ANA CAROLINA NERY TEIXEIRA

SISTEMAS DE RECUPERAÇÃO DE RIBOSSOMOS EM *Actinobacillus pleuropneumoniae*: PAPEL EM VIRULÊNCIA E RESPOSTA A DIFERENTES CONDIÇÕES DE ESTRESSE

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

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APROVADA: 27 de julho de 2018.

Ciro César Rossi

Míriam Teresinha dos Santos

Denise Mara Soares Bazzolli
(Orientadora)

Dedico este trabalho a minha avó Ida.
“The ones that love us never really leave us.” – J.K. Rowling

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A pleuropneumonia suína é uma doença respiratória causada pelo patógeno bacteriano *Actinobacillus pleuropneumoniae* (APP) cujo sucesso na colonização e permanência nos pulmões do suíno está relacionado à sua capacidade de resistência a diferentes condições de estresse e a inúmeros fatores de virulência. Para o sucesso do patógeno, a síntese proteica eficiente é um gargalo, uma vez que este processo pode ser um fator limitante durante o crescimento e replicação bacterianos, e para isso há uma expressiva necessidade de ribossomos ativos dentro da célula. Em condições de estresse, os ribossomos podem ficar parados em uma fita de mRNA quando um códon de parada não é detectado, precisando, portanto, de um resgate ativo em um processo chamado trans-tradução. Um RNA pequeno denominado tmRNA, ubíquo em bactérias, codificado pelo gene *ssrA*, é responsável por mediar o mecanismo de trans-tradução. Muitas bactérias ainda apresentam dois sistemas adicionais mediados pelo fator de resgate alternativo A (*alternative rescue factor* A), ArfA, descrito em outras bactérias como *Escherichia coli, Salmonella enterica* e *Yersinia pestis* e o fator de recuperação alternativo B (*alternative rescue factor* B), ArfB presente em 34% dos genomas bacterianos já sequenciados. Este trabalho teve como objetivos investigar qual(is) sistema(s) de recuperação de ribossomos está(ão) presente(s) em *A. pleuropneumoniae* e qual o envolvimento deste(s) na virulência e na resposta a diferentes condições de estresse. Análises *in silico* e *in vitro* foram conduzidas com este propósito. Nossos resultados *in silico* mostraram que *A. pleuropneumoniae* possui apenas dois sistemas de recuperação de ribossomos, e o fato de não conseguirmos a obtenção de uma linhagem duplo mutante (Δ*ssrA*_Δ*arfA*) valida nosso resultado. Para verificar o envolvimento destes genes em virulência e resposta a condições de estresse, linhagens mutantes denominadas Δ*ssrA* e Δ*arfA*, bem como linhagens complementadas para as respectivas linhagens, Δ*ssrA*C e Δ*arfA*C, respectivamente, foram construídas a partir da linhagem APP MIDG2331 WT (sorotipo 8). Estas linhagens (WT, mutantes e complementadas) foram investigadas quanto ao crescimento *in vitro*, adesão, sensibilidade a antibióticos e a diferentes condições de estresse (oxidativo, osmótico, temperatura e etanólico). Além

disso, a virulência destas linhagens foi analisada no modelo de infecção alternativo *Galleria mellonella*. Análises fenotípicas mostraram que o mutante Δ*ssrA* foi mais sensível antibióticos e a todas as condições de estresse se comparado à linhagem WT e ao mutante Δ*arfA*. Além disso, o mutante Δ*ssrA* apresentou menor capacidade de adesão e virulência atenuada em *G. mellonella* quando comparada com as linhagens WT e Δ*arfA*. A linhagem Δ*ssrA*C não mostrou uma restauração completa do fenótipo original. Entretanto, a linhagem Δ*arfA*C mostra uma restauração completa do fenótipo. Estes resultados mostram que o gene *ssrA* tem um papel importante na resistência ao estresse e na virulência em APP e a ausência do gene *arfA* não afetou o *fitness* da bactéria nas condições avaliadas. Diante desses resultados, podemos concluir que o sistema TmRNA/SmpB é o principal sistema de recuperação de ribossomos em *A. pleuropneumoniae* e que a falta do mesmo acarreta em atenuação do fenótipo de virulência e aumento da sensibilidade a condições de estresse.

ABSTRACT

TEIXEIRA, Ana Carolina Nery, M.Sc., Universidade Federal de Viçosa, July, 2018. **Ribosome recovery systems in *Actinobacillus pleuropneumoniae*: role in virulence and response to different stress conditions.** Advisor: Denise Mara Soares Bazzolli. Co-advisors: Hilário Cuquetto Mantovani, Janine Therésè Bossé and Marisa Vieira de Queiroz.

The porcine pleuropneumonia is a respiratory disease caused by the bacterial pathogen *Actinobacillus pleuropneumoniae*. The success of this bacterial pathogen on colonization and permanence in the pig's lungs is related to their capability of stress resistance as well its virulence. Efficient protein biosynthesis, the main rate-limiting factor during bacterial growth and replication, depends on the concentration of active ribosomes within the cell. Ribosomes may become stalled on an mRNA strand when a stop codon is not detected, and active rescue of the ribosome (a process called trans-translation) is required to release it. Almost all bacteria encode a small RNA (tmRNA), encoded by the *ssrA* gene, which mediates trans-translation. An alternative rescue factor protein, ArfA, has also been described in bacteria such as *Escherichia coli, Salmonella enterica*, and *Yersinia pestis*. A second alternative rescue factor protein, ArfB, has been found in 34% of the bacterial genomes already sequenced. Both the *ssrA* and *arfA* genes are present in the genome of *A. pleuropneumoniae*, and the aim of this work was to characterize the respective contributions of these factors to stress response and virulence of this important swine pathogen. For this, simple mutants named Δ*ssrA* and Δ*arfA,* as well complemented strains for the mutants named Δ*ssrA*C and Δ*arfA*C, respectively, were constructed from APP MIDG2331 WT (serotype 8). These mutants, complemented strains and their parental strain were tested for their growth (*in vitro* conditions), adhesion, sensitivity to antibiotics and different stress conditions (oxidative, osmotic, temperature and ethanolic). Besides that, their virulence was tested in *Galleria mellonella* larvae model. Our results showed that *A. pleuropneumoniae* has only two ribosome rescue systems since it was not possible to get a double mutant strain (Δ*ssrA*_Δ*arfA*) and there is a high conservation of these genes among the *A. pleuropneumoniae* serotypes (1-16) analyzed *in silico*. Phenotypic analyses showed that Δ*ssrA* mutant presented more sensitivity to the antibiotics and all stress conditions tested, had a minor adhesion capability and its virulence was more attenuated in *G. mellonella* when compared with the parental and the Δ*arfA* mutant strains. Furthermore, the Δ*ssrA*C did not show a complete phenotype restoration. This strain has it growth,

adhesion and virulence behavior very similar to the Δ*ssrA* mutant. Opposed to the Δ*ssrA*C, the Δ*arfA*C strain shows a complete phenotype restoration. These results show that the *ssrA* gene have an important role in the virulence and stress resistance on APP and the lack of the *arfA* gene did not affect the bacterial fitness. Then, we can conclude that the ribosome rescue system based on TmRNA/SmpB mediates the main ribosome rescue system in *A. pleuropneumoniae.*

1. INTRODUÇÃO GERAL

Em consonância com o aumento da demanda por carne e seus derivados, a produção mundial de carne suína tem apresentado um rápido crescimento nas últimas décadas. A China é responsável por mais de 56% da produção mundial, ocupa o primeiro lugar no *ranking* mundial, mas, consome praticamente tudo que produz (ABPA, 2017). Neste *ranking*, o Brasil se encontra em quarto lugar, sendo responsável por pouco mais de 3% da produção de carne suína em 2017 e o Estado de Minas Gerais é um importante polo, sendo o quarto estado brasileiro com maior produção (ABPA, 2017). O aumento na produção de carne suína está associado com o método de criação intensiva, onde um grande número de animais é mantido confinado em um pequeno espaço físico. No entanto, esse tipo de criação está associado a uma rápida transmissão de doenças entre os animais, principalmente doenças respiratórias infecciosas (Fournié et al., 2015).

Vários agentes etiológicos são capazes de causar doenças respiratórias em suínos. A maioria dos agentes virais causa pneumonia intersticial, sendo que o vírus da síndrome reprodutiva e respiratória suína, o vírus da influenza suína, o coronavírus respiratório e o circovírus suíno tipo 2 são os principais agentes etiológicos virais (Haimi-Hakala et al., 2017). Dentre os principais agentes bacterianos responsáveis por causar doenças respiratórias em suínos estão *Actinobacillus pleuropneumoniae, Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Haemophilus parasuis, Pasteurella multocida* e *Streptococcus suis.* Dentre estes agentes, *Actinobacillus pleuropneumoniae* é um patógeno pertencente à família Pasteurellaceae e o agente causal da pleuropneumonia suína (Haimi-Hakala et al., 2017). A pleuropneumonia suína (PPS) causada por *A. pleuropneumoniae* é uma importante doença respiratória que tem sido reportada em todos os países que praticam a criação intensiva de suínos (The Pig Site, 2013). A PPS causa grandes perdas econômicas para a indústria mundial de produção de carne suína, pois acomete suínos de todas as idades, gerando problemas respiratórios, febre e anorexia, que consequentemente fazem com que os animais afetados percam valor de mercado (Menzel et al., 2014).

A família Pasteurellaceae compreende bactérias Gram-negativas, com forma que varia de cocoide a bacilar, com tamanho usual de 0,2-2,0 µm, não formadoras de esporos e imóveis. Organismos pertencentes a esta família apresentam graus variáveis de microaerofilia e/ou são anaeróbios facultativos. São micro-organismos

quimiorganotróficos com temperatura ótima de crescimento a 37ºC. A maioria das espécies apresenta resultado positivo para as reações de catalase, oxidase e fosfatase alcalina, mas reações negativas podem ser detectadas em algumas espécies. Os membros dessa família são em sua maioria parasitas de vertebrados, principalmente mamíferos e aves e, frequentemente, podem colonizar as membranas mucosas dos tratos respiratórios, digestório e genital destes animais. De forma geral, o tamanho do genoma pode variar de 1,2 a 2,2 Mb e o conteúdo G+C do DNA pode variar entre 37-45% (Staley et al., 2005).

Actinobacillus pleuropneumoniae pode ser classificada em dois diferentes biotipos com base no seu requerimento por nicotinamida adenina dinucleotídeo (NAD): o biotipo 1 requer NAD e o biotipo 2 pode sintetizar NAD na presença de nucleotídeos específicos de piridina ou seus precursores (Bossé et al., 2002).

Atualmente, são relatados 18 sorotipos conhecidos de *A. pleuropneumoniae* e estes são diferenciados com base nas propriedades antigênicas dos polissacarídeos da cápsula (Sárközi et al., 2015; Bossé et al., 2017; Bossé et al., 2018). A distribuição desses sorotipos no Brasil varia amplamente de acordo com a região sendo o sorotipo 8 o mais prevalente no estado de Minas Gerais (Rossi et al., 2013).

A patogênese da pleuropneumonia suína é considerada multifatorial, portanto, ainda não é completamente entendida (Chiers et al., 2010; Pereira et al., 2018). Vários fatores de virulência são reconhecidos em *A. pleuropneumoniae*, sendo os principais: polissacarídeos capsulares, lipopolissacarídeos de membrana externa (LPS), atividade da urease, resistência a antibióticos, toxinas Apx e sistema de aquisição de ferro (sideróforos) (Klitgaard et al., 2012). Além desses fatores de virulência, *A. pleuropnemoniae* possui capacidade de transformação natural, o que pode contribuir para a aquisição de novos fatores de virulência (Bossé et al., 2009).

Estudos recentes têm mostrado que pequenos RNAs (sRNA – *small RNA*) constituem a maior classe de RNAs regulatórios em bactérias e estão envolvidos no controle dos mais diversos aspectos fisiológicos, inclusive na patogenicidade (Stortz et al., 2014). Pequenos RNAs regulatórios bacterianos podem variar em tamanho de 50 até 500 nucleotídeos (Updegrove et al., 2015). Os sRNAs bacterianos podem ser divididos em quatro classes principais, de acordo com seu mecanismo regulatório: RNAs *cis*-codificados, RNAs *trans*-codificados, CRISPRs (*clustered regulatory interspaced short palindromic repeats*) e RNAs que modulam a atividade de proteínas (Stortz et al., 2011).

RNAs *cis*-codificados são codificados na fita oposta ao seu RNA mensageiro (mRNA) alvo e são altamente alvos-específicos, pois compartilham mais de 75 nucleotídeos de complementariedade de bases com o seu alvo. Já os RNAs *trans*-codificados não possuem localização cromossomal relacionada ao seu gene alvo, além disso, possuem complementariedade de bases com o mRNA alvo limitada e, portanto, não apresentam comumente apenas um alvo-específico. O *locus* CRISPR quando transcrito produz pequenos RNAs CRISPR (crRNA) que através do pareamento de bases se ligam e marcam material genético exógeno para degradação. Em contraste com as três classes anteriores, RNAs que modulam a atividade de proteínas não dependem do pareamento de bases, podendo interagir diretamente com as proteínas alvo alterando sua atividade (Michaux et al., 2014).

Bactérias também possuem um número reduzido de sRNAs que possuem atividades conhecidas e que não envolvem pareamento de bases com um mRNA alvo ou regulação da atividade de proteínas (Gottesman et al., 2011). Um dos principais RNAs pequenos encontrado nas células bacterianas que possui atividade conhecida e que se mostra essencial para a maioria das espécies é o RNA mensageiro de transferência – tmRNA, também chamado SsrA (*stable small RNA*) ou RNA 10Sa, codificado pelo gene *ssrA* (Lee et al, 1978). A molécula de tmRNA foi primeiramente identificada como 10S RNA no final da década de 1970 em um estudo utilizando radiomarcação para identificar RNAs estáveis em *Escherichia coli* (Lee et al, 1978). A molécula de tmRNA associada à pequena proteína B – SmpB, reconhece especificamente e resolve complexos de tradução em que o ribossomo se encontra parado sobre o RNA mensageiro em uma reação conhecida como *trans*-tradução (Feaga et al., 2014). Homólogos de tmRNA e SmpB têm sido identificados em muitos genomas bacterianos sequenciados, incluindo aqueles com tamanho reduzido, como da bactéria endossimbionte *Tremblaya princeps* (Hayes et al., 2010). Essa conservação sugere que a *trans*-tradução evoluiu muito cedo no domínio Bacteria e promove uma significante vantagem evolutiva nos mais diversos ambientes (Hayes et al., 2010).

Os ribossomos são as moléculas responsáveis pela síntese de proteínas pela célula num processo chamado tradução, e dessa maneira, possuem um papel principal na determinação do perfil global da expressão gênica em uma célula (Starosta et al., 2014). O processo de tradução é muito dinâmico e envolve várias proteínas e RNAs que constantemente se ligam e se dissociam do ribossomo (Giudice et al., 2013). Qualquer interrupção neste processo, finamente regulado, irá acarretar prejuízos para a célula

bacteriana (Giudice et al., 2013). Quando a tradução de um mRNA por um ribossomo é interrompida, a célula dispõe de duas saídas possíveis: ou a elongação e a terminação eventualmente ocorrem ou o mRNA é clivado na porção 3' final posicionada no sítio A do ribossomo produzindo complexos de tradução sem um códon de parada que precisa ser resgatado pelo processo de *trans*-tradução ou uma via de recuperação alternativa (Keiler, 2015). O acúmulo de complexos de tradução sem um códon de parada pode ser deletério, pois reduz o número de ribossomos ativos e disponíveis que deveriam ser utilizados na tradução de outros mRNAs (Abo et al., 2014).

Sob severas condições de estresse como privação de nutrientes, alterações na temperatura, exposição a antibióticos e mudanças no potencial oxidativo do ambiente, a maioria dos mRNAs da célula são clivados na porção 3' final posicionada no sítio A do ribossomo gerando complexos de tradução sem códon de parada (Keiler, 2015). Quando isso ocorre, é necessário que os ribossomos sejam resgatados. A principal via de recuperação dos ribossomos é a *trans*-tradução, executada pela molécula de tmRNA associada a pequena proteína SmpB (Keiler, 2015). As extremidades 5' e 3' da molécula de tmRNA formam uma estrutura que se assemelha ao braço aceptor e ao braço TΨC da molécula de alanil-tRNA (Keiler et al., 2014). O braço aceptor do tmRNA é carregado com o aminoácido alanina pela alanil-tRNA sintetase e ligado pelo fator de elongação-Tu (EF-Tu) ao sítio A do ribossomo, da mesma forma que um $tRNA^{Ala}$ (Keiler et al., 2014). A molécula de tmRNA contém entre 2 e 4 pseudos nós e um quadro de leitura especifico codificando um pequeno peptídeo (Hayes et al., 2010). Além de EF-Tu, a molécula de tmRNA se liga à proteína SmpB cuja a estrutura mimetiza o braço do anti-códon de um tRNA e é essencial para a atividade do tmRNA (Hayes et al., 2010).

Durante a *trans*-tradução (Figura 1), a molécula de tmRNA associada à proteína SmpB reconhece especificamente um complexo de tradução sem códon de parada e se acomoda no sítio A do ribossomo (Keiler et al., 2014). A cadeia polipeptídica incompleta é transferida por transpeptidação para o resíduo de alanina localizado no tmRNA e o complexo tmRNA-SmpB é translocado para o sítio P do ribossomo (Brunel et al., 2016). Durante a translocação ocorre um giro na subunidade 30S do ribossomo que permite que o quadro de leitura presente no tmRNA entre no canal do mRNA no ribossomo (Keiler et al., 2014). O mRNA que não possui um códon de parada é liberado e degradado pela enzima RNase R, uma exoribonuclease que interage com o complexo de *trans*-tradução (Brunel et al., 2016). A tradução termina usando a porção mRNA do

tmRNA como um novo molde, liberando o ribossomo e uma proteína que apresenta uma marcação, o peptídeo *tag*, codificado pelo tmRNA na porção C terminal (Keiler et al., 2014). O peptídeo *tag* é reconhecido por múltiplas proteases na célula, garantindo que a proteína gerada seja rapidamente degradada (Keiler et al., 2014). Dessa maneira, a *trans*-tradução não somente recicla os ribossomos que se encontravam parados na célula como também ativa a degradação tanto do mRNA que fez parte do complexo de tradução sem códon de parada quanto do peptídeo por ele gerado (Keiler, 2015).

