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Programa de Pós-Graduação em Biotecnologia

Dissertação

Compostos indutores e genes regulando a expressão da bomba de efluxo Tap em *Mycobacterium bovis* BCG

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Pelotas, 2013

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Compostos indutores e genes regulando a expressão da bomba de efluxo Tap em *Mycobacterium bovis* BCG

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“A vida é assim mesmo. Para aqueles que não buscam nada, os dias são monotonamente tranquilos. Mas para os que buscam o mundo, os dias passam rapidamente, as vezes num ritmo caótico. Minha orientação para ti, que vives agora o turbilhão de compromissos e sentimentos, causados pelo teu sucesso, é a mesma de antes. Segue lutando…”

Silva P.E.A., 2012

RESUMO

FELIX, Carolina Rodrigues. **Compostos indutores e genes regulando a expressão da bomba de efluxo Tap em *Mycobacterium bovis* BCG**. 2013. 51f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Proteínas transportadoras relacionada ao efluxo de drogas desempenham um papel importante não só na aquisição de fenótipos resistentes aos fármacos, mas também na virulência de *M. tuberculosis*. O principal objetivo deste estudo foi analisar a regulação do gene *Rv1258c* codificador da proteína Tap considerando a expressão dos genes *Rv1255c* e *Rv1257c*. RNA foi extraído a partir de culturas de *M. bovis* BCG superexpressando *Rv1255c* e *Rv1257c,* bem como de uma cepa controle contendo o vector pVV16 realizando o qRT-PCR do gene *Rv1258c*. RT-PCR foi realizada com RNA previamente isolado de *M. tuberculosis* CDC1551, a fim de verificar se o *Rv1255c*, *Rv1256c*, *Rv1257c* e *Rv1258c* genes estão todos ligados em um transcrito. A cepa *M. bovis* BCG, expressando uma proteína fluorescente vermelha sob o controle do promotor do *Rv1258c* (pVVTapPro) foi cultivado na presença de estreptomicina e glicina para verificar a indução deste promotor. O qRT-PCR demonstrou uma regulação negativa do gene *Rv1258c* na cepa sobre expressando *Rv1257c*, mas não foi observada diferença na cepa sobre expressando *Rv1255c* em comparação com o controle. Um aumento de fluorescência foi observada na cepa contendo o promotor da Tap na presença de 1 mg/L de estreptomicina, em comparação com o controle. Uma indução de duas vezes do gene Tap foi também observada na presença de glicina 1,6 mM. Os resultados sugerem um papel para o gene *Rv1257c* na regulação da expressão Tap. Além disso, a indução da Tap por estreptomicina apoia a importância desta proteína na multidroga-resistência de *M. tuberculosis*. O efeito da glicina no promotor da Tap sugere um papel fisiológico de detoxificação celular para essa bomba de efluxo. Em conclusão este estudo reforça a necessidade de se obter um conhecimento mais aprofundado a respeito do operon da proteína Tap e sua regulação, de maneira a auxiliar no desenvolvimento de inibidores dessa bomba de efluxo e possivelmente de outros mecanismos de resistência induzida.

Palavras-chave: tuberculosis, resistência, efluxo.

ABSTRACT

FELIX, Carolina Rodrigues. **Inductors and genes that regulate expression of the Tap efflux pump in *Mycobacterium bovis* BCG**. In 2013. 51f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Transport proteins related to drug efflux play an important role not only in the acquisition of drug-resistant phenotypes, but also in virulence of *M. tuberculosis*. The main objective of this study was to analyze the regulation of genes encoding the protein Rv1258c Tap considering the expression of genes Rv1255c and Rv1257c. RNA was extracted from cultures of *M. bovis* BCG overexpressing Rv1255c and Rv1257c, as well as a control strain containing the vector pVV16, and qRT-PCR of the Rv1258c gene was performed. RT-PCR was performed using RNA isolated previously from *M. tuberculosis* CDC1551, in order to verify that Rv1255c, Rv1256c, Rv1257c and Rv1258c genes are all connected to a transcript. The strain *M. bovis* BCG, expressing a red fluorescent protein under control of the promoter Rv1258c (pVVTapPro) was grown in the presence of streptomycin and glycine to verify the induction of the promoter. The qRT-PCR showed a downregulation of the gene in the strain overexpressing Rv1258c and Rv1257c, but no difference was observed in the strain overexpressing Rv1255c compared with the control. An increase in fluorescence was observed in the strain containing the promoter of the tap in the presence of 1 mg/L of streptomycin in comparison with the control. A two-fold induction of gene Tap was also observed in the presence of 1.6 mM glycine. The results suggest a role for Rv1257c in the regulation Tap expression. Moreover, the induction of Tap by streptomycin supports the importance of this protein in multidrug-resistant *M. tuberculosis*. The effect of glycine on Tap promoter suggest a physiological role in cellular detoxification for this efflux pump. In conclusion, this study reinforces the need to obtain a deeper knowledge about the Tap protein operon and its regulation, in order to assist in the development of inhibitors of this efflux pump and possibly other mechanisms of induced resistance.

