription regulator Lmo0364 did not influence *L. monocytogenes* growth or *in vivo* virulence, pointing that probably it is not related to key virulence genes. Actually, when a protein BLAST (BLASTp, NCBI) was performed, 96% similarity was found between this transcriptional regulator and the DeoR regulator from *E. coli*. The *E.coli* family of DeoR regulators includes at least 14 members and they usually act as repressors in sugar metabolism (63). The Lmo0364 could then be related to *L. monocytogenes* sugar metabolism and not to the studied iron related operons. Either way, we showed that it might be transcribed when cells are grown in rich medium, since promoter activity was reported for *lmo0364* for both exponential and stationary phases.

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Appendix 1 – List of Primers

List of primers and Northern blot probes used in this study, including its sequences.

Primer	Sequence (5' – 3')[a]
Tat_A	CGTGTCGACCTGATAATGACCATTTCGTC
Tat_B	GCACGCGTCATTTCGATTTCCTCCATTC
Tat_C	CGACGCGTGTCTAAAATGAATTATTTATAC
Tat_D	TGTAGATCTTCGATTTGCCTTATTTAATG
TatA_fw	ATCGGACCAGGAAGTATTGC
TatA_rv	ATCATCCATCATGCCTTTGG
TatC_fw	GTCGCTGATAACACCAAACG
TatC_rv	CATTCGCCGGACAGTAAGC
Iap_fw	CAAACTGCTAACACAGCTACT
Iap_rv	GCACTTGAATTGCTGTTATTG
P1-lmo0364	GGGGGAATTCCGCACCAGTTCGTTTTAATTCC
P2-lmo0364	CATCGCCATCTCTTTTCTTTTTTC
P3-lmo0364	GAAAAAAGAAAAGAGATGGCGATGGAAATTGCTTCCAACGGTGT
P4-lmo0364	CCCCGGATCCCCATTCATCCTCCTTTCTAATTG
pAUL-1	ATGATTACCGCCCAAGCTTG
pAUL-2	CAGGACGTTGTAAAACGACG
PC1_lmo0364	GATAACTGCTCCAGCTGAC
PC2_lmo0364	CCCTAATGCACCTAATAGC
pTCV_lmo0361_fw	GGGGGAATTCGGAGCCGCCCTTGTGATTTT
pTCV_lmo0361_rv	GGGGGGATCCAGGTGTCCAGTGAGGCT
pTCV_lmo0362_fw	GGGGGAATTCAACCATGAAGCGATCTTTATTCGTG
pTCV_lmo0362_rv	GGGGGGATCCACAAGGGCGGCTCCGAC
pTCV_lmo0363_fw	GGGGGAATTCATCGCCGTACTTCGAGAAAT
pTCV_lmo0363_rv	GGGGGGATCCACAACATCTTTAAAAGACGAGGT
pTCV_lmo0364_fw	GGGGGAATTCTTTCCTTGCAGATTGCTTTC
pTCV_lmo0364_rv	GGGGGGATCCAGCATCATATCATTTAATCG
pTCV_lmo0365_fw	GGGGGAATTCAAATAAACATGGTTCAGCC
pTCV_lmo0365_rv	GGGGGGATCCATAACTCGACCTAAAAG
vLac-1	GTTGAATAACACTTATTCCTATC
vLac-2	CTTCCACAGTAGTTCACCACC
NB_lmo0361	GGCCATTACCCTACCAACTAGCTAATGCAC
NB_lmo0362	GTATCTCCAGCAGCTCTACCAAGTTCTGGCAG
NB_lmo0363	GTGAAGTGATAACTGCTCCAGCTGACTCGC
NB_16SrRNA	GGCCATTACCCTACCAACTAGCTAATGCAC

[a] **Underlined bases correspond to restriction sites.**

Appendix 2 – Plasmid Maps

Structure of plasmids pMAD, pAUL-A and pTCV-lac all used in this study.

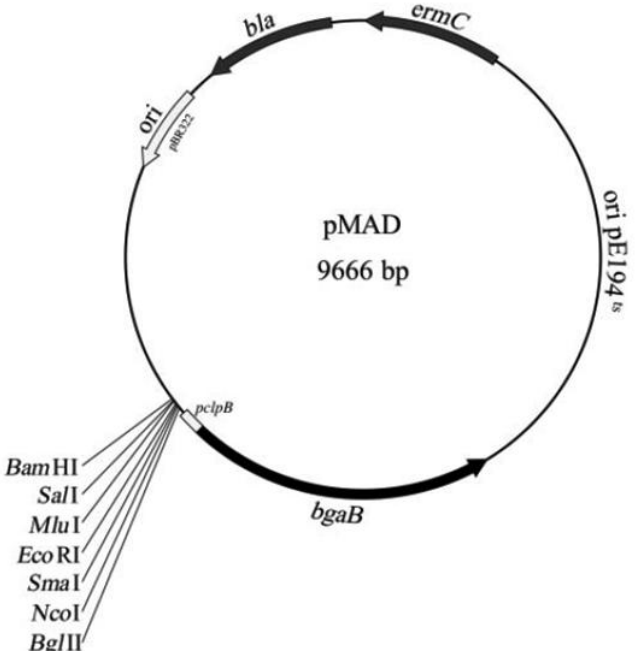

Figure 2. 1. Structure of pMAD plasmid (44).