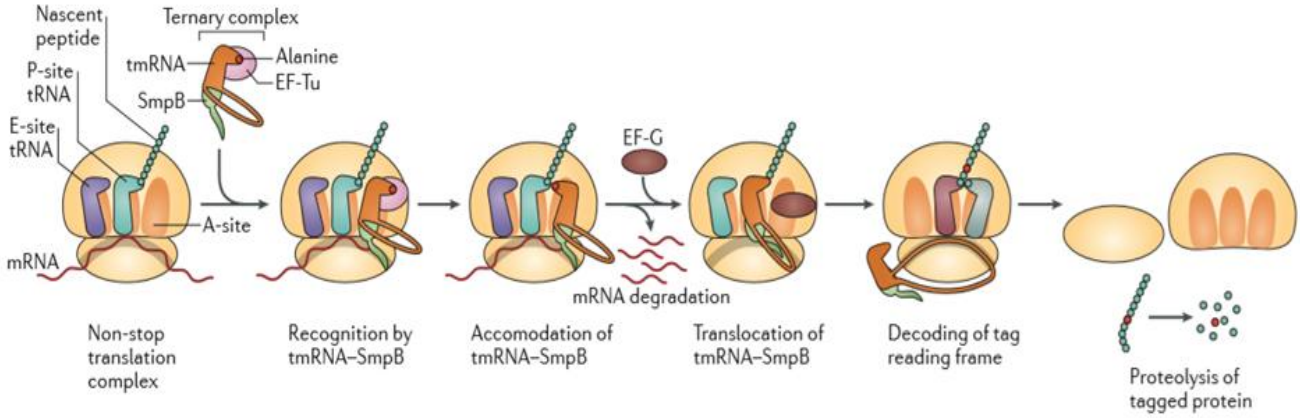

Figura 1: Recuperação de ribossomos pelo sistema de *trans*-tradução. O complexo ternário formado por tmRNA, SmpB, fator de elongação Tu (EF-Tu) e uma molécula de GTP (tmRNA-SmpB-EF-Tu-GTP) reconhece um ribossomo parado em um complexo de tradução sem códon de parada quando a cauda carboxi terminal de SmpB se liga ao canal vazio do mRNA (sítio A). O complexo tmRNA-SmpB é acomodado de forma que o domínio RNA transportador do tmRNA seja posicionado no centro peptidil transferase e EF-Tu seja liberado. A reação de transferência da cadeia polipeptídica resulta na transferência do polipeptídeo nascente para a alanina ligada ao domínio RNA transportador do tmRNA e o peptidil-tmRNA-SmpB é translocado para o sítio P do ribossomo. Este movimento posiciona o domínio RNA mensageiro do tmRNA no canal do mRNA no ribossomo. O mRNA original é removido do ribossomo e degradado. A tradução recomeça usando o quadro de leitura da sequência codificadora do peptídeo tag presente no domínio mRNA do tmRNA e, como essa sequência contém um códon de parada, a terminação ocorre e o ribossomo e a proteína marcada são liberados. A proteína marcada é rapidamente degradada por proteases, como ClpXP, ClpAP, Lon, HflB e Tsp. Retirado de Keiler, 2015.

Apesar do tmRNA e da proteína SmpB apresentarem grande conservação e distribuição no domínio Bacteria, deleções tanto da proteína SmpB quanto da molécula de tmRNA não necessariamente conferem fenótipos letais (Starosta et al., 2014). Isto sugere que em bactérias onde o tmRNA não é essencial para o crescimento, exista um sistema alternativo para a recuperação de ribossomos (Liu et al., 2010). De fato, são descritos dois sistemas alternativos envolvidos com a recuperação de ribossomos, presentes em bactérias cuja ausência do tmRNA não se manifesta em um fenótipo letal,

denominados *alternative rescue factors* (fatores de resgate alternativos) A e B – ArfA e ArfB (Starosta et al., 2014).

O fator de resgate alternativo A – ArfA, foi descoberto em uma triagem genética realizada em *Escherichia coli* com o objetivo de encontrar mutações que se mostravam sinergicamente letais quando combinadas com a deleção do gene *ssrA* (Chadani et al., 2010). Diferentemente do tmRNA, ArfA é uma pequena proteína com cerca de 72 aminoácidos codificada pelo gene *arfA* (Garza-Sanchez et al., 2011). A proteína ArfA é sintetizada a partir de um mRNA sem códon de parada, portanto, tem sua tradução regulada pela via de resgate ribossomal baseada na molécula de tmRNA (Janssen et al., 2012). Em células em que o gene *ssrA* se encontra ativo, teoricamente todo mRNA de ArfA é degradado pelo sistema de resgate de ribossomos baseado no tmRNA (Janssen et al., 2012). O gene *arfA* tem sido identificado em muitas espécies pertencentes à classe Gammaproteobacteria e algumas espécies pertencentes à classe Betaproteobacteria (Schaub et al. 2012).

Para que a recuperação de ribossomos por meio do fator de resgate alternativo A ocorra, é necessário o recrutamento do fator de liberação 2 (*release factor 2* – RF2) pela proteína ArfA (Shimizu 2012) (Figura 2a). ArfA reconhece o complexo de tradução sem um códon de parada e se liga próximo ao canal do mRNA no ribossomo (Keiler, 2015). Após a ligação de ArfA, RF2 é recrutado ao complexo formado e se liga ao sítio A do ribossomo, permitindo uma mudança conformacional que irá resultar na hidrólise do peptidil-tRNA catalisada pelo próprio RF2 levando à liberação do ribossomo, do mRNA e da cadeia polipeptídica (Chadani et al. 2012). Diferente do que ocorre no processo de *trans*-tradução, o resgate ribossomal através de ArfA somente leva a liberação do ribossomo, não causando a marcação do peptídeo ou do mRNA para posterior degradação (Keiler, 2015). Interessantemente, a proteína ArfA somente é expressa quando o processo de *trans*-tradução tem sua atividade limitada, reforçando que este sistema funciona somente como uma via alternativa de resgate ribossomal (Keiler, 2015). Embora a sua descoberta não seja recente (Chadani et al., 2010), ainda há necessidade de um melhor entendimento do mecanismo de ação via ArfA como um sistema alternativo de resgate de ribossomos em bactérias (Ma et al., 2016).

Um terceiro sistema de recuperação de ribossomos, chamado fator de resgate alternativo B – ArfB, foi identificado como um supressor de letalidade multi-cópia em uma célula mutante de *Escherichia coli* em que os genes *ssrA* e *arfA* haviam sido deletados (Chadani et al. 2011). Homólogos de ArfB tem sido encontrados em apenas

34% dos genomas bacterianos já sequenciados (Feaga et al., 2014). Em contraste com o fator de resgate alternativo A, que tem sido encontrado apenas nas classes Betaproteobactéria e Gammaproteobacteria do domínio *Bacteria*, homólogos de ArfB também já foram descritos no domínio Eukarya (Schaub et al., 2012).

O resgate de ribossomos realizado por ArfB (Figura 2b) ocorre quando a proteína ArfB se liga ao sítio A do ribossomo e catalisa a hidrólise do pepetidil-tRNA (Keiler, 2015). Semelhante a ArfA, ArfB apenas gera a liberação do ribossomo preso em um complexo de tradução sem códon de parada, não levando a marcação do peptídeo ou do mRNA para degradação (Keiler, 2015). Ainda não há um completo entendimento do papel fisiológico de ArfB, supõe-se que ArfB possua outras funções e que só realiza o resgate de ribossomos presos em complexos de tradução sem códon de parada na ausência dos outros dois sistemas de resgate (Keiler, 2015). Além disso, especula-se que, diferentemente de ArfA, sua síntese não esteja relacionada a atividade de *trans*-tradução (Chadani et al., 2011; Keiler et al., 2014; Huter et al., 2017).

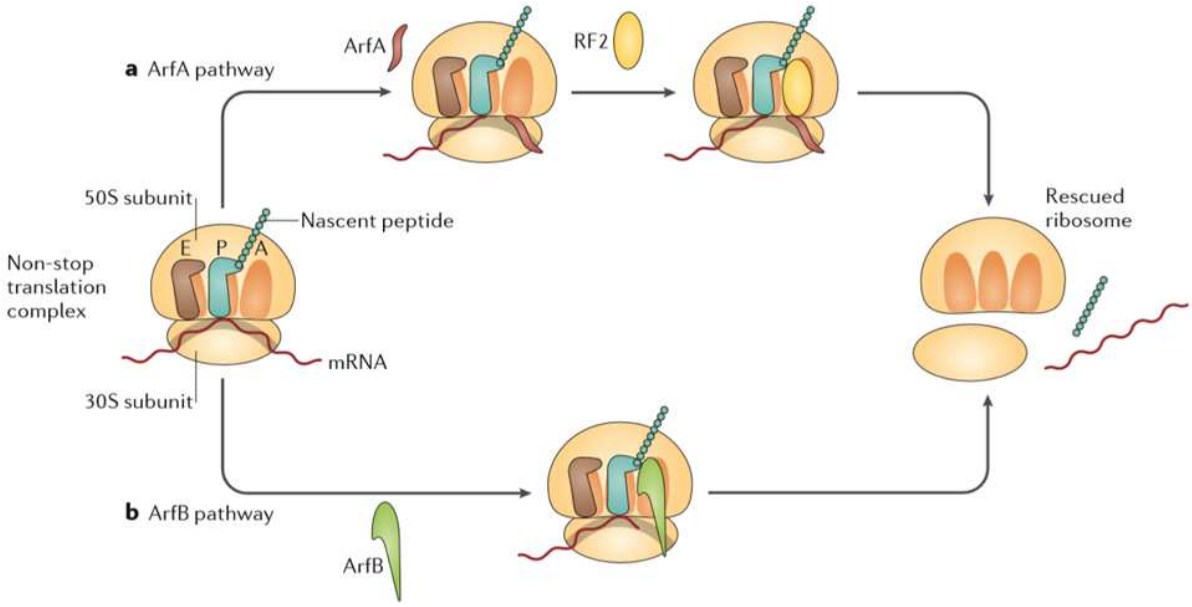

Figura 2: ArfA e ArfB: vias alternativas para a recuperação de ribossomos. Quando a *trans*-tradução é prejudicada, os fatores alternativos de resgate de ribossomos A (ArfA) e B (ArfB) funcionam como sistemas de reserva para a recuperação de ribossomos em complexos de tradução sem códon de parada. **a**: ArfA reconhece o complexo de tradução sem códon de parada e liga-se ao canal do mRNA vazio ou próximo dele, o que facilita a ligação do fator de liberação da cadeia peptídica 2 (RF2) ao sítio de ligação aminoacil-tRNA (sítio A). O RF2 catalisa a hidrólise da ligação peptidil-tRNA, que resulta na libertação do ribossomo em conjunto com o péptido nascente e o mRNA, nenhum dos dois é alvo de degradação. **b**: A cauda terminal carboxil de ArfB liga-se ao canal do mRNA vazio, alinhando o seu motivo GGQ para catalisar diretamente a hidrólise da ligação peptidil-tRNA. Similarmente ao ArfA, o

ribossomo, o peptídeo nascente e o mRNA são liberados. Tanto o mRNA quanto o peptídeo não são degradados. Retirado de Keiler, 2015.

Nos últimos anos vários estudos utilizando-se células em que o gene *ssrA* foi de alguma forma modificado ou até mesmo deletado têm sido realizados com a finalidade de se entender o impacto dessa alteração para o fenótipo da célula sob condições de estresse. (Ramadoss et al., 2013). Mutantes que apresentam alterações no funcionamento do sistema de *trans*-tradução exibem uma ampla gama de fenótipos relacionados à regulação da fisiologia celular, ciclo celular, resposta ao estresse e virulência (Himeno et al., 2014).

Um estudo utilizando *E. coli* como modelo, mostrou que linhagens Δ*ssrA* são mais sensíveis a antibióticos de diferentes classes em comparação à linhagem parental (Li et al., 2013). Um segundo estudo também realizado em *E. coli,* mostrou que o estresse gerado devido à limitação de aminoácidos disponíveis no meio utilizado para o crescimento resulta na interrupção da tradução (Li et al., 2008). Neste mesmo estudo foi mostrado que células em que o gene *ssrA* foi deletado, tinham seu crescimento inibido de maneira significativa diante deste tipo de estresse quando comparado à linhagem parental, tal alteração se dá devido ao acúmulo de complexos de tradução ineficientes (Li et al., 2008).

Com o objetivo de se entender melhor o papel da *trans*-tradução na fisiologia do patógeno *Helicobacter pylori*, uma linhagem mutante para o gene *ssrA* foi submetida a diferentes condições de estresse (Thibonnier et al., 2008). Os resultados obtidos indicam que os mutantes foram mais susceptíveis a diferentes classes de antibióticos bem como mais sensíveis a estresse oxidativo, além disso, apresentaram capacidade de transformação natural reduzida (Thibonnier et al., 2008). Da mesma forma, a inativação do gene *ssrA* de *Francisella tularensis* resultou em um fenótipo de crescimento lento, maior sensibilidade a choque térmico e frio, além de maior susceptibilidade ao estresse oxidativo e a antibióticos em relação à linhagem parental (Svetlanov et al. 2012). Além disso, a linhagem mutante de *F. tularensis* se mostrou avirulenta e foi capaz de promover imunidade efetiva em camundongos (Svetlanov et al., 2012).

Em 2003, em um estudo realizado com a linhagem referência do sorotipo 1 de *A. pleuropneumoniae,* tendo como finalidade identificar genes requeridos para a sobrevivência da bactéria *in vivo*, foram construídas diferentes linhagens mutantes utilizando-se o transposon Tn10 (Sheehan et al., 2003). Entre essas linhagens, o mutante identificado como 14D5, que possui o transposon Tn10 interrompendo o gene *ssrA*,

mostrou-se altamente atenuado *in vivo* sugerindo a importância da funcionalidade deste gene para *A. pleuropneumoniae* (Sheehan et al., 2003).

Na família Pasteurellaceae ainda não existem relatos sobre o impacto da ausência do sistema de recuperação de ribossomos baseado na molécula de tmRNA associada à proteína SmpB, bem como no sistema de resuperação alternativo baseado na proteína ArfA na virulência e resposta ao estresse. Em estudo recente realizado em *A. pleuropneumoniae,* o gene *ssrA* foi identificado no genoma de linhagens referência de 11 sorotipos e 6 isolados clínicos desta espécie e também de espécies pertencentes à família Pasteurellaceae: *A. succinogenes, A. suis, Aggregatibacter actinomycetemcomitans*, *A. aphrophilus*, *Gallibacterium anatis, Haemophilus ducrey, H. influenzae, H. parainfluenzae, H. parasuis, H. somnus, Mannheimia haemolytica, M. succiniciproducens*, *M. varigena e Pasteurella multocida* (Rossi et al., 2016). Este mesmo estudo mostra que o gene *ssrA* é expresso constitutivamente na linhagem referência do sorotipo 5b de *A. pleuropneumoniae* (Rossi et al., 2016). Com base neste primeiro relato da presença e expressão do gene *ssrA* em *A. pleuropneumoniae* e a ausência de estudos sobre a trans-tradução, assim como outros sistemas de recuperação de ribossomos neste patógeno, o objetivo deste trabalho é investigar e determinar o papel de cada um destes sistemas na virulência e resistência ao estresse de *A. pleuropneumoniae.*

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3. HIPÓTESE

Existem sistemas de recuperação de ribossomos em *A. pleuropneumoniae* e estes são importantes para a virulência e a resistência deste patógeno bacteriano a diferentes condições de estresse.

4. OBJETIVOS

4.1 Objetivo Geral

Identificar gene(s) envolvido(s) em sistemas de recuperação de ribossomos em *A. pleuropneumoniae* e investigar o papel deste(s) na virulência e sensibilidade a condições de estresse

4.2 Objetivos específicos

4.2.1 Identificar e caracterizar *in silico* os genes envolvidos com sistemas de recuperação de ribossomos a partir de genomas de *A. pleuropneumoniae* de diferentes sorotipos e outras espécies pertencentes à família Pasteurellaceae sequenciados e disponíveis no banco de dados GenBank;

4.2.2 Obter linhagens de *A. pleuropneumoniae* sorotipo 8 MIDG2331 mutantes (simples e/ou duplos) para o(s) gene(s) identificados;

4.2.3 Obter linhagens de *A. pleuropneumoniae* sorotipo 8 MIDG2331 complementadas para a(s) respectiva(s) linhagem(ns) mutante(s) obtida(s);

4.2.4 Caracterizar as linhagens mutantes obtidas em relação ao crescimento, formação de biofilme, sensibilidade a diferentes condições de estresse e virulência em *Galleria mellonella*.

CHAPTER 1:

RIBOSOME RESCUE SYSTEMS IN *Actinobacillus pleuropneumoniae* AND THEIR ROLE IN STRESS RESISTANCE AND VIRULENCE

1. INTRODUCTION

Actinobacillus pleuropneumoniae is a Gram-negative, anaerobic facultative and coccobacillary bacteria belonging to the Pasteurellaceae family. This bacterium is the etiological agent of porcine pleuropneumonia, a respiratory disease that causes significative economic losses in the swine industry around the globe (Gottschalk et al., 2012). The pathogenesis of *A. pleuropneumoniae* is multifactorial and not totally comprehended yet (Chiers et al., 2010; Pereira et al., 2018).

The main problem that could happen during the bacterial translation is the absence of a stop codon in the messenger RNA that cause the interruption of this tight regulated process (James et al., 2016). Stress conditions, such as low or high temperature, antibiotics, and ethanol could increase the number of truncated mRNA in the cell and the absence of a functional ribosome rescue system leads to a decrease in bacterial fitness (Huter et al., 2017).

The transfer-messenger RNA – tmRNA in association with a small protein B – SmpB, plays an important role in translation quality control and bacterial physiology by rescuing ribosomes that cannot finish the translation due to some problem, often lacking of a stop codon, in the mRNA sequence, in a process called trans-translation (Keiler., 2015). The tmRNA/SmpB complex mimics a transfer RNA and enters the ribosome allowing the continuation of translation. The polypeptide chain translated contains a peptide tag that will send it to degradation (Himeno et al., 2015). Despite being the main ribosome rescue system in bacteria, the trans-translation system in not the only one present in the Bacteria domain (Liu et al., 2010). In fact, there are two other ribosome rescue systems named alternative ribosome rescue system A and B (Starosta et al., 2014). Both systems are based on proteins known as ArfA and ArfB (Himeno et al., 2015). As suggested by their names, this alternative ribosome rescue systems are only useful in the absence of a functional trans-translation system (Keiler., 2015).