Keywords: tuberculosis, drug resistance, efflux.

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1. INTRODUÇÃO

Atualmente, estima-se que 8,7 milhoes de novos casos de tuberculose (TB) ocorram por ano no mundo. Esse fardo é agravado pelo crescente número de casos de tuberculose multi-droga resistente (TB-MDR), o qual chega a quase 4% do número total de casos anuais (~0,5 milhões de novos casos/ano). Segundo a Organização Mundial da Saúde, 60% dos novos casos de TB-MDR em 2011 ocorreram em cinco países, entre eles, o Brasil. (OMS, 2012)

Os bacilos alcool-ácido resistentes que pertencem ao complexo *Mycobacterium tuberculosis* são os notórios patógenos causadores desta infecção, predominantemente pulmonar. Uma característica clássica da fisiopatogenia da TB é a formação do granuloma que ocorre como consequencia da resposta imune do hospedeiro ao bacilo de Koch (Gengenbacher & Kaufmann, 2012).

O granuloma consiste em um aglomerado de células do sistema imune no sítio de infecção, sendo marcado particularmente, pela presença de macrófagos. Tradicionalmente o granuloma é descrito como uma estrutura de proteção do hospedeiro, que seria formada no sentido de limitar a disseminação de bacilos. Entretanto, recentemente tem sido relatado que o *M. tuberculosis* poderia promover a formação dessa estrutura para seu próprio benefício. Através da proteína ESAT- 6 (*6KDa early secretory antigentic target)*, o *M. tuberculosis* induz a secreção da mataloproteinase – 9 da matriz extracelular (MMP9) de células

epiteliais, acarretando no recrutamento de macrófagos ao sítio da infecção. Além disso, há evidências que a bacteria modula a formação do granuloma, retardando a montagem de uma resposta imune adaptativa e recrutamento de linfócitos T secretores de interferon-γ (IFN- γ) que ativa os macrófagos intensificando a atividade antimicrobiana. Desta forma, é possível que os estágios iniciais do granuloma sejam, na verdade, permissivos à proliferação e disseminação do *M. tuberculosis* no pulmão do hospedeiro (Tsai et al., 2006, Russell, 2007, Ramakrishnan, 2012, Silva Miranda et al., 2012, Shaler et al., 2013).

Estima-se que apenas 10% dos indivíduos infectados pelo *M. tuberculosis* irão desenvolver a doença ativa, enquanto nos 90% restante a bacteria pode ser eliminada completamente do organismo, ou entrar em um estado de dormência, e permanecer por anos no hospedeiro sem manifestar quaisquer sinais e/ou sintomas (Cardona & Ruiz-Manzano, 2004, Chao & Rubin, 2010, Gengenbacher & Kaufmann, 2012).. Durante esse estágio o *M. tuberculosis* reduz drasticamente a sua atividade metabólica e replicativa (Wayne & Hayes, 1996). Ocorre também espessamento da parede celular do bacilo, acúmulo de partículas lipídicas no citosol da bactéria e perda gradual da álcool-ácido resistência. A característica talvez mais importante desenvolvida pelo bacilo na fase de latência é a tolerância aos antmicrobianos de primeira linha rifampicina (RIF) e isoniazida (INH) (Garton et al., 2008, Deb et al., 2009) Baek e colaboradores (Baek et al., 2011) demonstraram que o bacilo passa a acumular triacilglicerol através da atividade da enzima triacilglicerol sintase 1 (tgs1) a qual sequestra acetil-CoA do ciclo do ácido cítrico.