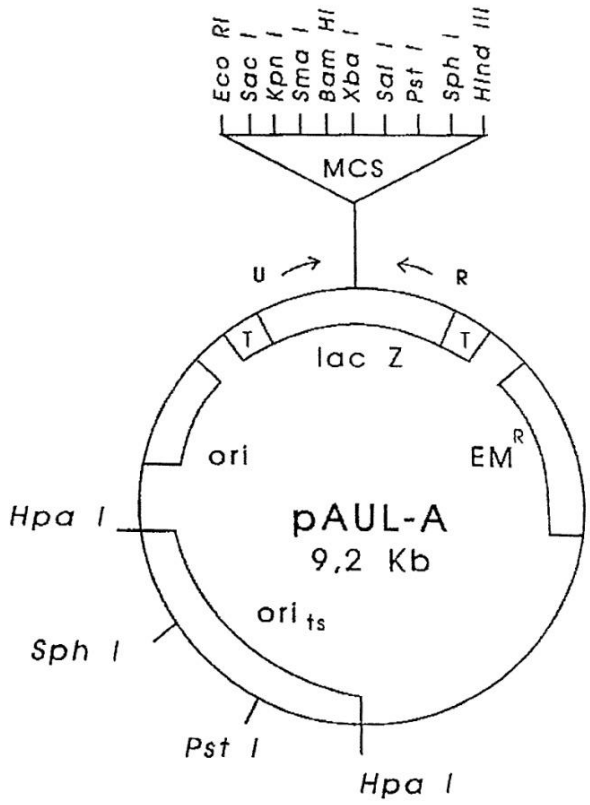

Figure 2. 2. Structure of pAUL-A plasmid (43). MCS, multi cloning site.

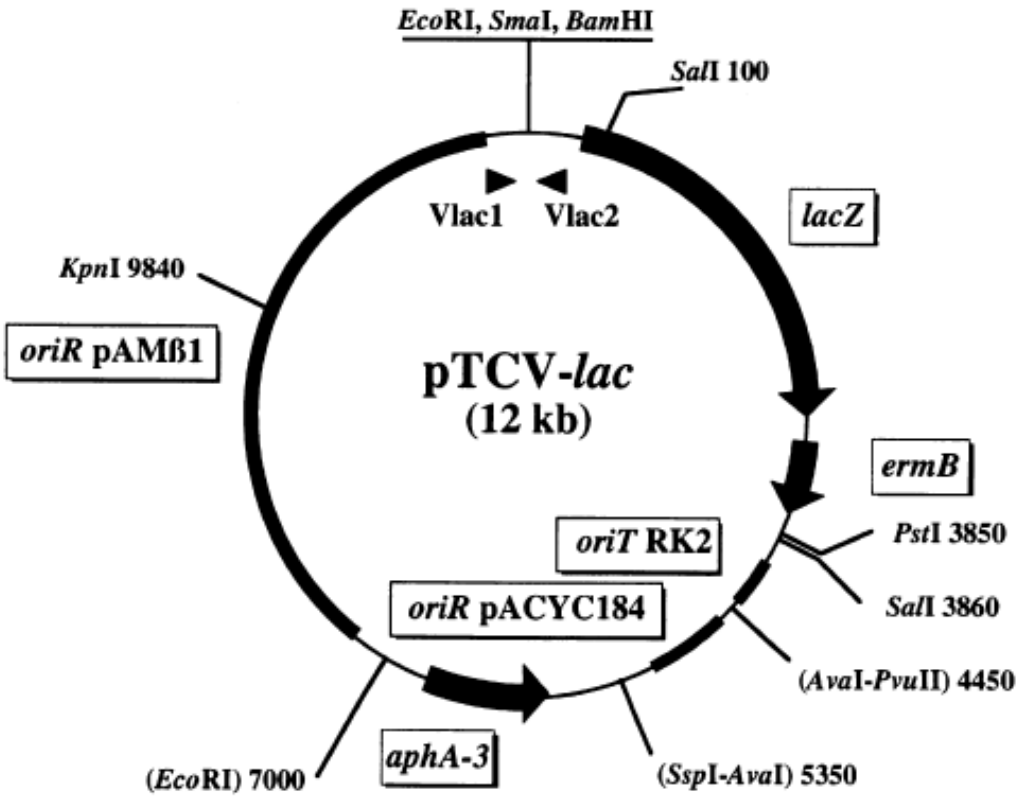

Figure 2. 3. Structure of pTCV-lac plasmid. (45)