Besides its role in translation quality control and cell physiology, the tmRNA plays a regulatory role by controlling the translation of specific genes, like the *arfA* gene that encode the ArfA protein (Janssen et al., 2012). The tmRNA shows an essential role in virulence since the tmRNA gene mutation in *Yersinia pseudotuberculosis* and *Y. pestis* (Okan et al., 2006 and 2010), *Fancisella tularensis* (Svetlanov et al., 2012) and *Escherichia coli* (Mu et al., 2013) results in attenuation of virulence by decrease fitness face to stressful conditions (Huter et al., 2017).

In *A. pleuropneumoniae*, a work made in 2003 with the reference strain of the serotype 1, showed that a tmRNA mutant (named 14D5) was attenuated in pigs when compared with the wild type strain (Sheehan et al., 2003). In 2016, Rossi and colleagues showed that the tmRNA gene is present in all *A. pleuropneumoniae* serotypes and it is constitutively expressed in the reference strain of the serotype 5b (Rossi et al., 2016).

As far as we know this work is the first report on ribosomes rescue systems in the Pasteurellaceae family. Besides that, this study is an additional step in the effort to better understand the fitness of *A. pleuropneumoniae*. Based on these facts, the aim of this work is to identify *in silico* the ribosome rescue systems presents in *A. pleuropneumoniae* and others bacterial species of the Pasteurellaceae family and to investigate their role in the virulence and stress resistance of *A. pleuropneumoniae*.

2. MATERIALS AND METHODS

2.1 Identification *in silico* of ribosome rescue systems in members of the Pasteurellaceae family

2.1.1 Characterization of *A. pleuropneumoniae ssrA*, *smpB* and *arfA* genes

In silico analysis were conducted on veterinary bacterial pathogens from Pasteurellaceae family (Table 1) to identify genes evolved in ribosome rescue systems as *ssrA* and *smpB* (trans-translation) and *arfA* (alternative rescue factor A). The genomic organization, the number of copies and sequence conservation of the genes identified in all *A. pleuropneumoniae* genomes sequenced available at the GenBank database plus the genome of the *A. pleuropneumoniae* strain HS143 belonging to the serotype 15 (Table 1) were evaluated using BLAST (Basic Local Alignment Search Tool). The conservation of the nucleotides sequence of *ssrA* and *arfA* genes and amino acid sequences of the *smpB* in others species belonging to the Pasteurellaceae family was analyzed using the BLAST tool followed by a multiple sequence alignment with the online software ClustalW and a phylogenetic tree was constructed using the maximum likelihood method with a Bootstrap of 5000 and the best model generated by MEGA6, Kamura 2 parameters + Gamma distributed for *ssrA* gene, LG model for the *smpB* gene and Kamura 2 parameters + Gamma distributed with Invariant sites (G+I) for the *arfA* gene (Tamura et al., 2013). The *A. pleuropneumoniae* secondary structure of the *ssrA* gene product was determined by the RNAfold software according to Gruber et al., 2008 (http://rna.tbi.univie.ac.at). The software Phyre² was used to determinate the structure of ArfA and SmpB proteins from *A. pleuropneumoniae* according to Söding, (2005).

Table 1: Pasteurellaceae genomes used in the *in silico* analyses

Strains	Serotype	NCBI access number	Pathogen host
Actinobacillus pleuropneumoniae			
Shope 4074	1	NZ_AACK0000000.1	
S1536	2	NZ_ADOE00000000.1	
JL03	3	NC_010278	
M62	4	NZ_ADOF00000000.1	
L20	5b	NC_009053.1	
Femø	6	NZ_ADOG00000000.1	
AP76	7	NC_010939.1	
MV 460	8	NZ_JSVG00000000.1	
MIDG2331	8	NZ_LN908249.1	
MV 518	8	NZ_JSVZ00000000.1	Pig
MV 597	8	NZ_JSVX00000000.1	
MV 780	8	NZ_JSVV00000000.1	
MV 1022	8	NZ_JSVF00000000.1	
MV 5651	8	NZ_JSVY00000000.1	
CVJ13261	9	NZ_ADOI00000000.1	
D13039	10	NZ_ADOJ00000000.1	
56153	11	NZ_ADOK00000000.1	
1096	12	NZ_ADOL00000000.1	
N273	13	NZ_ADOM00000000.1	
HS143	15	-	
KL 16	16	CP022715.1	
Actinobacillus suis			
NCTC12996	-	LT906456.1	
ATCC 33415	-	CP009159.1	Pig
H91-0380	O2	CP003875.1	
Actinobacillus equuli			
19392	-	CP007715.1	Equine
Glaesserella parasuis			
SH0165	5	NC_011852.1	
SH03	5	NZ_CP009158.1	
KL0318	1	NZ_CP009237.1	
SC1401	11	NZ_CP015099.1	Pig
CL120103	12	NZ_CP020085.1	
Haemophilus influenzae			
PittGG	Non-typeable	CP000672.1	
11P6H	b	CP020014.1	Human
6P32H1	Non-typeable	CP020013.1	
6P24H2	Non-typeable	CP020012.1	
Rd KW20	d	L42023.1	
Aggregatibacter aphrophilus			
NJ8700	-	CP009230.1	
W10433	-	CP012067.1	Human
Aggregatibacter			

actinomycetemcomitans			
D7S1-1	a	NC_017846	
D11S-1	c	CP001733	Human
ANH9381	b	CP003099	
Pasteurella multocida			
ATCC 43137	A	NZ_CP008918.1	Pig
Pm70	F	NC_002663.1	Chiken
36950	A	NC_016808.1	Bovine
HN06	D	NC_017027.1	Pig
3480	A	NC_017764.1	Pig
Mannheimia haemolytica			
M42548	A1	NC_021082.1	
USDA-ARS-USMARC-183	A1	NC_020833.2	
USDA-ARS-USMARC-185	A6	NC_020834.2	Bovine
D174	A6	NC_021739.1	
D153	A1	NC_021743.1	

* "-"denotes non-available.

2.2 *In vitro* analyses

2.2.1 Microorganisms used, culture and maintenance conditions

The strains used in this work (Table 2) were grown in Brain Heart Infusion-BHI (Becton Dickinson, USA) supplemented with nicotinamide adenine dinucleotide - NAD 10µg.mL^{-1} (Sigma Aldrich, UK) at 37ºC with 5% CO_2. In specific growth conditions, the antibiotics trimethoprim at the concentration of 10 µg/ml (Sigma Aldrich, UK), chloramphenicol 1 ug/ml (Sigma Aldrich, UK) was added to the medium. For the growth in BHI broth, the strains were incubated at 37ºC, 180 rpm until the desired growth phase. The strains were maintained in BHI broth with glycerol 20% and kept at -80ºC.

Table 2: Bacterial strains used in the *in vitro* analyses

Specie/strain	Serotype	Description	Reference or source
A. pleuropneumoniae			
MIDG2331	8	Clinical isolate from UK	Bossé et al., 2016
Δ*ssrA*	8	*ssrA* mutant of MID2331	This study
Δ*arfA*	8	*arfA* mutant of MIDG2331	This study
Δ*ssrA*C	8	*ssrA* mutant complemented strain	This study
Δ*arfA*C	8	*arfA* mutant complemented strain	This study
L20	5b	Reference strain of serotype 5b	Foote et al., 2008

2.2.2 Obtainment of *A. pleuropneumoniae* serotype 8 MIDG2331 Δ*ssrA* and Δ*arfA* strains

The DNA knockout cassettes for the *ssrA* and *arfA* genes were constructed based on the *A. pleuropneumoniae* serotype 8 MIDG2331 Wild type (WT) genome (Table 1). These DNA cassettes are composed of a sequence containing 500 nucleotides upstream and 500 nucleotides downstream to the target genes, those sequences are necessary for the double recombination at the chromosome, as described by Bossé et al., (2014). The DNA knockout cassettes were designed with MacVector Version 12.6 software 2012 (Olson, 1994), synthesized by Eurofins. The mutants were obtained by natural transformation according to Bossé et al., (2014) and confirmed by PCR using primers pair 5 and 18 for the *ssrA* mutant strain and primers pair 1 and 18 for the *arfA* mutant strain (Table 3).

2.2.3 Complementation of the Δ*ssrA* and Δ*arfA* strains

To obtain the A. *pleuropneumoniae* complemented Δ*ssrA* strain the overlapping extension (OE) PCR strategy was used according to Shevchuk et al., (2004). The *ssrA* gene was amplified from the parental *A. pleuropneumoniae* MIDG 2331 strain genome using specific primers (primers 6-11) (Table 3). All the PCR reactions were made using Platinum Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific) according to manufacturer. The PCRs products generated were cleaned up with the QIAquick PCR Purification Kit (Qiagen) and then combined in a single PCR reaction (Shevchuk et al., 2004). The DNA cassette obtained was used to transform *A. pleuropneumoniae* Δ*ssrA* strain as described by Bossé et al., (2014). The DNA cassette for the complementation of the Δ*arfA* was constructed using MacVector Version 12.6 software 2012 (Olson, 1994), synthesized by Eurofins and amplified by PCR using primers 3 and 4 (table 3). This DNA cassette contains the *arfA* gene of the *A. pleuropneumoniae* MIDG 2331 WT strain, a *cat* gene used as the selection marker for the complemented strain and the *A. pleuropneumoniae* DUS (DNA Uptake Sequence) 5' ACAAGCGGT 3'. This DNA cassette was used in a natural transformation process using the Δ*arfA* strain as the receptor strain. The complemented strains were selected in BHI medium supplemented with NAD and chloramphenicol (1µg/ml). The complementation was confirmed by PCR using the primers 5 and 9 for the Δ*ssrA* complemented strain and primers 1 and 9 for the Δ*arfA* complemented strain (Table 3).

Table 3: Primers used in this work

Primer	Identification	Sequence (5'-3')
1	arfA_up	TGAGTGAGTAAAAAACCGCCC
2	arfA_down	CCAAATGTACGAAAACACACCG
3	arfA_comp_for	AGGTAATTTGGTCGCATTTGCAC
4	arfA_comp_rev	CAAGGTATCGAACGTTGGCG
5	tmRNA_up	CAGTAAAACGGCAAGAGTGAGG
6	tmRNA_left_for	TGCCCCTAAACAGATCGCAAAC
7	tmRNA_left_revcat	GTATGCATAATGGCGGTATACAAATTTGGTGGAGCTGG
8	cat_tmRNA_for	ACCAAATTTGTATACCGCCATTATGCATACTACG ATTCAC
9	cat_tmRNA_rev	TTTGGTGGAGCTGGGGACAAGGGATTTTTTTATG CCAAACCG
10	tmRNA_rt_for_cat	AAAAAATCCCTTGTCCCCAGCTCCACCAAATTTGT ATAC
11	tmRNA_rt_rev	ATCTTTTATGGTTACAACATCAACACC
12	2051_for	ATTATGGTCACAAACGGTTGGG
13	2051_rev	AAACCCATCGTCGTCATCATTG
14	2053_for	GCTATTGCACTTAGTAGTTTTGGTG
15	2053_rev	ATTCAGAGTTAGAGAGGATTTGCG
16	00781_for	ACAAAATGACGATGCGGAACA
17	00781_rev	GCTACAAACTGCAACAAAGCC
18	dfrA5'_out	CACGGTTCTCATCCTAATTCCTCC

2.3 Phenotypic characterization of the *A. pleuropneumoniae* strains

2.3.1 Non-polar mutation

To evaluate a possible polar mutation due to strategy used in this study, the total RNA of the Δ*ssrA* e Δ*arfA* strains grown in BHI/NAD medium for 12 hours was extracted using the miRNeasy Mini Kit (Qiagen), including a step of mechanical lyses using Lysing Matrix B 2 ml tubes (MP Biomedicals) with acid phenol and an extra step of chloroform extraction of the aqueous phase. A total of 10 µg of the RNA extracted was treated with TURBO DNase (AM2238 -Thermo Fisher Scientific) according to the manufacturer's instructions. The treated RNA was used as template in a reverse transcription PCR using random primers with the IMPROM-II™ Reverse Transcription System Kit (Promega, USA) according to the manufacturer's instructions. The cDNA generated was submitted to a PCR reaction using primers pairs 12/13 and 14/15 (Table 3) to analyze the expression of the genes localized upstream and downstream to the *ssrA* gene and the primer pair 16/17 (Table 3) was used to analyze the expression of the gene localized downstream to the *arfA* gene. The *A. pleuropneumoniae* MIDG2331 WT strain was used as control.

2.3.2 Growth curve

After 24 hours of growth in BHI medium *A. pleuropneumoniae* Δ*ssrA*, Δ*arfA*, Δ*ssrA*C, Δ*arfA*C and the WT strains were suspended in BHI broth and the optical density ($O.D._{600}$) was adjusted to 0.01. An aliquot of 200µl of the suspension was inoculated in a 96 wells microplate (Kasvi K12-096) and incubated in a Multiskan™ GO Microplate Spectrophotometer at 37ºC (Thermo Fisher Scientific) and the reads were done each 15 minutes (10 minutes of incubation and 5 minutes of agitation) followed by optical density measured at D.O. 600nm in a total period of 24 hours. The experiment was conducted in biological and experimental triplicates. The specific growth rate of each strain was calculated and submitted to ANOVA followed by Tuckey test with a p-value ≤ 0.05 using the R software (R Development Core Team 2008).

2.3.3 Biofilm formation analysis

The biofilm formation by *A. pleuropneumoniae* Δ*ssrA*, Δ*arfA*, Δ*ssrA*C, Δ*arfA*C and the WT strains was evaluated in biological and experimental triplicates using a 96 wells polystyrene microplates (Kasvi K12-096) after an incubation of 24 hours at 37ºC according to Grasteau et al., (2011). For this, 200µl of cell suspension at $D.O._{600}$ 0.01 were inoculated in the wells (experimental triplicates). The biofilm formed was visualized using the cristal violet staining according to Grasteau et al., (2011) except for the stain-releasing solution, which consisted of a 30% acetic acid solution. The *A. pleuropneumoniae* serotype 5b L20 strain was used as a positive control and was considered here as 100% and the others strains had their adhesion capacity compared with it. The data were analyzed using ANOVA followed by Tukey test and a p-value $\leq$ 0.05 with the R software (R Development Core Team 2008).

2.3.4 Analysis of the sensitivity to different stress conditions

2.3.4.1 Antibiotics susceptibility

The susceptibility of the *A. pleuropneumoniae* Δ*ssrA*, Δ*arfA*, Δ*ssrA*C, Δ*arfA*C and the WT strains to the antibiotics Kanamycin (Sigma-Aldrich), Tilosyn (Sigma-Aldrich) and Ampicillin (Sigma-Aldrich) was investigated in biological and experimental triplicates using the microdilution method in a 96 wells microplate (Kasvi K12-096). The concentration range used for the antibiotics Ampicilin and Tylosin was 8 to 0.062 mg/ml; to Kanamycin the range used was 128 to 1 mg/ml. The cells viability was revealed using the resazurin sodium salt, an oxidoreduction indicator used in MIC tests (Elshikh et al., 2016).

2.3.4.2 Abiotic stress conditions

The *A. pleuropneumoniae* Δ*ssrA*, Δ*arfA*, Δ*ssrA*C, Δ*arfA*C and the WT strains were tested in biological and experimental triplicates regarding their susceptibility to different stress conditions: osmotic stress using 0.1 M potassium chloride; oxidative stress using 0.1 mM hydrogen peroxide; ethanolic stress 4% ethanol and the temperature at 42ºC. All the stressors agents were added to the BHI-NAD medium. The cells suspensions with initial OD_{600} of 1.0 were serially diluted to 10^{-7} and an aliquot of 10 µl of each dilution was spotted in square plates (Greiner Bio-one 688102). All plates were incubated at 37ºC, 5% CO_2, except the temperature stress. As control, the strains were inoculated in BHI-NAD.

2.3.5 Virulence in *Galleria mellonella*

The virulence of the *A. pleuropneumoniae* Δ*ssrA*, Δ*arfA*, Δ*ssrA*C, Δ*arfA*C and the WT strains was determined in the alternative infection model *Galleria mellonella* as described by Pereira et al., 2015. The experiment was conducted in biological and experimental triplicates. The larvae were incubated at 37ºC and the survival rates were evaluated after 24, 48, 72 and 96 hours. The survival curves were constructed with the Kaplan-Meyer method and differences in the survival were calculated using the Log-rank test with a p-value ≤ 0.05 in the SigmaPlot software version 12.0 (Systat Software, San Jose, CA).

3. RESULTS

3.1 *In silico* analyses

3.1.1 Identification of ribosome rescue systems in *A. pleuropneumoniae*

From the *in silico* analyses it was possible to identify in the investigated *A. pleuropneumoniae* genomes the genes related to the ribosome rescue system called trans-translation, the *ssrA* gene that encodes the tmRNA and the *smpB* gene that encodes the small protein B. In addition to the components of the trans-translations system we also found the *arfA* gene that encodes the ArfA protein that is responsible by the alternative ribosome rescue system A. The second alternative ribosome rescue system called ArfB was not founding in *A. pleuropneumoniae* indicating that this species only has two ribosomes rescue system. The same occurs in the genomes of members of the Pasteurellaceae family, that also only present two ribosome rescue systems: the one based on the tmRNA/SmpB and the one based on the ArfA protein.

Following the genes' identification, a characterization of these genes and their organization was done. The sequences of the *ssrA* and *arfA* genes in the genomes belonging to *A. pleuropneumoniae* available at the GenBank (Table 1) were characterized according following parameters: size, copy number and flanking genes. The identity and coverage were determined using the BLAST tool (Altschul et al., 1990). Both genes are present as a single copy and presenting the same size, 365 bp for the *ssrA* gene and 243 bp for the *arfA* gene, in all genomes evaluated. The *ssrA* flanking genes in all evaluated *A. pleuropneumoniae* genomes are an upstream MFS transporter and a downstream hypothetical protein. For the *arfA* gene the flanking genes in all evaluated *A. pleuropneumoniae* genomes are a Val-tRNA upstream and a class I SAM-dependent methyltransferase downstream except for the *A. pleuropneumoniae* strain AP76 that has a hypothetical protein upstream instead of a Val-tRNA. Besides that, the *ssrA*, *smpB* and *arfA* genes shows a great conservation among the 16 serotypes of *A. pleuropneumoniae* (Table 4, 5 and 6).