A reduzida atividade replicativa do microrganismo durante a fase quiescente é, ao menos parcialmente, responsável pela antibiótico-tolerância adquirida, e vem sendo intensamente estudada. Entretanto, existem outros mecanismos envolvidos

com a resistência aos antimicrobianos, como a inativação das drogas por enzimas microbianas, alteração de permeabilidade e o efluxo ativo de farmacos que também podem desempenhar um papel importante neste fenótipo de resistência (Silva & Palomino, 2011).

Nos isolados clínicos de *M. tuberculosis* a resistência adquirida aos antimicrobianos ocorre essencialmente devido a mutações no gene codificador do alvo ou ativador do pró-fármaco. As mutações encontradas com maior frequência nas bactérias resistentes à RIF estão concentradas em uma região de 81 pares de bases do gene *rpoB* codificador da subunidade beta da enzima RNA polimerase (Spies et al., 2013; Silva & Palomino, 2011). Em relação a pró-droga INH, mutações no gene *katG*, codificador da catalase-peroxidase, são bastante abundantes, essa enzima realiza a ativação da INH. O alvo de ação da INH ativa é a enzima codificada pelo gene *inhA*, envolvida na extenção de ácidos graxos, fazendo parte do complexo enzimatico da ácido graxo sintase II (FAS-II). Mutações nesse gene, e em seu promotor também são encontradas em cepas INH-resistentes. Entretanto, as mutações no *katG*, são bem mais frequentes, provavelmente por conferirem um menor custo biológico à bacteria (Banerjee et al., 1994, Pym et al., 2002).

O alto grau de resistência conferido por mutações genômicas ocorre geralmente após o bacilo ser exposto à concentrações subinibitórias dos antimicrobianos. No entanto, é importante considerar que esses microrganismos são particulamente difíceis de eliminar do organismo mesmo quando sensíveis aos antibióticos. Isso é devido, em parte, a resistência intrínseca do *M. tuberculosis* a vários antimicrobianos, a qual é conferida principalmente por uma barreira de permeabilidade (parede celular micobacteriana). Além disso, alguns mecanismos intrinsecos a bactéria regulam funções fisiológicas em resposta a ambientes hostis.

Dessa forma, estes também contribuem para a aquisiçao de genótipos e consequentes fenótipos de resistência clinicamente detectável. As etapas anteriores ao desfecho de resistência clínica envolvem complexos circuitos de regulação da expressão gênica, que atuam permitindo ao microrganismo perceber e produzir resposta à danos provocados por antimicrobianos.

As proteínas WhiB, aparentemente exclusivas dos Actinomicetos, são reguladores de expressão gênica contendo um domínio conservado de ligação à regiões ricas em A/T no DNA. O regulador transcricional whiB7 está relacionado a resistência a vários fármacos com mecanismos de ação e estruturas bastante diversificadas. A expressão da WhiB7 é induzida na presença de tetraciclinas, aminoglicosídeos e lincosamidas. Além disso, foi demonstrado que esse regulador é sobre-expresso em diversas outras condições como por exemplo, após choque térmico, em meio com baixa quantidade de ferro ou em um ambiente redutor. Isso sugere que o whiB7 possui funções fisiológicas para a bactéria além de promover antibiótico-resistência (Morris et al., 2005, Burian et al., 2012).

Ainda que o mecanismo de resistência intrínseca relacionado ao gene *whiB7* não esteja completamente esclarecido, sabe-se que a indução desse regulador não é devido a interação do promotor do gene com o fármaco em si, mas sim em resposta à stress celular decorrente da sua atividade antimicrobiana. Sabe-se também que a resistência intrínseca ocorre devido a outras proteínas, cuja expressão é ativada por este regulador transcricional. Três genes com expressão induzida pelo WhiB7 possuem funções diretamente vinculadas à resistência: Rv2416c que codifica para a proteína Eis; *Rv1988*, codifica para a proteína Erm e o

gene *Rv1258c*, codificador da bomba de efluxo Tap, a qual é o foco do presente estudo. (Morris & Nguyen et al., 2005, Geiman et al., 2006)

As bombas de efluxo são proteínas envolvidas no transporte ativo de substâncias através da membrana. Esses sistemas vêm sendo estudados, pois possuem afinidade por um amplo espectro de antimicrobianos. Essas proteínas são divididas em dois grandes grupos: os transportadores da família ABC (ATP-*binding-cassette*) os quais tem como fonte a energia livre proveniente da hidrólise do ATP; e os transportadores secundários, os quais utilizam o gradiente eletroquímico de prótons para extrusão de drogas. Este grupo está subdividido em 4 famílias: MFS (*Majos Facilitator Family*), RND (*Resistance-nodulation Division*), MATE (*Multudrug an toxic compound extrusion*), e SMR (*Small Multidrug Resistance*) (Putman et al., 2000, Li & Nikaido, 2009).