Appendix 3 – Schematic representation of gene deletion

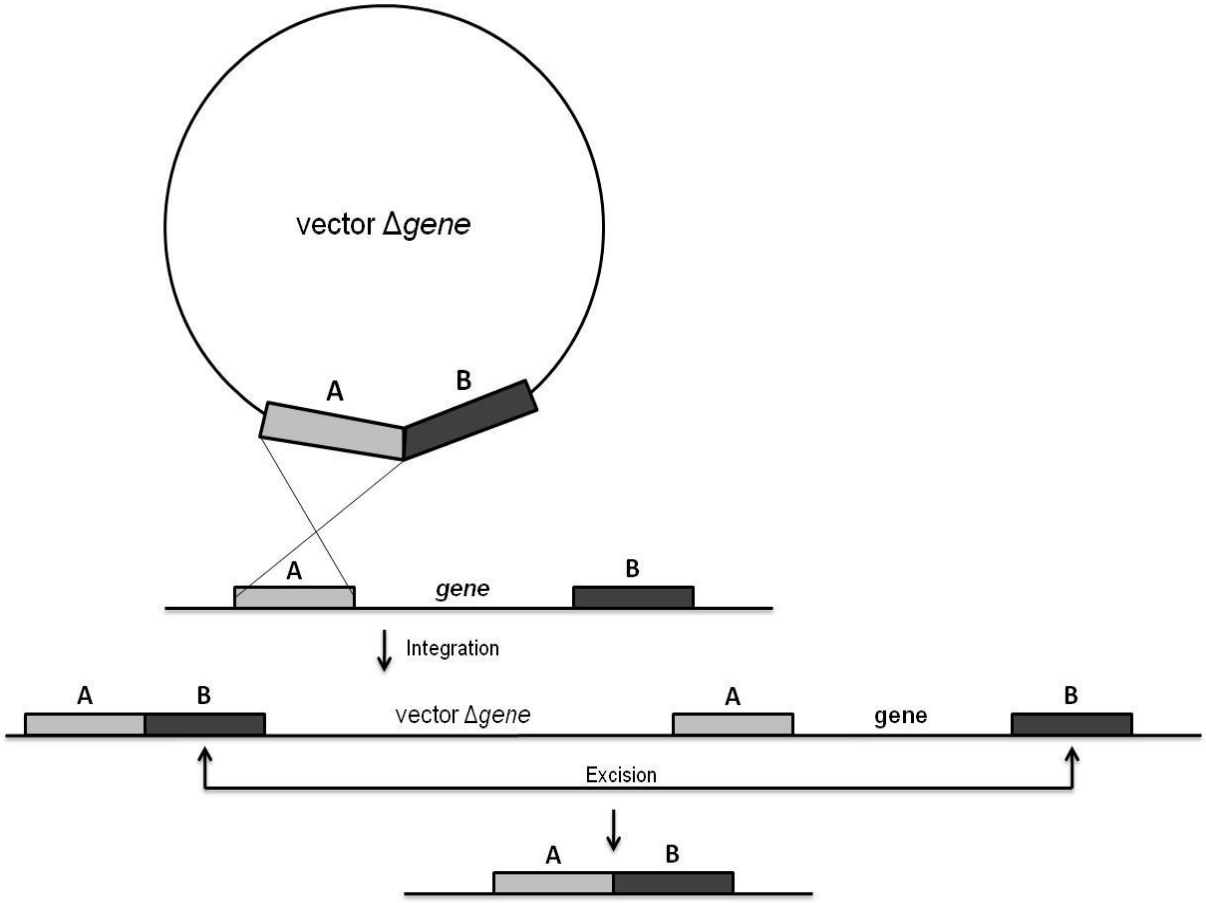

Figure 3. 1. Schematic representation of a two step procedure used to obtain gene deletion by recombination.

Areas labeled A and B represent DNA sequences located upstream and downstream from *gene.* The crossed lines indicate crossover events. The first step is integration of the vector via homologous sequences, it can take place in area A or B. Depending on whether the second recombination event occurs between the two homologous sequences in area A or B, the gene will either remain in the chromosome (area A) or be excised along with the vector (area B). Gene deletion occurs only if the second recombination event occurs in area B, as shown.