Table 4: Features of the *ssrA* gene in 16 *A. pleuropneumoniae* serotypes

Serotype	Strain	Access number (GenBank)	Genome position (bp)	Identity (%)	Coverage (%)	Strand
1	Shope 4074	NZ_AACK0000000.1	2101365-2101730	100	99	+
2	S1536	NZ_ADOE00000000.1	25161-25526	100	99	+
3	JL03	NC_010278	2021278-2021643	100	99	+
4	M62	NZ_ADOF00000000.1	16965-17330	100	99	+
5	L20	NC_009053.1	2059595-2059960	100	99	+
6	Femo	NZ_ADOG00000000.1	25273-25638	100	99	+
7	AP76	NC_010939.1	2116748-2117113	100	99	+
8	MIDG2331	NZ_LN908249.1	2118118-2118483	100	100	+
8	MV 460	NZ_JSVG00000000.1	25269-25634	100	100	+
8	MV 518	NZ_JSVZ00000000.1	25269-25634	100	100	+
8	MV 597	NZ_JSVX00000000.1	25243-25608	100	100	+
8	MV 780	NZ_JSVV00000000.1	25269-25634	100	100	+
8	MV 1022	NZ_JSVF00000000.1	17826-18191	100	100	+
8	MV 5651	NZ_JSVY00000000.1	25253-25618	100	100	+
9	CVJ13261	NZ_ADOI00000000.1	25291-25656	100	99	+
10	D13039	NZ_ADOJ00000000.1	25651-26016	100	99	+
11	56153	NZ_ADOK00000000.1	25204-25569	100	99	+
12	1096	NZ_ADOL00000000.1	25271-25636	100	99	+
13	N273	NZ_ADOM000000001	25337-25702	100	99	+
15	HS143	-	5214-5579	100	99	_
16	KL 16	CP022715.1	907369-907733	100	99	+

* ”- “ means that the strain HS143 genome is not available at the GenBank.

The *A. pleuropneumoniae ssrA* gene has all the necessary elements for its activity *in vivo:* as the sequence coding peptide tag ANDEQYALAA whose five last amino acids are well conserved in the Bacteria domain, the resume codon GCA for the amino acid alanine that is also well conserved in the domain Bacteria; besides that the link between the tRNA and mRNA parts, the acceptor stem and the 3’ end characteristic of tRNA molecules as shown in its predicted secondary structure in the Figure 1A. The secondary structure of the *Escherichia coli* K12 tmRNA is show in the Figure 1B for comparative purposes since it is an already well established structure.

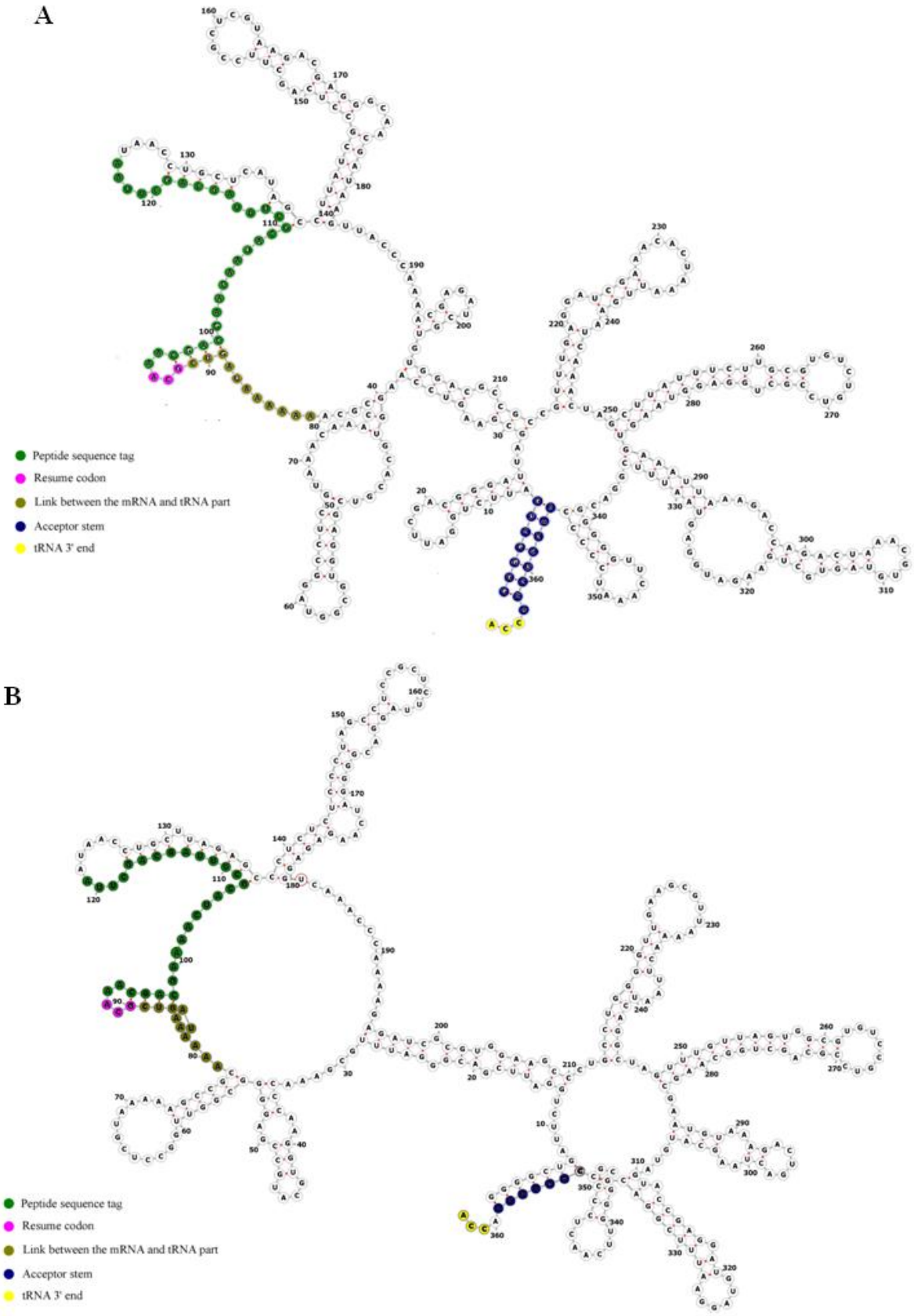

Figure 1. A*: Actinobacillus pleuropneumoniae* tmRNA secondary structure predicted by RNAfold (Gruber et al., 2008). **B:** *Escherichia coli* K12 tmRNA secondary structure predicted by RNAfold (Gruber et al., 2008). The colored sequences represented important regions of the messenger RNA and transfer RNA portions of the tmRNA.

Table 5: Features of the *smpB* gene in 16 *A. pleuropneumoniae* serotypes

Serotype	Strain	Access number (GenBank)	Genome position (bp)	Identity (%)	Coverage (%)	Strand
1	Shope 4074	NZ_AACK0000000.1	23302-23781	100	100	+
2	S1536	NZ_ADOE00000000.1	70603-71082	100	100	+
3	JL03	NC_010278	971889-972368	100	100	+
4	M62	NZ_ADOF00000000.1	1936-2415	100	100	+
5	L20	NC_009053.1	1006549-1007028	100	100	+
6	Femo	NZ_ADOG00000000.1	113624-114103	100	100	+
7	AP76	NC_010939.1	1039797-1040276	100	100	+
8	MIDG2331	NZ_LN908249.1	975532-976011	100	100	-
8	MV 460	NZ_JSVG00000000.1	263433-263912	100	100	+
8	MV 518	NZ_JSVZ00000000.1	188179-188658	100	100	+
8	MV 597	NZ_JSVX00000000.1	263424-263903	100	100	+
8	MV 780	NZ_JSVV00000000.1	188160-188639	100	100	+
8	MV 1022	NZ_JSVF00000000.1	188176- 188655	100	100	+
8	MV 5651	NZ_JSVY00000000.1	263437-263916	100	100	+
9	CVJ13261	NZ_ADOI00000000.1	51176-51655	100	100	+
10	D13039	NZ_ADOJ00000000.1	118541-119020	100	100	+
11	56153	NZ_ADOK00000000.1	263437-263916	100	100	+
12	1096	NZ_ADOL00000000.1	204082-204561	100	100	+
13	N273	NZ_ADOM000000001	115177-115656	100	100	+
15	HS143	-	311502-311981	100	99	+
16	KL 16	CP022715.1	2172040-2172519	100	99	+

* ”- “means that the strain HS143 genome is not available at the GenBank.

To predict the tertiary structure formed by the product of the *A. pleuropneumoniae smpB* gene we have used the software Phyre² (Kelley et al., 2015) shown in Figure 2. This structure obtained was very similar to tertiary structure formed by the SmpB protein of *Escherichia coli* strain K12 that was determined using crystallography.

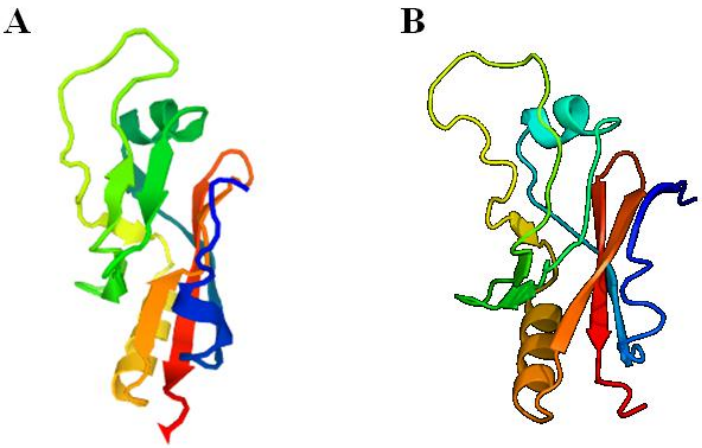

Figure 2. A: Tertiary structure of the SmpB protein of *Escherichia coli* strain K12 determined using crystallography. **B:** Tertiary structure of the SmpB protein of *A. pleuropneumoniae* strain MIDG2331 predicted by Phyre[2]

Table 6: Features of the *arfA* gene in 16 *A. pleuropneumoniae* serotypes

Serotype	Strain	Access number (GenBank)	Genome position (bp)	Identity (%)	Coverage (%)	Strand
1	Shope 4074	NZ_AACK0000000.1	883357-884112	99	100	+
2	S1536	NZ_ADOE00000000.1	33632-33874	99	100	+
3	JL03	NC_010278	835523-835765	99	100	+
4	M62	NZ_ADOF00000000.1	46424-46666	99	100	+
5	L20	NC_009053.1	870918-871160	99	100	+
6	Femo	NZ_ADOG00000000.1	131064-131306	99	100	+
7	AP76	NC_010939.1	913579-913821	99	100	+
8	MIDG2331	NZ_LN908249.1	842285-842527	100	100	-
8	MV 460	NZ_JSVG00000000.1	130213-130455	100	100	+
8	MV 518	NZ_JSVZ00000000.1	54954-55196	100	100	+
8	MV 597	NZ_JSVX00000000.1	130199-130441	100	100	+
8	MV 780	NZ_JSVV00000000.1	54931-55173	100	100	+
8	MV 1022	NZ_JSVF00000000.1	54951-55193	100	100	+
8	MV 5651	NZ_JSVY00000000.1	130213-130455	100	100	+
9	CVJ13261	NZ_ADOI00000000.1	16406-16468	100	99	+
10	D13039	NZ_ADOJ00000000.1	162814-163056	100	99	+
11	56153	NZ_ADOK00000000.1	12545-12787	100	99	+
12	1096	NZ_ADOL00000000.1	71250-71492	100	98	+
13	N273	NZ_ADOM000000001	69879-70121	99	100	+
15	HS143	-	175370-175312	100	99	+
16	KL 16	CP022715.1	907369-907733	100	99	+

* ”- “means that the strain HS143 genome is not available at the GenBank.

To predict the tertiary structure formed by the product of the *A. pleuropneumoniae arfA* gene we have used the software Phyre² (Kelley et al., 2015) shown in Figure 2. This structure obtained was very similar to tertiary structure formed by the ArfA protein of *Escherichia coli* strain K12 that was determined by Ma et al., 2017 using cryo-electron microscopy.

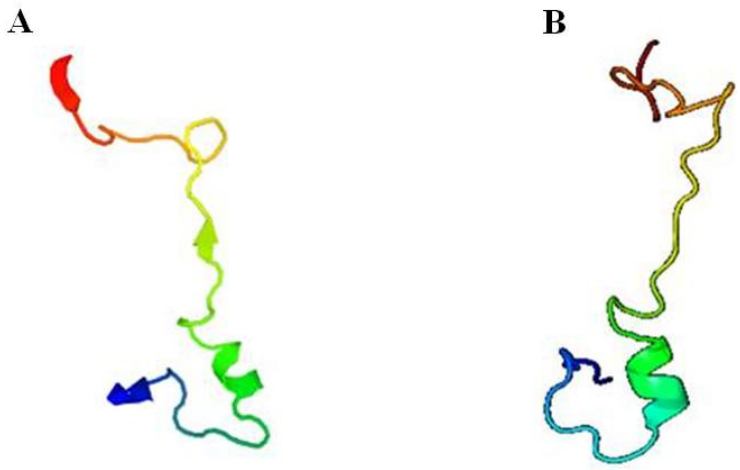

Figure 3. A: Tertiary structure of the ArfA protein of *Escherichia coli* strain K12 determined using cryo-electron microscopy by Ma et al., 2017. **B:** Tertiary structure of the ArfA protein of *A. pleuropneumoniae* strain MIDG2331 predicted by Phyre[2].

The nucleotide sequences of the *ssrA* and *arfA* genes and the amino acid sequences of the *smpB* gene of *A. pleuropneumoniae* strain MIDG2331 and other species belonging to the Pasteurellaceae family where compared by multiple sequence alignments that was used to build two phylogenetic trees, one per gene. The phylogenetic tree constructed with the *ssrA* gene sequences (Figure 4) shows that the *ssrA* gene is very conserved between the species of the Pasteurellaceae family, but present specificities.

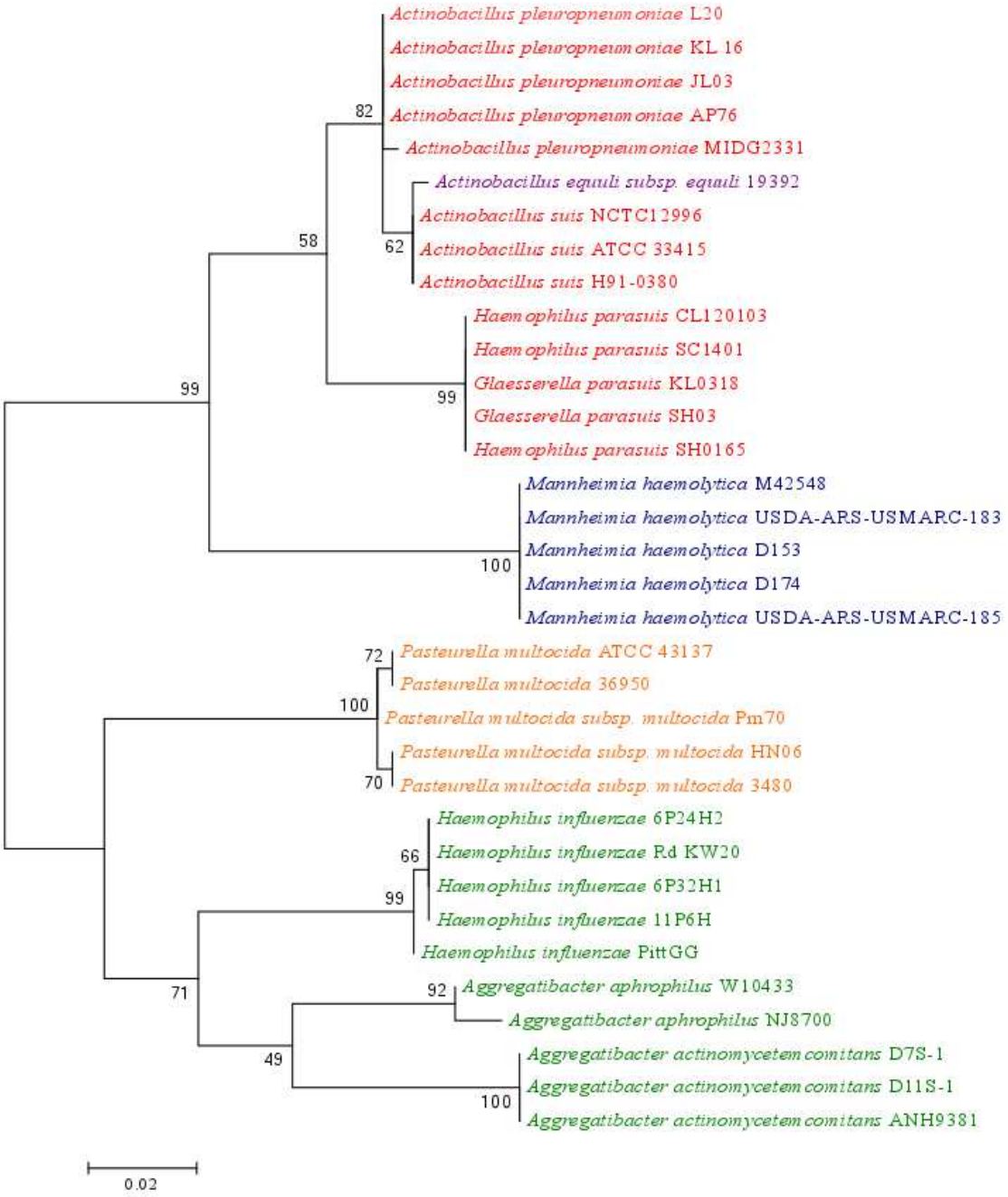

Figure 4. Phylogenetic tree showing the relationship between nine different bacterial species belonging to the Pasteurellaceae family based on the sequence of the *ssrA* gene. The tree shows that the *ssrA* gene separated the nine species into two large groups. One of them grouped the swine pathogens (red) belonging to the *Actinobacillus* genus and *Haemophilus* (*Glaesserella*) *parasuius* as well the horse pathogen (purple) of the *Actinobacillus* genus and the cattle pathogen (blue) *Mannheimia haemolytica*. The other one grouped the species that infect humans (green) and the *Pasteurella multocida* that have multiples hosts (orange).