Sistemas de efluxo foram descritos em bactérias gram-positivas, gram-negativas, micobactérias e células eucariotas (Nikaido, 1998). Diversos sistemas de efluxo já foram descritos em *M. tuberculosis,* a maioria conferindo baixos níveis de resistência a uma gama variada de antimicrobianos.(De Rossi et al., 2002; Louw *et al.,* 2009*;* Silva *et al.,* 2011). Dentre aquelas descritas em micobactérias estão as bombas de efluxo Tap, P55 e Lfra. As bombas Tap, descrita em *Mycobacterium fortuitum* e *M. tuberculosis*, e P55 descrita em *M. tuberculosis*, conferem resistência a aminoglicosídeos (De Rossi & Arrigo et al., 2002). Em *M. smegmatis* o gene *lfra* codifica para uma bomba de efluxo a qual confere resistência a fluoroquinolonas (Liu et al., 1996).

A relevância desses sistemas para o *M. tuberculosis* tem sido amplamente discutida, visto que seu papel na resitencia clínica do bacilo ainda precisa ser

esclarecido . Recentemente foi demonstrado que o efluxo de drogas em *M. tuberculsis* pode estar produzindo níveis subinibitórios de farmacos como INH dentro da células micobacterianas. Dessa forma, o efluxo favoreceria o surgimento de mutações nos alvos da INH e consequentemente, de resistência completa a esse antibiótico de primeira linha (Machado et al., 2012).

O foco maior de atenção as bombas de efluxo é devido à propriedade bem caracterizada dessas proteínas em fazer a extrusão ativa de antimicrobianos e portanto conferir resistência aos mesmos para a bactéria. No entanto, alguns estudos vem descrevendo outras funções dessas proteínas, sugerindo uma relevância para esses sistemas na sobrevivência e interação do patógeno com o hopedeiro. BacA é uma bomba de efluxo de *M. tuberculsis* codificada pelo gene *Rv1819* a qual não produz resistência a farmacos, mas possui um importante papel no estágio latente da TB. Em um estudo com camundongos, observou-se sobrevivência por um período mais longo dos animais infectados com a cepa de *M. tuberculosis* contendo deleção do gene *Rv1819* do que daqueles infectados com a cepa parental (Domenech et al., 2009).

A bomba de efluxo P55, codificada pelo gene *Rv1410c*, foi inicialmente caracterizada por Silva e colaboradores como um transportador secundário da família MFS (*Major Facilitator Superfamily*) conferindo resistência a tetraciclina e aminoglicosídeos, entre eles a estreptomicina. A concentração mínima inibitória dessas drogas (estreptomicina e tetraciclina) aumentou oito vezes quando a P55 foi sobre-expressa em *M. smegmatis* em relação a cepa parental. Outros estudos demonstraram que a deleção dessa bomba de efluxo em *M. bovis* BCG torna essa bactéria mais sensível à rifampicina que a cepa parental. Funções não relacionadas à resistência também foram descritas para essa bomba de efluxo. A cepa de *M.*

bovis BCG contendo a deleção da P55 possui uma desvantagem de crescimento quando cultivada em meio líquido, e é mais sensível à alguns compostos redutores. Além disso, Zhu e colaboradores observaram a forte atividade imunogênica de peptídeos derivados da proteína P55, sugerindo que esses são bons candidatos para produção de uma vacina de subunidade contra TB (Silva et al., 2001, Ramon-Garcia et al., 2009, Zhu et al., 2011).

A proteína Tap de *M. tuberculosis* tem sido vinculada a fenótipos relevantes para diversos aspectos tanto de infecção quanto de resistência a fármacos. Esta foi primeiramente descrita em *M. fortuitum* como uma bomba de efluxo dependente do gradiente de prótons para a sua atividade. A Tap do *M. fortuitum* tem aminoglicosideos e tetraciclina entre seus substratos conhecidos. Um alto grau de homologia existe entre as proteínas Tap de *M. fortuitum* e *M. tuberculosis*, sendo supostamente transportadoras de açucares da família MFS, e conferindo baixos níveis de resistência aos antimicrobianos mencionados acima. Alguns estudos realizados com cepas clínicas multidroga-resistentes de *M. tuberculosis* demonstraram maior expressão do gene *Rv1258c* na presença de rifampicina (Ainsa et al., 1998, Siddiqi et al., 2004, Jiang et al., 2008).

Ultimamente, tem se observado que a bomba de efluxo Tap provavelmente possui um impacto maior que o previsto no âmbito da infecção. Em um estudo recente, Adams e colaboradores observaram menor crescimento em macrófagos de cepas de *M. tuberculosis* contendo esse gene interrompido por um transposon. Notou-se também que tolerância a antimicrobianos como INH e RIF ocorre em *M. tuberculosis* em estágio replicativo dentro do macrófago, e não apenas em bacterias quiescentes. Nesse trabalho foi demonstrado que as bacterias se tornam tolerantes em resposta ao ambiente intramacrofágico. O mecanismo utilizado pelo *M.*

tuberculosis para que isso aconteça é através da atividade da bomba de efluxo Tap. Entretanto a antibiótico-tolerância devido a esse sistema de efluxo ocorreu apenas para RIF e não para INH (Adams et al., 2011).

A importância desse sistema de efluxo foi reforçada recentemente em um estudo com *M. bovis* BCG. Utilizando uma cepa de BCG contendo esse gene deletado do cromossomo, foi possível detectar menor crescimento durante a fase estacionaria, em relação a cepa parental. Além disso, diversos genes foram diferencialmente expressos durante essa fase de crescimento na cepa *knockout*. Baseado nesses resultados os autores sugerem que a proteína Tap pode desempenhar alguma função no estágio de dormência da bactéria. Uma hipótese a respeito da função fisiológica dessa proteína também foi esboçada, sugerindo que a Tap pode estar envolvida na extrusão de compostos tóxicos acumulados durante a fase estacionária, como aminoaçúcares advindos da biossíntese da parede celular bacteriana (Ramon-Garcia et al., 2012). No entanto, é importante notar que não foi realizada a complementação do gene *Rv1258c* na cepa knockout de *M. bovis* BCG . Isso deve ser considerado já que a deleção do gene *Rv1258c* pode ter afetado outros genes *downstream*, os quais poderiam ser os verdadeiros responsáveis pelos fenótipos observados nesse estudo.

Baseado nesta revisão da literatura, é possível observar que existe uma ampla quantidade de informação a respeito da bomba de efluxo Tap de *M. tuberculosis*, além de sua importância, como já mencionado, em diversos aspectos da TB. Neste sentido o melhor entendimento a respeito de sinais que regulam a expressão dessa bomba de efluxo vão ajudar a esclarecer o papel fisiológico dessa proteína. Além disso, essa informação poderia facilitar o desenvolvimento de inibidores não apenas para a Tap como também para outras bombas de efluxo. Os

inibidores atualmente disponíveis são tóxicos ao homem por terem alvos com forte homologia a proteínas e mecanismos existentes em eucariotos. Sendo assim, poder-se-ia buscar inibidores dos mecanismos regulatórios que induzem a expressão das bombas de efluxo . Dessa forma seriam específicos à sistemas de efluxo bacterianos, pouco tóxicos ao homem e agiriam reduzindo não só o efluxo como possivelmente outros mecanismos de resistência intrínseca. Isso poderia ser atingido através de compostos que possam inibir circuitos mais amplos de regulação gênica relacionada antibiótico-resistência, como o do regulador *whiB7*.

A expressão do gene tap/*Rv1258c* é ativada pelo regulador transcricional WhiB7, cepas que sobre-expressam o *whiB7* também apresentam maior expressão do *Rv1258c* e consequente resistência a estreptomicina (Reeves et al., 2013). O fato de que a indução do *whiB7* antecede a do *Rv1258c* (assim como outros genes) após o *M. tuberculosis* ser exposto a antimicrobianos, é mais um indicativo de que a transcrição da bomba Tap está sob o controle do regulador transcricional *whiB7* (Morris & Nguyen et al., 2005).