The phylogenetic tree based on the amino acid sequence of the *smpB* gene belonging to nine different species of the Pasteurellaceae family (Figure 5) shows a similar pattern when compared to the phylogenetic tree constructed based on the *ssrA* gene.

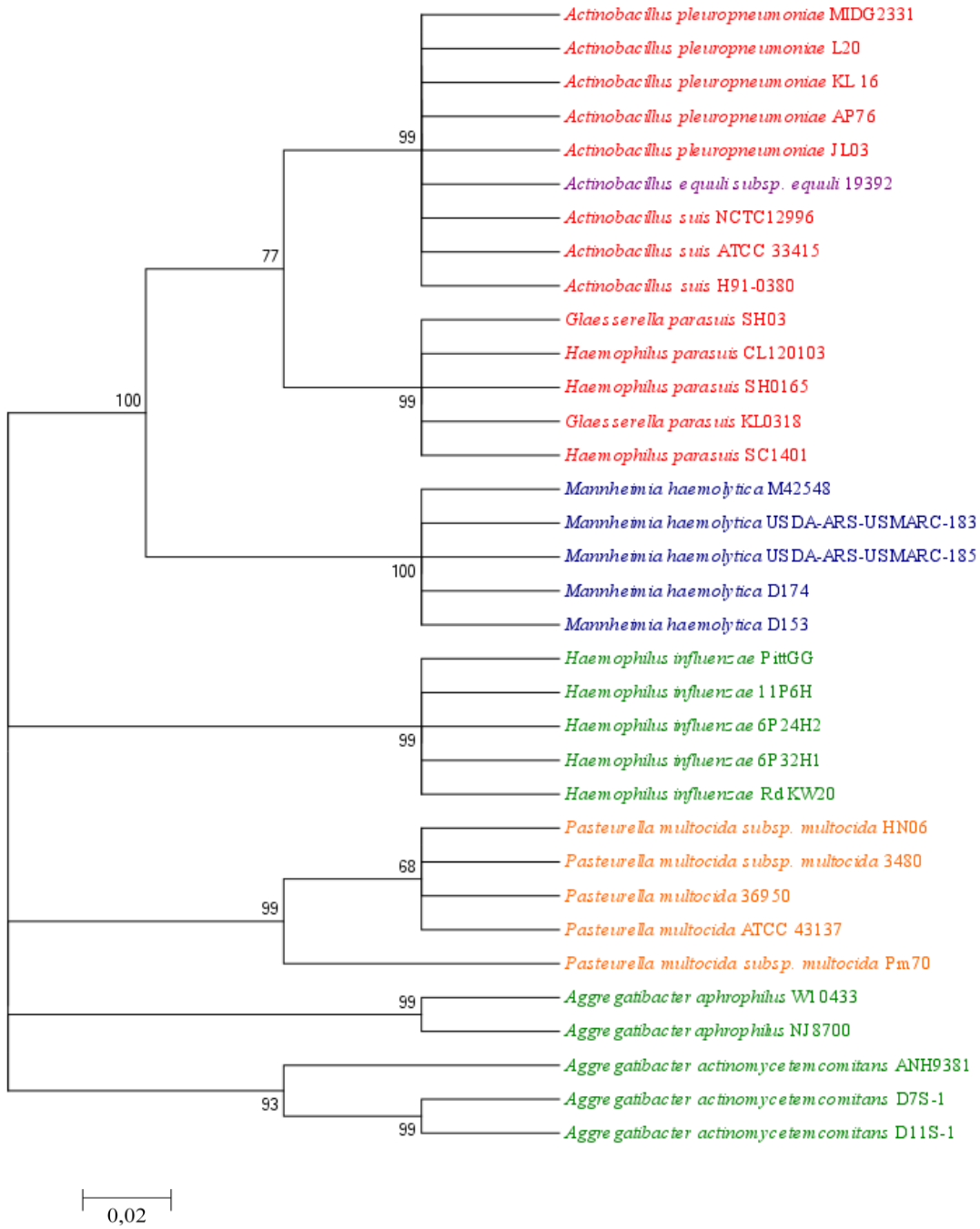

Figure 5. Phylogenetic tree showing the relationship between nine different bacterial species belonging to the Pasteurellaceae family based on the amino acid sequence of the *smpB* gene. The tree shows that the *smpB* gene separated the nine species into two large groups. One of them grouped the swine pathogens (red) belonging to the *Actinobacillus* genus and *Haemophilus* (*Glaesserella*) *parasuius* as well the horse pathogen (purple) of the *Actinobacillus* genus and the cattle pathogen (blue) *Mannheimia haemolytica*. The other one grouped the species that infect humans (green) and the *Pasteurella multocida* that have multiples hosts (orange).

On the *arfA* gene its size and sequence has more difference between the members of Pasteurellaceae family if compared with *ssrA* gene. However the gene sequence shows a great conservation between strains belonging to the same species. The sequences belonging to human pathogens are more similar with each other than with the pig pathogens, as in Figure 6.

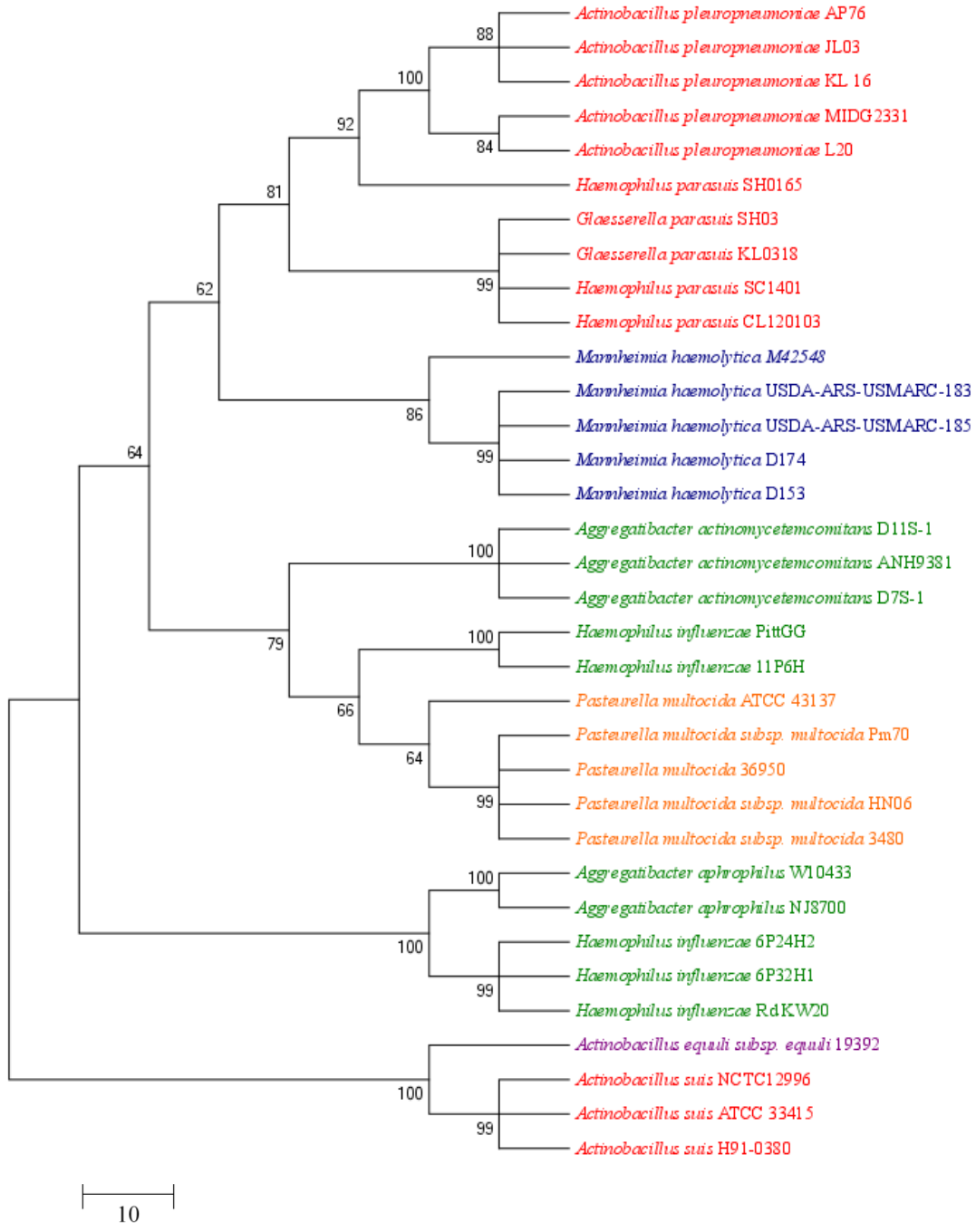

Figure 6. Phylogenetic tree showing the relationship between nine different bacterial species belonging to the Pasteurellaceae family based on the sequence of the *arfA* gene. The tree shows that the *arfA* gene separated these nine species into two groups. The species that infect swine's are represented in red, the specie that infect cattle is in blue, the specie that infect multiple animals are in orange and the species that infect humans are in green.

3.2 *In vitro* analyses

3.2.1 Obtainment of *A. pleuropneumoniae* serotype 8 MIDG2331 mutant and complemented strains for the *ssrA* and *arfA* genes

The strategies used to construct the *A. pleuropneumoniae* Δ*ssrA* and Δ*arfA strains* are shown in the Figure 7. The sequences of the DNA cassette used are in the supplementary material.

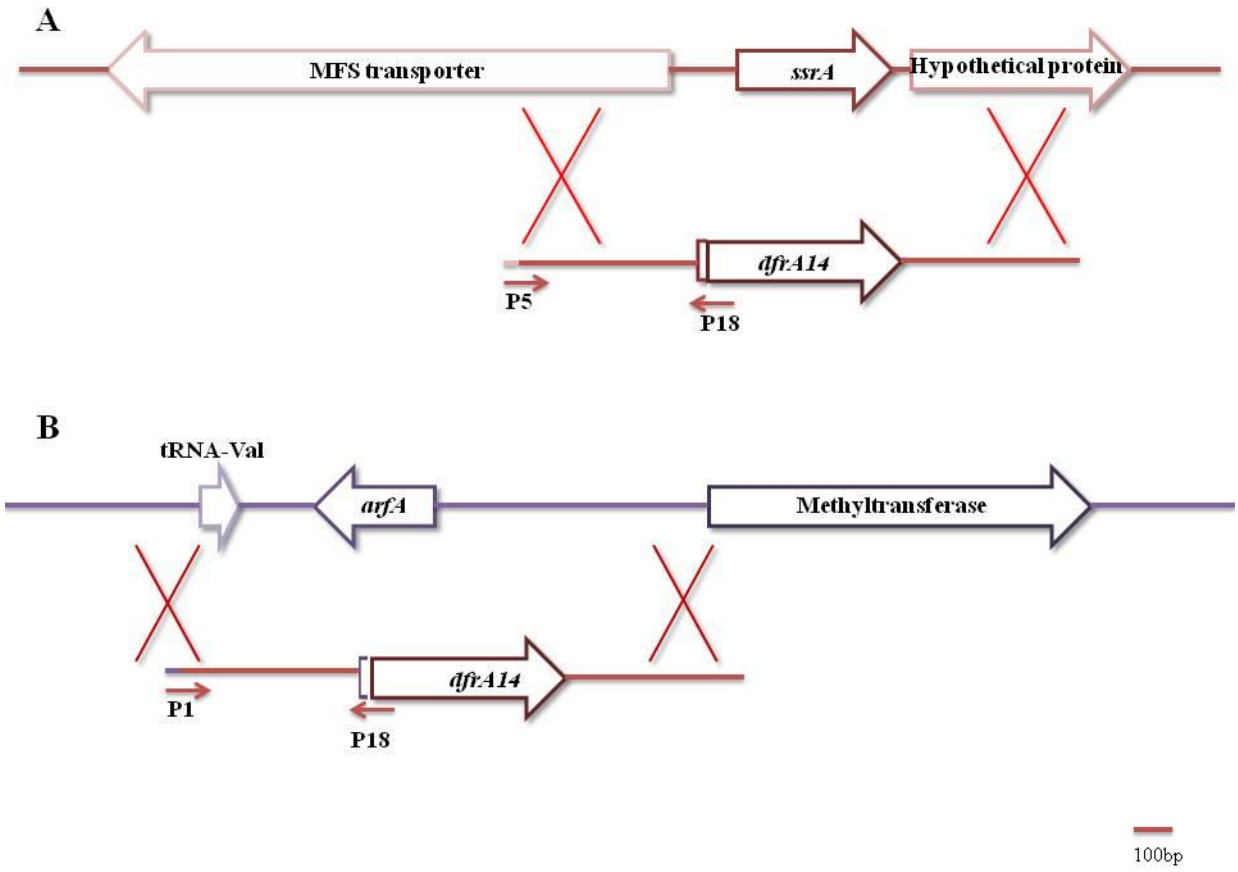

Figure 7. Strategy used to obtain the *A. pleuropneumoniae* MIDG2331 Δ*ssrA* and Δ*arfA* strains. **A**: Genomic context of the *ssrA* gene in *A. pleuropneumoniae* MIDG2331 WT and DNA knockout cassette used to make the recombination at the chromosome. **B**: Genomic context of the *arfA* gene in *A. pleuropneumoniae* MIDG2331 WT and DNA knockout cassette used to make the recombination at the chromosome.

The mutants strains obtained were confirmed by PCR using primers 5 and 1, for the *A. pleuropneumoniae* Δ*ssrA* and Δ*arfA* respectively and the primer 18 that anneal at the *dfrA14* gene that confers resistance to the antibiotic trimethoprim and was used to knockout the target genes and as selection marker for differentiate the mutants strains from the WT strain (Tabela 3). The amplicon obtained contains a small piece of the target gene that remains in the mutants cells and part of the *dfrA14* gene (Figure 8). The

Δ*ssrA* amplicon is 648 bp long and the Δ*arfA* amplicon is 699 bp long. The genomic DNA from WT strain was used as negative control.

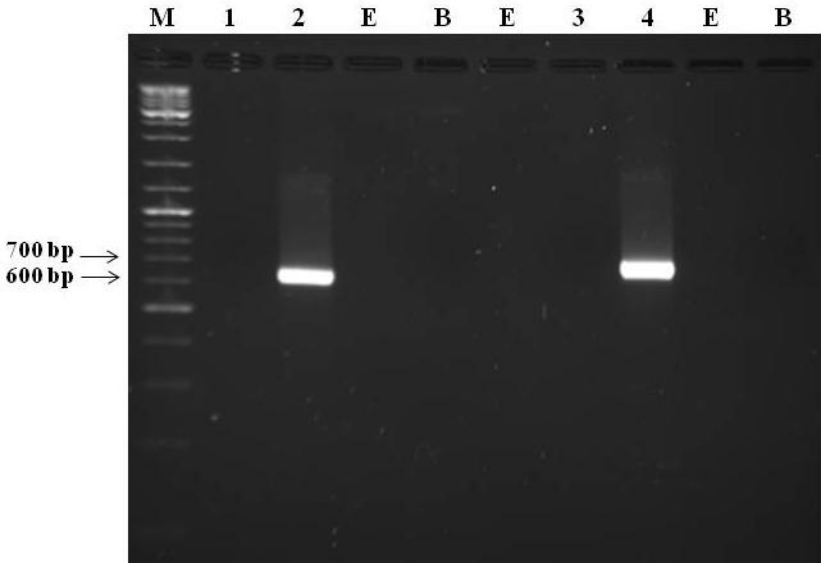

Figure 8. Confirmation of the *A. pleuropneumoniae* MIDG2331 Δ*ssrA* and Δ*arfA* strains. **M**: O'GeneRuler DNA Ladder Mix (Thermo Scientific), **1**: genomic DNA (gDNA) from *A. pleuropneumoniae* MIDG2331 WT with primers to confirm the Δ*ssrA* strain, **2**: gDNA from *A. pleuropneumoniae* Δ*ssrA* strain, **E**: empty well, **B**: blank of the reaction, **3**: gDNA from *A. pleuropneumoniae* MIDG2331 WT with primers for confirm the Δ*arfA* strain, **4**: gDNA from *A. pleuropneumoniae* Δ*arfA* strain.

The complemented strains were constructed by replacing the knocked out gene in the chromosome with the original gene and a different selection marker, the *cat* gene using a different DNA cassette (Suplementary material and Figure 9).