O *M. bovis* BCG possui o gene *Rv1258c,* no entanto, os genes *Rv1257c, Rv1256c* e *Rv1255c* estão deletados nessa bactéria, fazendo parte da RD13. Resultados não publicados por Silva e colaboradores, demonstraram que a expressão da bomba de efluxo Tap é mais elevada no *M. bovis* BCG quando comparado ao *M. tuberculosis*. Esses resultados sugerem que algum repressor da expressão dessa bomba de efluxo está ausente do cromossomo do *M. bovis* BCG. O gene *Rv1255c* codifica para um suposto regulador transcricional (Cole et al., 1998), o qual poderia estar desempenhando esta função. Baseado nisso é possível inferir que o gene *Rv1258c* tem sua expressão inibida pelo regulador transcricional codificado pelo *Rv1255c*. Alternativamente, a inibição poderia estar ocorrendo

através de um mecanismo resultante da atividade dos genes *Rv1257c* ou *Rv1256c,* também ausentes do cromossomo do *M. bovis* BCG. O gene *Rv1256c* não foi testado pois codifica para um enzima bem caracterizada da familia P450, e possui uma função não relacionada à bomba de efluxo Tap.

O objetivo geral deste trabalho foi estudar a regulação do gene que codifica para a bomba de efluxo Tap (*Rv1258c*) de *M. tuberculosis,* considerando a expressão dos genes *Rv1255* e *Rv1257*, além de caracterizar a organização desses genes no cromossomo de *M. tuberculosis*.

Objetivos Específicos

1 – Verificar se a expressão da proteína fluorescente vermelha varia sob o controle do promotor do gene *Rv1258c* na presença de compostos indutores.

2 – Verificar os níveis de expressão do gene *Rv1258c* em *M. bovis* BCG artificialmente expressando os genes *Rv1257c* e *Rv1255c* ou não.

3 – Verificar se os genes *Rv1255c, Rv1256c, Rv1257c, Rv1258c* estão conectados eem um mesmo mRNA através de RT-PCR

Artigo

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Inductors and genes regulating Tap efflux pump expression in *M. bovis* BCG

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ABSTRACT

Efflux proteins are thought to play a role not only in the acquisition of drug-resistant phenotypes but also in virulence of *M. tuberculosis.* The main goal of this study was to analyze the regulation of the Tap gene considering the expression of the *Rv1255c* and *Rv1257c* genes. RNA was extracted from cultures of *M. bovis* BCG overexpressing *Rv1255c* and *Rv1257c* as well as a control strain containing the vector pVV16. qRT-PCR of the *Rv1258c* gene was performed. RT-PCR was performed on RNA previously isolated from *M. tuberculosis* CDC1551 in order to verify if the *Rv1255c, Rv1256c, Rv1257c* and *Rv1258c* genes are all linked in one transcript. A *M. bovis* BCG strain expressing a red fluorescent protein under the control of the *Rv1258c* promoter (*pVV*Tap*Pro*) *was* grown in the presence of Streptomycin and Glycine to verify the induction of this promoter using a fluorescent readout. The qRT-PCR showed a two-fold downregulation of *Rv1258c* in the *Rv1257c* over expressing strain, but no difference was observed in the *Rv1255c* over expressing strain in comparison to the control. An increase in fluorescence was observed in the Tap promoter reporter strain in the presence of 1mg/l streptomycin in comparison to the control. A two-fold induction of the Tap gene was also observed in the presence of 1.6mM glycine. The data from this study suggests a role for the *Rv1257c* gene in the regulation of Tap expression. Also, induction of Tap by streptomycin supports the importance of this protein in inducible drug resistance of *M. tuberculosis.*

ABREVIATURAS

NOMES DAS CONSTRUÇÕES

INTRODUCTION

Mycobacterium tuberculosis is estimated to cause disease in approximately 9 million people every year. To further worsen this burden, these bacteria are becoming increasingly drug resistant. The multidrug-resistant (MDR) strains of *M. tuberculosis* are, by definition, resistant to at least isoniazid (INH) and rifampicin (RIF), two front line drugs used to treat tuberculosis (TB)(1).