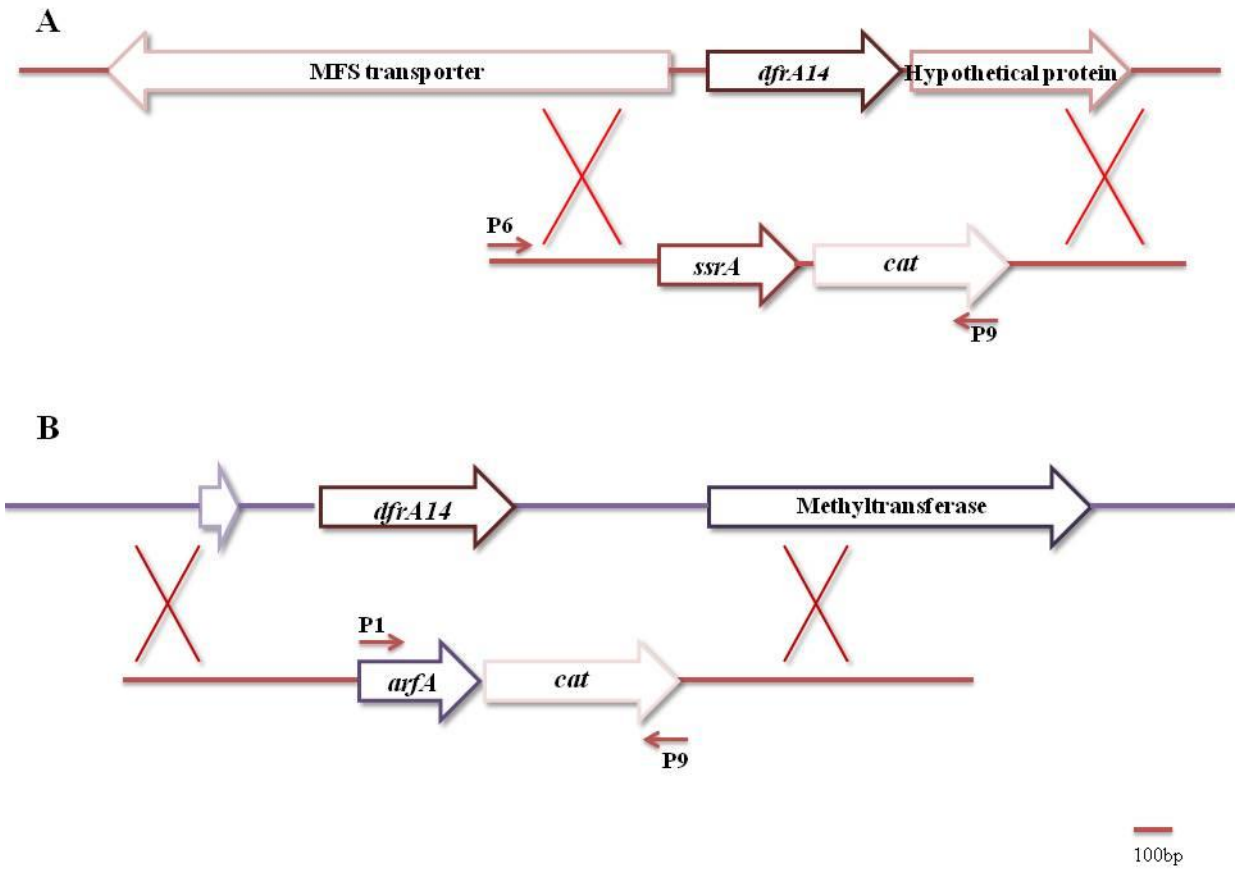

Figure 9. Strategy used to obtain the *A. pleuropneumoniae* MIDG2331 Δ*ssrA*C and Δ*arfA*C (Complemented strains). **A**: Genomic context of the *A. pleuropneumoniae* MIDG2331 Δ*ssrA* and DNA cassette used to make the recombination to get complementation at the chromosome. **B**: Genomic context of the *A. pleuropneumoniae* MIDG2331 Δ*arfA* and DNA knockout cassette used to make the recombination to get complementation at the chromosome.

This different selection marker allows differentiating the complemented strains from both the mutants and the WT strains. The PCR reaction was used to confirm the complementation using a primer that anneals at the beginning of the *ssrA* complementation DNA cassette (primer 6) or *arfA* gene (primer 1) and in the *cat* gene (primer 9). The WT and mutants strains were used as negative control (Figure 10).

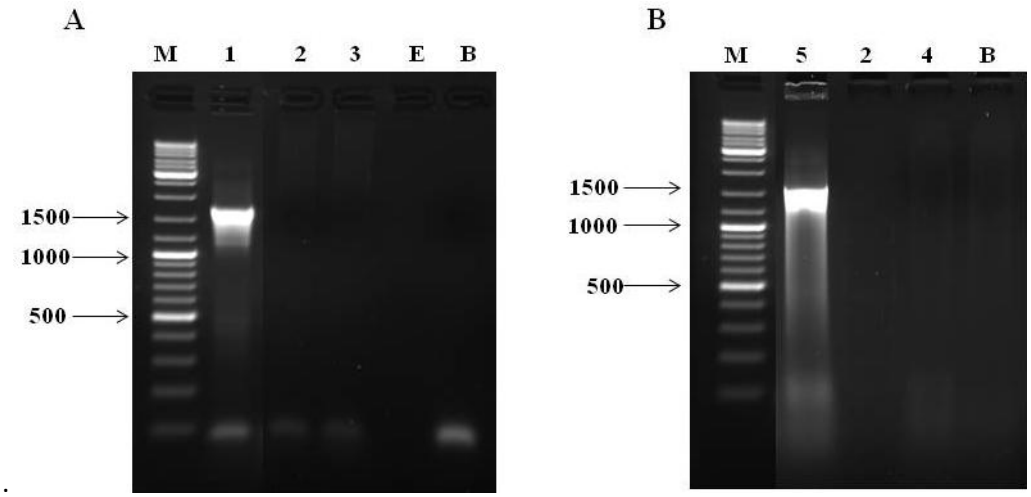

Figure 10. Confirmation of the *A. pleuropneumoniae* MIDG2331 Δ*ssrA*C **(A)** and Δ*arfA*C **(B)**. **M**: O'GeneRuler DNA Ladder Mix (Thermo Scientific). **1** and **5**: gDNA from *A. pleuropneumoniae* MIDG2331 Δ*ssrA*C and Δ*arfA*C, respectively. **2**: gDNA from *A. pleuropneumoniae* MIDG2331 WT. **3** and **4**: gDNA from *A. pleuropneumoniae* MIDG2331 Δ*ssrA* and Δ*arfA*, respectivelly. **E**: empty well. **B**: blank of the reaction.

3.2.2 Non-polar mutation

The analyses to evaluate a possible polar mutation due the mutation strategy to obtain the mutants and the complemented strains showed that the genes localized downstream to the *ssrA* and *arfA* genes were not affected. The expression of the gene upstream to *ssrA* gene in the Δ*ssrA strain* was also analyzed and did not show any change (Supplementary material Figure S1 and S2). Those genes maintain their expression as in the WT strain in the conditions evaluated.

3.2.3 Phenotypic characterization

3.2.3.1 Growth curve

A graphic representing three biological replicates of the growth curve of all strains (WT, Δ and ΔC) is shown in Figure 11. The data show that the Δ*arfA*, Δ*ssrA*C and Δ*arfA*C have a growth similar to the WT strain while the Δ*ssrA* strain shows a slower exponential growth. The growth rate was calculated (Table 7) and the values obtained were submitted to ANOVA and Tuckey test. The statistical tests show there is significant difference between the Δ*ssrA* and the WT strain (p-value < 0.01) and between Δ*ssrA* and Δ*ssrA*C (p-value < 0.01).

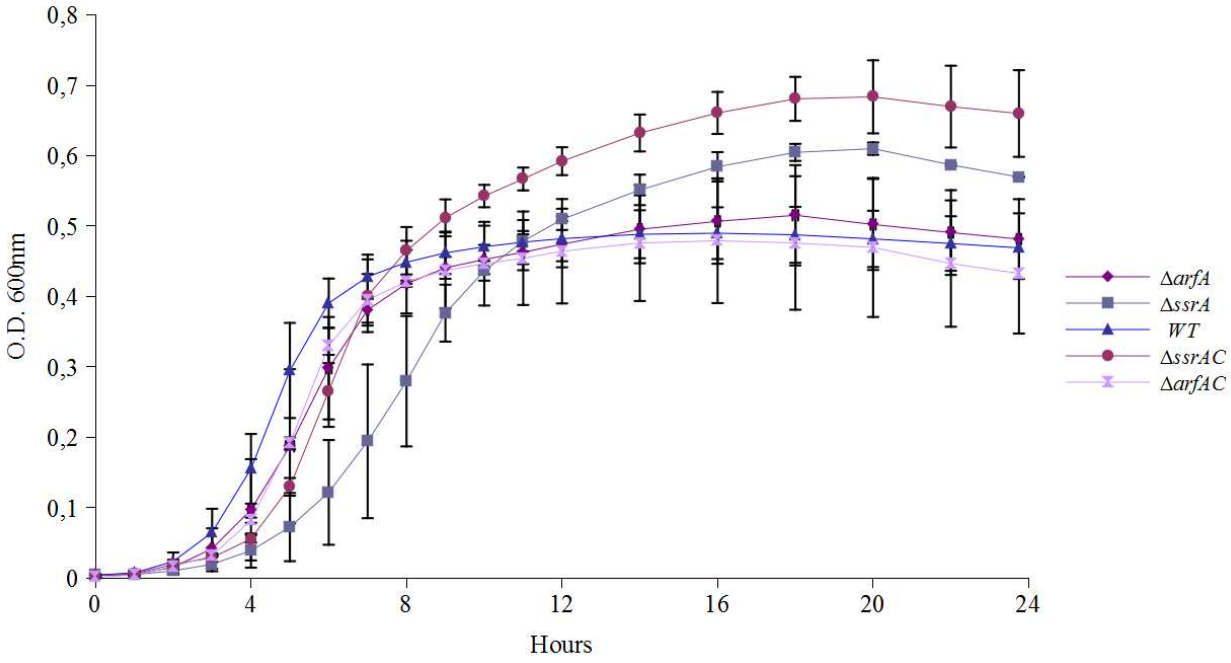

Figure 11. Growth curves of *A. pleuropneumoniae* MIDG2331 WT, Δ*ssrA*, Δ*arfA*, Δ*ssrA*C and Δ*arfA*C strains in BHI/NAD broth at 37ºC, aerobic conditions.

Table 7: Specific growth rate of the *A. pleuropneumoniae* MIDG2331 WT, Δ*ssrA*, Δ*arfA*, Δ*ssrA*C and Δ*arfA*C strains

Strain	Growth rate (μ)
WT	0,116
Δ*ssrA*	0,0811
Δ*arfA*	0,0963
Δ*ssrAC*	0,1411
Δ*arfAC*	0,1078

3.2.3.2 Biofilm

The biofilm formation capacity of the *A. pleuropneumoniae* MIDG2331 WT, Δ*ssrA*, Δ*arfA*, Δ*ssrA*C and Δ*arfA*C strains were evaluated (Figure 12). The results show that the Δ*ssrA* and the Δ*ssrA*C strains have lower biofilm formation ability when compared to the WT strain. These results suggest that the trans-translation system influences in the *A. pleuropneumoniae* biofilm formation capacity since its absence descreases significantly the biofilm formed by the *A. pleuropneumoniae* Δ*ssrA* strain. The result also shows that the *A. pleuropneumoniae* Δ*ssrA*C strain was not capable of to restore the WT phenotype regarding the biofilm formation capacity.

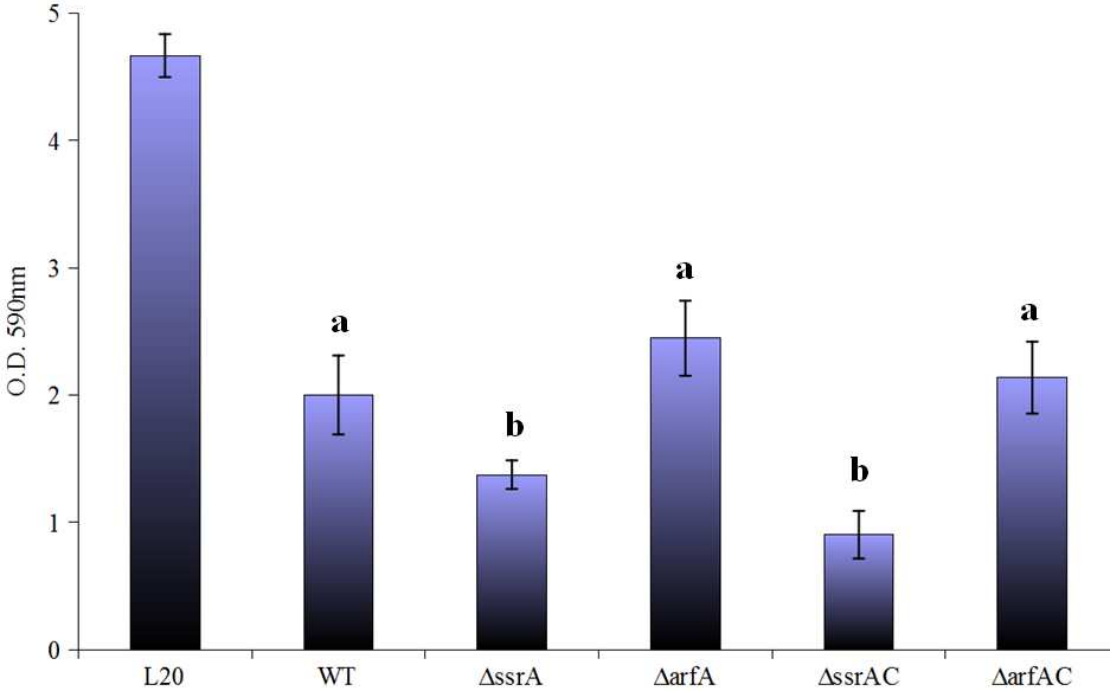

Figure 12. *A. pleuropneumoniae* L20, MIDG2331 wild-type, mutants and complemented strains biofilm formation capacity in polystyrene microplates. The letter a represents absence of significant statistical difference between WT and Δ*arfA* and Δ*arfA*C strains. The letter b represents significant difference between WT and Δ*ssrA* (p-value < 0.05) and WT and Δ*ssrA*C (p-value <0.01) in ANOVA follow by Tuckey test.

3.2.3.3 Antibiotics susceptibility

The antibiotics kanamycin and tylosin, respectively, binding to the 30S and 50S ribosomes sub-units and the absence of an efficient ribosome rescue system will lead to a decrease in this antibiotics resistance. Ampicillin acts inhibiting cell wall formation a process that also depends on an efficient ribosome rescue system due the high demand for proteins. Our results showed (Table 8) that the *A. pleuropneumoniae* Δ*ssrA* strain is at least ten times more (one dilution) sensitive to the antibiotics ampicillin, kanamycin and tylosin when compared to the WT, Δ*arfA*, Δ*ssrA*C and Δ*arfA*C strains.

Table 8: *A. pleuropneumoniae* antibiotics susceptibility in mg/L

***A. pleuropneumoniae* strain**	**Ampicilin**	**Kanamycin**	**Tylosin**
MIDG2331 WT	2	32	2
Δ*ssrA*	1	16	1
Δ*arfA*	2	32	4
Δ*ssrA*C	2	32	4
Δ*arfA*C	1	32	4

3.2.3.4 Stress susceptibility

The ability to deal with and resist to different stress conditions is an important feature of a mammalian pathogenic bacterium. The results of all stress susceptibility tests in Figure 13 show that the *A. pleuropneumoniae* Δ*ssrA* strain is more sensitive to all stress condition investigated here when compared to the WT, Δ*arfA,* Δ*ssrA*C and Δ*arfA*C strains. Different from what could be observed in the biofilm assay, in all stress conditions investigated the *A. pleuropneumoniae* Δ*ssrA*C showed a phenotype very similar to the WT strain. The incapacity of the *A. pleuropneumoniae* Δ*ssrA* strain to resist and grow under all abiotic and biotic stress used here reinforces that the ribosome rescue system based on the tmRNA/SmpB has a central role in *A. pleuropneumoniae* fitness.

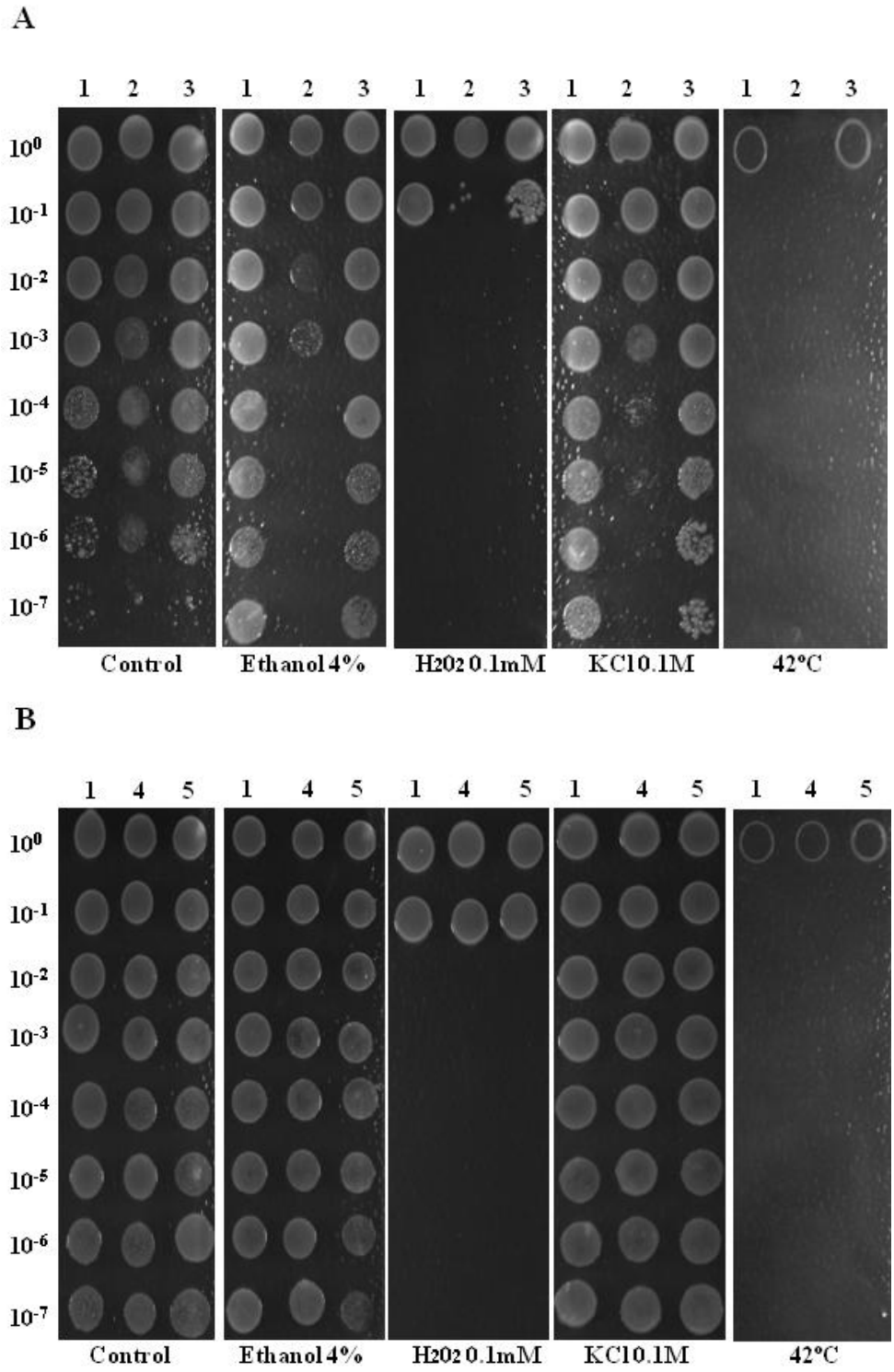

Figure 13. Susceptibility to four different stresses: ethanolic (ethanol 4%), oxidative (H_2O_2 0,1mM), osmotic (KCl 0,1M) and high temperature (42ºC) of the *A. pleuropneumoniae* MIDG2331 WT, mutants and complemented strains. **A:** Stress susceptibility of the *A. pleuropneumoniae* Δ*ssrA* and Δ*ssrA*C in comparison with the parental strain. **1**: *A. pleuropneumoniae* WT strain. **2**: *A. pleuropneumoniae* Δ*ssrA*. **3**: *A. pleuropneumoniae* Δ*ssrA*C. **B**: Stress susceptibility of the *A. pleuropneumoniae* Δ*arfA* and Δ*arfA*C in comparison with the parental strain. **1**: *A. pleuropneumoniae* WT strain. **4**: *A. pleuropneumoniae* Δ*arfA*. **5**: *A. pleuropneumoniae* Δ*arfA*C. The numbers in the left side of the figure represent the cell dilutions that were spotted in each line.

3.2.3.5 Virulence in *Galleria mellonella*

Figure 14 represents three biological replicates of the assays in *Galleria mellonella* to evaluate the virulence of the *A. pleuropneumoniae* Δ*ssrA*, Δ*arfA*, Δ*ssrAC* and Δ*arfAC* strains compared with the virulence of the WT strain. The results show that the *A. pleuropneumoniae* Δ*arfA* and Δ*arfAC* strains have a similar virulence to the WT strain. Unlike these strains, the *A. pleuropneumoniae* Δ*ssrA* and Δ*ssrAC* strains showed a highly attenuated phenotype when compared to the WT strain. Again the *A. pleuropneumoniae* Δ*ssrAC* strain does not show a restored phenotype. The attenuation of the *A. pleuropneumoniae* Δ*ssrA* strain showed here in the alternative infection model *G. mellonella* is an indication that this strain has a potential to be used in further studies as a candidate to a live attenuated vaccine for the swine pleuropneumonia. The same attenuated phenotype observed here are in accordance with previous findings of the attenuation of *A. pleuropneumoniae* serotype 1 Shope 4074 Δ*ssrA* strain in pigs.

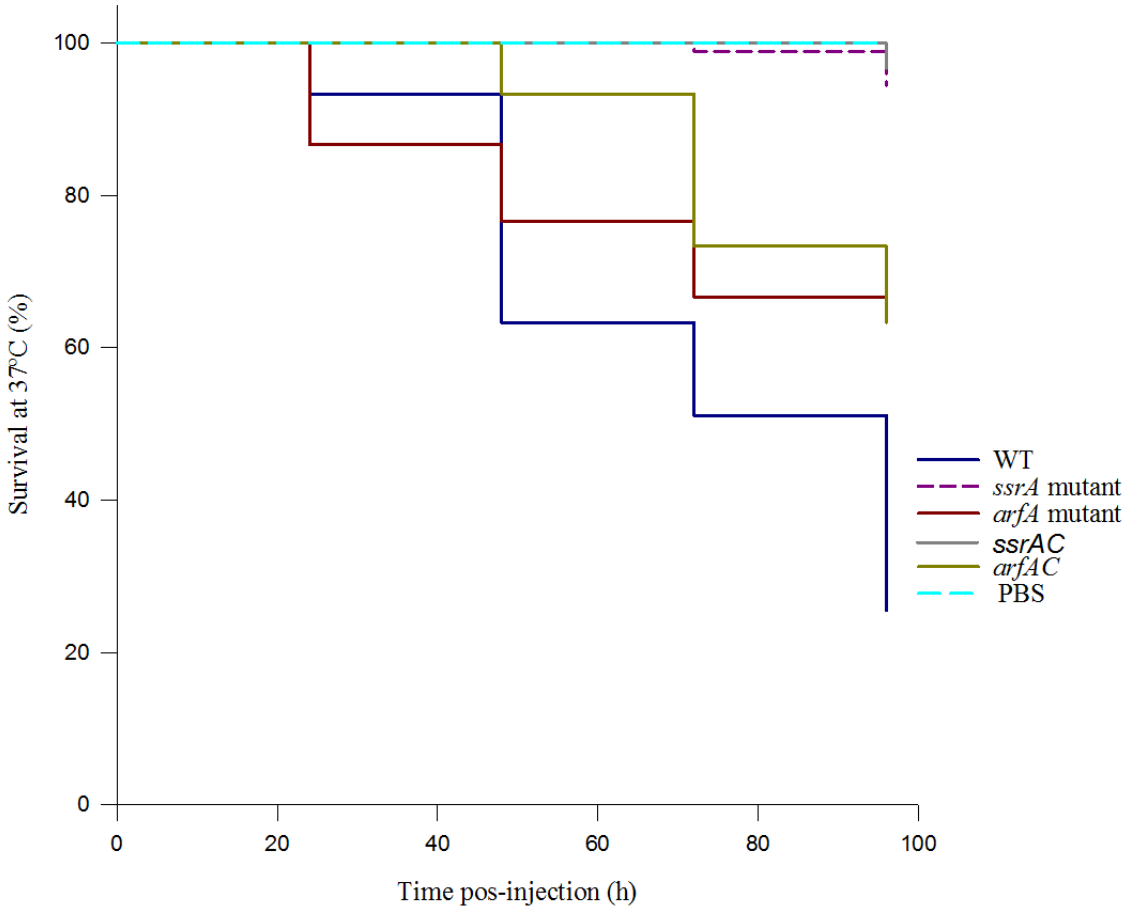

Figure 14. *Galleria mellonella* killing by *A. pleuropneumoniae* MIDG2331 WT, mutants and complemented strains.

4. DISCUSSION

The *in silico* analyses performed in this study shows that the *ssrA* gene as well the *smpB* gene are present and has a great conservation between the *A. pleuropneumoniae* genomes of different serotypes and even between different species belonging to the *Pasteurellaceae* family. This is in accordance to the fact that the ribosome rescue system based on the tmRNA/SmpB is the main system found in almost all bacteria known so far, even in symbiotic species like *Carsonella rudii* and in the minor genomes of *Mycoplasma* species (Macé et al., 2016; Huter et al., 2017). This ubiquity and the great conservation of the *ssrA* and *smpB* gene are related to its important and oftentimes essential function, especially in pathogens species where its absence affect the virulence and viability (Macé et al., 2017). Different from the *ssrA* and *smpB* genes, the *arfA* gene shows itself well conserved in the *A. pleuropneumoniae* different serotypes and more diverse within the *Pasteurellaceae* family. The fact that the *arfA* gene is responsible for a backup ribosome rescue system could explain this less conservation since its function it is not essential for cell survival. Besides that, this gene has a limited distribution in the Bacteria domain, being restricted to β- and γ-proteobacteria (Schaub et al. 2012). Both the secondary structure of the tmRNA molecule and the tertiary structure of the SmpB and ArfA protein are important for their function *in vivo* and the ones found here for *A. pleuropneumoniae* are very similar to those structures formed by the same molecules of *Escherichia coli* K12 (Giudice et al., 2014 and Ma et al., 2017).

The phenotypic characterization of the *A. pleuropneumoniae* serotype 8 MIDG2331 Δ*ssrA* in this work indicates that the presence of the tmRNA is essential for the adaptation to different stress conditions and virulence of this swine pathogen. The first big difference that could be noticed in the Δ*ssrA* in comparison to the parental strain is the slower exponential growth. This characteristic has already been described in other bacteria such as *Streptococcus pneumoniae* (Brito et al., 2017), *Escherichia coli* (Wurihan et al., 2016) and *Francisella tularensis* (Svetlanov et al., 2012). This probably occurs because during the exponential growth the great majority of ribosomes are at a near maximum elongation rate (Dai et al., 2016) and the formation of non-stop translation complex probably occurs. The absence of the main and most efficient ribosome rescue system will delay the translation process as well the cell growth.

A proper biofilm production is an important factor for a successful host colonization by *A. pleuropneumoniae* (Chiers et al., 2010), and also needs an efficient

translation which in ensured by a functional ribosomes rescue system. Is well known that for *A. pleuropneumoniae* the biofilm formation in polystyrene microplates and other surfaces is dependent of the production of the polymer poly-N-acetyl-glucosamine (PGA) whose biosynthesis depends on the translation of the *pgaABCD* operon (Grasteu et al., 2011; Hathroubi et al., 2017). Any problems in this process could affect a proper biofilm formation by *A. pleuropneumoniae* as we see in the Δ*ssrA* strain in this work. The lower biofilm formation by the *A. pleuropneumoniae* Δ*ssrA* strain is very interesting because the biofilm/adhesion capacity is an important feature that helps the pathogen to colonize and stay in the pig's lung and suggest that the trans-translation system is essential to this process.

Antibiotics are commonly used as feed additives in animal agriculture and this could lead to the increase of bacterial strains resistant to them (Chattopadhyay, 2014). The presence of antibiotics, especially the ones used here which are widely used in veterinary medicine, is a problem that *A. pleuropneumoniae* faces in the field. The phenotype of increased antibiotics sensitivity observed in the *A. pleuropneumoniae* Δ*ssrA* strain it is also reported in other bacteria, especially in *Escherichia coli* (Luidalepp et al., 2005; Li et al., 2013). A bigger sensitivity to antibiotics that target the ribosome is expected since the Δ*ssrA* strain has a problem to deal with stalled ribosomes. Antibiotics that inhibit the protein synthesis like kanamycin, which binds to the ribosome sub-units 30S and 50S, and tylosin that targets the ribosome 50S sub-unit will generate stalled ribosomes that need to be rescued (Lalaouna et al., 2014). The Δ*ssrA* strain also showed itself more sensitive to the antibiotic ampicillin which was not expected since the ampicillin target the cell wall. In *E. coli* it was reported that the trans-translation system regulates the translation of the mRNA encoding the SecM protein. This protein regulates the expression of the SecA protein that is an ATPase necessary for the function of the protein translocation system SecYEG, a general secretory pathway (Collier et al., 2004). It is supposed that the absence of the trans-translation could affect the transport, by this pathway, of necessary proteins to respond to stresses at the cell wall (Luidalepp et al., 2005). The *A. pleuropneumoniae* Δ*ssrA* strain increased sensitivity to the antibiotics investigated here indicates that the trans-translation process plays an important role in *A. pleuropneumoniae* capacity to deal with this kind of stress.

The stress response plays an important role in *A. pleuropneumoniae* during host infection (Bossé et al., 2002; Sheehan et al., 2003; Bossé et al., 2010). The mammalian

immune defense systems are versatile and count with many strategies to eliminate the pathogen, like the generation of oxidizing substances by the neutrophils and macrophages. Inside the host, the pathogen also faces changes in osmolarity and temperature and the capacity to resist to them is crucial for the pathogen survival. Here we show that the *A. pleuropneumoniae* Δ*ssrA* strain is more sensitive than the parental and Δ*arfA* strains to four different stress conditions: oxidative, osmotic, ethanol and temperature. This phenotype of stress sensitivity is also seen in other bacteria lacking the trans-translation system as *Francisella tularensis, Helicobacter pylori, Escherichia coli* (Svetlanov et al., 2012; Thibonnier et al., 2008; Li et al., 2013). This occurs because the number of defective mRNAs, usually cleaved in the ribosome A-site by RelE or RNases increases under stress conditions in an attempt to save energy, and consequently the trans-translation process becomes necessary (Keiler, 2015). The incapacity to adapt and grow under stress conditions has a huge impact in the bacteria fitness, especially in pathogenic species like *A. pleuropneumoniae* that constantly face stress conditions created by the host immune defense mechanisms.

A previous study with *A. pleuropneumoniae* serotype 1 strain 4074 transposons mutants reported that a mutant, identified as 14D5, showed itself much attenuated in pigs. Later it was discovered that the mutated gene was the *ssrA* gene (Sheehan et al., 2003). Our results show that the *A. pleuropneumoniae* Δ*ssrA* strain has a much-attenuated phenotype in the *Galleria mellonella* larvae when compared to the parental strain. The virulence attenuation is a common phenotype observed in other bacteria whose the *ssrA* gene was mutated, like *Fransicella tularensis* (Svetlanov et al., 2012), *Helicobacter pylori* (Thibonnier et al., 2008), *Salmonella enterica* serovar Typhimurium (Julio et al., 2000), *Streptococcus pneumoniae* (Wilson et al., 2015), *Yersinia pseudotuberculosis* and *Y. pestis* (Okan et al., 2006 and 2010), and *Escherichia coli* (Mu et al., 2013). The attenuation observed in the *A. pleuropneumoniae* Δ*ssrA* in comparison with the virulence of the WT strain shows that the ribosome rescue system based on trans-translation has a critical role in *A. pleuropneumoniae* physiology since its absence has a huge impact in the virulence of this pathogen. The attenuated phenotype of *A. pleuropneumoniae* Δ*ssrA* in *G. mellonella* indicates that this strain has a potential for further studies in the direction to create a live attenuated vaccine with it.

The *A. pleuropneumoniae* Δ*ssrA* still has the alternative ribosome rescue based on the ArfA but this is not enough to keep the parental phenotype since this backup system is not as efficient as the tmRNA/SmpB system (Shimizu, 2012). The two main

aspects that make the ArfA system less efficient is that efficiency decreases with the increasing length of the mRNA at the 3' end at the ribosome A site (Shimizu, 2012 and Zeng et al., 2015) and the ArfA system does not tag for degradation the defective protein generated by the mRNA that cause the stalled ribosome (Keiler, 2015).

The decreased exponential growth, the small adhesion capacity, the greater sensitive to all stresses including the antibiotics and the reduction in the virulence that it is seen in the *A. pleuropneumoniae* Δ*ssrA* is not observed in the Δ*arfA* strain. This mutant still has a functional trans-translation system and therefore has its behavior very similar to the parental strain. Besides that, in the wild type cell containing both ribosome rescue systems, the *arfA* mRNA is cleaved by RNase III generating a non-stop mRNA that will cause the formation of a non-stop translation complex which will be rescued by the tmRNA/SmpB system (Chadani et al., 2011).

Like in *E. coli* (Chadani et al., 2010) a double mutant Δ*ssrA*_Δ*arfA* is lethal for *A. pleuropneumoniae*. This result is in accordance with the genome analyses that did not find the third known ribosome rescue system called ArfB (alternative ribosome rescue factor B) in *A. pleuropneumoniae*. Based on these facts and in the phenotypes presented by the *A. pleuropneumoniae* Δ*ssrA* and Δ*arfA* we can conclude that the tmRNA/SmpB ribosome rescue system has the main role in the *Actinobacillus pleuropneumoniae* physiology.

5. CONCLUSION

This work is the first report of the presence of ribosome rescue systems in the *Pasteurellaceae* family and in a pathogen of veterinary importance as *Actinobacillus pleuropneumoniae.* This is also the first report to confirm the importance of the trans-translation system in the stress resistance and virulence of *A. pleuropneumoniae*. The great phenotype modifications observed in the *A. pleuropneumoniae* Δ*ssrA* strain reflects the importance of a ribosome rescue system for perfect cell functioning. Besides that, previous bioinformatics analyses show that only two ribosomes rescue systems exist in *A. pleuropneumoniae*, the one based on the tmRNA/SmpB and the other based on the ArfA protein suggesting that a double knockout of this genes would be lethal.

The phenotype observed in the *A. pleuropneumoniae* Δ*arfA* strain reinforces that for *A. pleuropneumoniae* the ribosome rescue system based on the tmRNA/SmpB and not in the ArfA protein has the main role in this pathogen's fitness. The much-attenuated virulence profile shown in this work by the *A. pleuropneumoniae* Δ*ssrA* strain in the *Galleria mellonella* larvae is the first step for further studies of this strain as a candidate to reach an attenuated live vaccine to control the porcine pleuropneumonia in the future.

6. REFERENCES

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SUPPLEMENTAL MATERIAL

Non-polar mutation analyses: *A. pleuropneumoniae* MIDG2331Δ*ssrA*

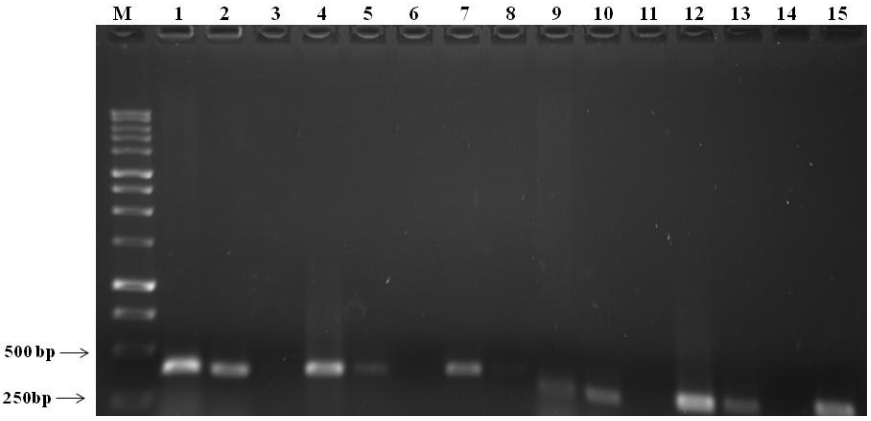

Figure S1: Analysis of non-polar mutation of the upstream and downstream genes to the knockout target gene of the *A. pleuropneumoniae* MIDG2331 Δ*ssrA* strain. **M**: 1 Kb DNA Ladder (Promega), **1**: *A. pleuropneumoniae* MIDG 2331 WT genomic DNA amplification of the upstream gene, 2051, with primer pair 12 and 13, **2**: WT RNA without DNase treatment, **3**: WT RNA with DNase treatment, **4**: WT cDNA amplification of the upstream gene, **5**: *A. pleuropneumoniae* Δ*ssrA* strain RNA without DNase treatment, **6**: Δ*ssrA* strain RNA with DNase treatment, **7**: Δ*ssrA* cDNA amplification of the upstream gene, **8**: blank, **9**: *A. pleuropneumoniae* MIDG 2331 WT genomic DNA amplification of the downstream gene, 2053, with the primer pair 14 and 15. **10**: WT RNA without DNase treatment, **11**: WT RNA with DNase treatment, **12**: WT cDNA amplification of the downstream gene, **13:** *A. pleuropneumoniae* Δ*ssrA* strain RNA without DNase treatment, **14**: *A. pleuropneumoniae* Δ*ssrA* strain RNA with DNase treatment, **15**: Δ*ssrA* cDNA amplification of the downstream gene.

Non-polar mutation analyses: *A. pleuropneumoniae* MIDG2331 Δ*arfA*

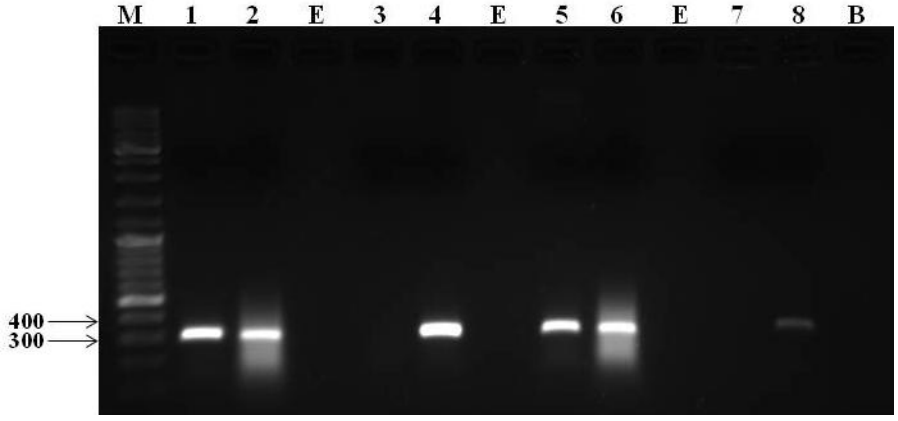

Figure S2: Analyze of non-polar mutation in the downstream (0781) gene to the knockout target gene of the *A. pleuropneumoniae* MIDG2331 Δ*arfA* strain. All the PCR reactions were realized with the primer pair 16 and 17. **M**: O'GeneRuler DNA Ladder Mix (Thermo Scientific). **1**: *A. pleuropneumoniae* parental strain genomic DNA. **2**: *A. pleuropneumoniae* parental strain total RNA without DNase treatment. **E**: empty. **3**: *A. pleuropneumoniae* parental strain total RNA with DNase treatment. **4**: *A.*

pleuropneumoniae parental cDNA. **E**: empty. **5**: *A. pleuropneumoniae* Δ*arfA* strain genomic DNA. **6**: *A. pleuropneumoniae* Δ*arfA* strain total RNA without DNase treatment. **E**: empty. **7**: *A. pleuropneumoniae* Δ*arfA* strain total RNA with DNase treatment. **8**: *A. pleuropneumoniae* Δ*arfA* strain cDNA. **B**: blank.

DNA Cassette for *A. pleuropneumoniae ssrA* gene knockout constructed by Eurofins

sodC Promoter region in light green text; dfrA14 gene in light blue text; APP DNA uptake sequence underlined; terminator sequence in italics; Primer 5 in purple; primer 18 in bold.

CAGTAAAACGGCAAGAGTGAGGATCGAAGTATGGTAGAAACCGATCGTAA
TACAAATCACGGCGCTTGCCCCTAAACAGATCGCAAACAATTTACGTAAAT
CGACACGATCGCCCAATGCGCCCATATAAAATTTTGCCAAGCCGTAAGAAA
TGGTTAAACAGGAAAGTAACATCGCAATATCCGCTTTTTCCCAACCGTTTGT
GACCATAATGGATTTAGACATTAATTTAAAATTGTTACGGACTAAATAAGC
GCAGACATAGCCGAGAAACGCTACGATAAAGATCATTTTTTGGCGTTTTAG
AAAAGTCGCAAATTTTTCCGGCTCTTTTTGTTGATAGGCGAAAGTCATTGTG
GACTCCTTTAATAAGAAAGTGGAAGATTTGTTCGAATTACAATCAATCGAA
GTTTATTTGTCAAAATAAAAAAATGAAATCTTTGAACTACTCCACAAATTAA
TTGAGCAAGCGGTTAAATTTACAAATTTTTTTGCAAAATCAACCGCTTTCCA
TTAGAATTATAAGACTTTTGGGGCTGATTCTGGATTTAGACTATTTAAATAA
TATTATTTAAATTCTTTACTATAGTGTACAATACACACAGTCCATTAACCAA
AATAAAA**GGAGGAATTAGGATGAGAACCTTG**AAAGTATCATTGATAGCT
GCGAAAGCGAAAAACGGCGTGATTGGTTGCGGTCCAGACATACCCTGGTCC
GCGAAAGGGGAGCAGCTACTTTTTAAAGCATTGACCTACAATCAGTGGCTT
CTGGTGGGTCGCAAGACGTTTGAATCTATGGGCGCACTCCCCAATAGGAAA
TACGCGGTCGTTACCCGCTCAGGTTGGACATCAAATGATGACAATGTAGTTG
TATTTCAGTCAATCGAAGAGGCCATGGACAGGCTAGCTGAATTCACCGGTC
ACGTTATAGTGTCTGGTGGCGGAGAAATTTACCGAGAAACATTACCCATGG
CCTCTACGCTCCACTTATCGACGATCGACATCGAGCCAGAGGGGGATGTTTT
CTTCCCGAGTATTCCAAATACCTTCGAAGTTGTTTTTGAGCAACACTTTACTT
CAAACATTAACTATTGCTATCAAATTTGGAAAAAGGGTTAACAAGCGGTAG
GATAGTAG*CCGCCTAATGAGCGGGCTTTTTTTT*TTCAAATCCCCCCAGCTCCAC
CAAATTTGTATACTTTTAATAGGTTATTTAAAATGAAATTCCACCAAAAACT
AAATAGAACTGCCAAAACTGCAGTGGCTATTGCACTTAGTAGTTTTGGTGTA
TTAGATATGTCTTCATTAGATAATGTCACTCAATCTATTGAAAATATAAGAA
TATTATCCTCTCAAAAAAATAATATTAATTTTGTTTTCTTAAACGTTAAAGA
ATTATCGAAAGAGATAGAAAATATTAGCCAAGCGATTAATGCTAATTTAGA
TAAAGTTAATATAAAAGAATTTAATCATTTAGTATTAATTAATCAACTTATC
AATTTTTCATTTTATATGTTGAAAAATGAATATAAAACGCAAATCCTCTCTA
ACTCTGAATATAAATTAAGTTTTAGATATTTTTCTAACGCTAAAAATAACTT
TGAGAATTCTTTACAACGTATCAAAGAAAATATTAATGGTGTTGATGTTGTA
ACCATAAAAGAT

DNA Cassette for *arfA* gene knockout constructed by Eurofins

sodC Promoter region in light green text; dfrA14 gene in light blue text; APP DNA uptake sequence underlined; terminator sequence in italics; Primer 1 in purple; primer 18 in bold.

ATGCCAAAGAAGGATTTTTTACTTATTTTTGGCTACAAACTGCAACAAAGCC
TAGTTTATGTTCAGGATTATTAAAGGTTTGGAACATTTTTTTGAATGTTTCAC
GGTTTTCGGGTTTTAGCGCATTACCGACGATTTTCATTGCACCTAAAACGCC
TTCGTCGTAGATCAGACCTTTCGGCGAAAGCAGCGTCATCTCTCCTGAAAAT
ACATCGACATTACGGAAACCGCATTGATGGAAAGTGTTTTTCCACCCTTCTT
TAGTAAGCGGCGTTACACTAACGTTAATCGCTTCACGTAAAGCGACTAAAA
CTTGTTCCGCATCGTCATTTTGTAACATCACATCGTGAGTGAGTAAAAAACC
GCCCGGTTTTAAGACTCTGAAATATTCACGAATCGCTTTCTCTTTAGCTTCA
AGAGGAAGCATAGTCAGCATCGCTTCATTGATGACAATATCAAAACTGTTG
TCTTCAAAAGGTAATTTGGTCGCATTTGCACGTTGAACTTGAATGAAGTCTT
CCAAGTTATTTTCTTTTATATTACTGCGCGCTTTATCTAAGGCTTCTTCGTCC
AGATCAATCCCTGTAATATGGCAACCGTACTGTTTAGCAATTTGTATTGCAG
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GTTTTTCCTAAACGAGATAAGAAATTATGTCCTATTTCATCTTTCTTCATAGA
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TGCGAATGATTATACAGAAAATTTCAGTTTTCTTATATACAAAGAGCGTAAA
TTTCGGCATTATGCATACTACGATTCACATATTTTGTACAGTTAAAATAGAG
GAACTAAAAAATCTGATAAATTTATTATAATAAATTTAAGAAATGATATCA
CATGTTATGTGTGTCAGGTAATTGGTTTTATTTT**CCTCCTTAATCCTACTCT**
TGGAACTTTCATAGTAACTATCGACGCTTTCGCTTTTTGCCGCACTAACCAA
CGCCAGGTCTGTATGGGACCAGGCGCTTTCCCCTCGTCGATGAAAAATTTCG
TAACTGGATGTTAGTCACCGAAGACCACCCAGCGTTCTGCAAACTTAGATA
CCCGCGTGAGGGGTTATCCTTTATGCGCCAGCAATGGGCGAGTCCAACCTGT
AGTTTACTACTGTTACATCAACATAAAGTCAGTTAGCTTCTCCGGTACCTGT
CCGATCGACTTAAGTGGCCAGTGCAATATCACAGACCACCGCCTCTTTAAAT
GGCTCTTTGTAATGGGTACCGGAGATGCGAGGTGAATAGCTGCTAGCTGTA
GCTCGGTCTCCCCCTACAAAAGAAGGGCTCATAAGGTTTATGGAAGCTTCA
ACAAAAACTCGTTGTGAAATGAAGTTTGTAATTGATAACGATAGTTTAAAC
CTTTTTCCCAATTGTTCGCCATCCTATCATC*GGCGGATTACTCGCCCGAAAAAA*
*AA*AAGGTATCGAACGTTGGCGATATATTATCAACGTATTTACCCGTATGCGT
TTTTGCTATGCCGACAAGCGGTTGGATTTTGCTTGTAAATTACCTGTTGAAG
ACGCACCAAACGAATTAAAACCTTGGTTTAAATTAGATAATCCGTTATTTCA
TCAACAAGACATTATTTTCGGTCATTGGGCAAGTTTAATGGGTAAAGCGGAT
AAACCGAATATCTACGCCTTAGACACCGGCTGTGCTTGGGGTAACCATCTCA
CCATGATCCGATGGGAAGATAAACAAATTTTCACCCAAGAACGCTTAAAAT
AAAATCAAACGGAATACGGGGTCTTGCTATCCTCCGCATTTTCGATTATAAT
TCTTCCCATCTATGCGACTATAGCTCAGTTGGTTAGAGCACCACCTTGACAT

GGTGGGGGTCACTGGTTCGAGTCCAGCTAGTCGCACCATAAAATTCCAATC
CCTTGTCGAAAGACAAGGGATTTTTTTATGCCAAATTAATTACTGAATGCTA
ATAAAAAAGCCCATTTAAAATTGCTCGTCGGAACATTTTTAAATGGTCTTTC
GCTTCTCTCATAACCCGCTTTACGGTGTTTTGCGTTTCGTTGATAACTCCCTT
TGCCTTTTCGATTGTTCTCAATCCGAGTACGAAACAATTTATCCGTTACAAG
TGCTTTTATCGCACTTTCTCGGATCTCGCCTCTTTGGTGCTCATAAACCGTAT
TTTGTGCTTGTTTCAT

DNA cassette for complementation of the *A. pleuropneumoniae* Δ*ssrA* strain constructed by Overlaping PCR

Orange: Primer 6; *Italic*: Primer 7; Red: *ssrA* gene; Underlined: Primer 8; Green: *cat* gene; *Purple:* Primer 9; Underlined: Primer 11; Gray: Primer 10; Blue: APP DUS.

TGCCCCTAAACAGATCGCAAACAATTTACGTAAATCGACACGATCGCCCAA
TGCGCCCATATAAAATTTTGCCAAGCCGTAAGAAATGGTTAAACAGGAAAG
TAACATCGCAATATCCGCTTTTTCCCAACCGTTTGTGACCATAATGGATTTA
GACATTAATTTAAAATTGTTACGGACTAAATAAGCGCAGACATAGCCGAGA
AACGCTACGATAAAGATCATTTTTTGGCGTTTTAGAAAAGTCGCAAATTTTT
CCGGCTCTTTTTGTTGATAGGCGAAAGTCATTGTGGACTCCTTTAATAAGAA
AGTGGAAGATTTGTTCGAATTACAATCAATCGAAGTTTATTTGTCAAAATAA
AAAAATGAAATCTTTGAACTACTCCACAAATTAATTGAGCAAGCGGTTAAA
TTTACAAATTTTTTTGCAAAATCAACCGCTTTCCATTAGAATTATAAGACTTT
TGGGGCTGATTCTGGATTCGACGGGATTAGCGAAGTCCAAGGTGCACGTCG
AGGTGCGGTAGGCCTCGTAAACAAACCGCAAAAAAATAGTCGCAAACGAC
GAACAATACGCTTTAGCAGCTTAATAACCTGCTCATAGCCTTATCGCCTCAG
CTTCCGCTCGTAAGACGAGGGCAACGATAAGTTACCCAAAACGAGATCGTG
TGGACGCCGCCGTTTGAGGATCGAAACACTAAATTGAATCAAACTAGCTTA
TTTCTTGCGTGTCTGTCCGCTGGAGGTAAGTGAAATTAAAGACCAGACTAAA
CGTGTAGTGCTGAAGATGGAGTAATTTCGGACGGGGGTTCAAATCCC*CCCA*
GCTCCACCA<u>*AATTTGTATACCGCCATTATGCATAC*</u>TACGATTCACATATTTTGTA
CAGTTAAAATAGAGGAACTAAATTGAGACCTTTAAATTCATAACTATAGTG
TACAATACACACAGTCCATTAACCAAAATAAAAGGAGGAATTAGGATGAAC
TTTAATAAAATTGATTTAGACAATTGGAAGAGAAAAGAGATATTTAATCAT
TATTTGAACCAACAAACGACTTTTAGTATAACCACAGAAATTGATATTAGTG
TTTTATACCGAAACATAAAACAAGAAGGATATAAATTTTACCCTGCATTTAT
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AATAGCGACGGAGAGTTAGGTTATTGGGATAAGTTAGAGCCACTTTATACA
ATTTTTGATGGTGTATCTAAAACATTCTCTGGTATTTGGACTCCTGTAAAGA
ATGACTTCAAAGAGTTTTATGATTTATACCTTTCTGATGTAGAGAAATATAA
TGGTTCGGGGAAATTGTTTCCCAAAACACCTATACCTGAAAATGCTTTTTCT
CTTTCTATTATTCCATGGACTTCATTTACTGGGTTTAACTTAAATATCAATAA
TAATAGTAATTACCTTCTACCCATTATTACAGCAGGAAAATTCATTAATAAA
GGTAATTCAATATATTTACCGCTATCTTTACAGGTACATCATTCTGTTTGTGA
TGGTTATCATGCAGGATTGTTTATGAACTCTATTCAGGAATTGTCAGATAGG

CCTAATGACTGGCTTTTATAACAAG*CGGTTTGGCATA*<u>*AAAAAATCCCTTGTCCC*</u>
<u>*CAGCTCCACCAAA*TTTGTATAC</u>TTTTAATAGGTTATTTAAAATGAAATTCCAC
CAAAAACTAAATAGAACTGCCAAAACTGCAGTGGCTATTGCACTTAGTAGT
TTTGGTGTATTAGATATGTCTTCATTAGATAATGTCACTCAATCTATTGAAA
ATATAAGAATATTATCCTCTCAAAAAAATAATATTAATTTTGTTTTCTTAAA
CGTTAAAGAATTATCGAAAGAGATAGAAAATATTAGCCAAGCGATTAATGC
TAATTTAGATAAAGTTAATATAAAAGAATTTAATCATTTAGTATTAATTAAT
CAACTTATCAATTTTTCATTTTATATGTTGAAAAATGAATATAAAACGCAAA
TCCTCTCTAACTCTGAATATAAATTAAGTTTTAGATATTTTTCTAACGCTAAA
AATAACTTTGAGAATTCTTTACAACGTATCAAAGAAAATATTAATGGTGTTG
ATGTTGTAACCATAAAAGAT

DNA cassette for complementation of the *A. pleuropneumoniae* Δ*arfA* strain constructed by Eurofins

Green: *arfA* gene; Yellow: cat gene ; Blue: APP DUS; Orange: Primer 3; Purple: Primer 4.

CAAGGTATCGAACGTTGGCGATATATTATCAACGTATTTACCCGTATGCGTT
TTTGCTATGCCGACAAGCGGTTGGATTTTGCTTGTAAATTACCTGTTGAAGA
CGCACCAAACGAATTAAAACCTTGGTTTAAATTAGATAATCCGTTATTTCAT
CAACAAGACATTATTTTCGGTCATTGGGCAAGTTTAATGGGTAAAGCGGAT
AAACCGAATATCTACGCCTTAGACACCGGCTGTGCTTGGGGTAACCATCTCA
CCATGATCCGATGGGAAGATAAACAAATTTTCACCCAAGAACGCTTAAAAT
AAAATCAAACGGAATACGGGGTCTTGCTATCCTCCGCATTTTCGATTATAAT
TCTTCCCATCTATGCGACTATAGCTCAGTTGGTTAGAGCACCACCTTGACAT
GGTGGGGGTCACTGGTTCGAGTCCAGCTAGTCGCACCATAAAATTCCAATC
CCTTGTCGAAAGACAAGGGATTTTTTTATGCCTTATAAAAGCCAGTCATTAG
GCCTATCTGACAATTCCTGAATAGAGTTCATAAACAATCCTGCATGATAACC
ATCACAAACAGAATGATGTACCTGTAAAGATAGCGGTAAATATATTGAATT
ACCTTTATTAATGAATTTTCCTGCTGTAATAATGGGTAGAAGGTAATTACTA
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