The most common mechanism by which *M. tuberculosis* becomes resistant to antibiotics is through drug target modification. Acquiring and accumulating mutations in the genome is a process occurring over a certain period of time. There are several intrinsic mechanisms of drug resistance that are known to come into play in the stages preceding the development of resistance phenotype(2). Recent data by Machado and coworkers suggested that active efflux is contributing to the development of these resistant mutants. By extruding compounds from the intracellular space, this mechanism generates sub inhibitory concentrations of drugs inside the mycobacterial cell. This allows them to grow even in the presence of the antimicrobials, thus favoring the highly resistant mutant genotypes which arise during bacterial replication(3).

Bacterial efflux pumps are trans membrane proteins which are known to confer resistance to a wide variety of antimicrobial compounds(4, 5). In *M. tuberculosis* many of these proteins have been described, most of them conferring low levels of drug resistance. Moreover, some have been shown to have a variety of roles in infection and virulence of the bacillus(6).

The Tap efflux pump of *M. tuberculosis,* coded by *Rv1258c*, was first described in *M. fortuitum* with aminoglycosides and tetracycline as known substrates. This protein is a sugar transporter which uses the proton-motive force for its activity (7, 8).

The relevance of the Tap efflux pump has been broadened since a study by Adams and coworkers showed decreased growth of a Tap transposon mutant of *M. tuberculosis* infecting macrophages(9). Also, in *M. bovis* BCG it was shown that Tap mutants have defective growth during stationary phase, suggesting a possible role

for this efflux pump in latency(10). Furthermore, this efflux pump is induced in the presence drugs such as rifampin and isoniazid in multidrug-resistant strains(11, 12).

The Tap gene is organized in a putative two-gene operon with *Rv1257c*, an uncharacterized oxidoreductase. Also, upstream are the genes *Rv1256c,* coding for an enzyme in the Cytochrome P450 superfamily*,* and *Rv1255c,* a putative transcriptional regulator(13)*.* In *M. bovis* BCG these three genes (*Rv1257c-Rv1256c-Rv1255c*) are deleted from the chromosome as a part of RD13. Unpublished data by Silva and coworkers showed that Tap expression is higher in *M. bovis* BCG than in *M. tuberculosis*. Considering this data we hypothesized that one of the deleted genes are affecting the expression of *Rv1258c* causing the observed down regulation in *M. tuberculosis*.

In this work we establish the correlation between these four genes and also their importance in the regulation of the Tap efflux pump gene. Morris and colleagues (ANO) have shown that the expression of *Rv1258c* is activated by the transcriptional regulator WhiB7 in response to antibiotics such as streptomycin and tetracycline(14). A better understanding of the regulation of *Rv1258c* expression could aid the development of more effective and specific efflux inhibitors than the ones currently available, which are toxic to humans. By targeting the expression of these genes and not the protein itself, the issue of homology with human systems, and consequent toxicity of compounds, would be minimized. The main goal of this study was to analyze the regulation of the Tap gene considering the expression of the *Rv1255c* and *Rv1257c* genes.

MATERIALS AND METHODS PADRONIZAR UNIDADES

Bacterial Strains

M. bovis BCG Pasteur was grown at 37°C on Middlebrook 7H9 enriched with 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.05% Tween 80 or in solid media Middlebrook 7H10 OADC. *M. smegmatis* mc^2 was cultured on LB 1.5% Agar plates or in LB Broth containing 0.05% Tween 80. *Escherichia coli* was cultivated in LB or LB Agar plates. In order to cultivate bacterial strains containing plasmids, 250mg/L Hygromicin or 50mg/L Kanamycin were added to the media depending on the vector.

Plasmid Constructs

In this study all the inserts were cloned into the shuttle vector pVV16 which contains the groEL promoter from *M. tuberculosis* (15). A derivative of this plasmid, pVVmCh, containing RFP under the control of the Smyc promoter was used to build the promoter fusions. The Rv1255c gene was amplified using primers on Table 1, digested with *BamHI* and *HindIII* restriction enzymes (New England Biolabs® Inc., Ipswich, MA, USA) and ligated with linearized pVV16 vector using FastLink™ DNA Ligation Kit (Epicentre®, Madison, WI, USA) following manufacturer instructions. The pVV1257c and pVVmCh:Tap*Pro* constructs were built using a digest- and ligation-independent method known as FastCloning(16). FastCloning entails Phusion High Fidelity DNA Polymerase (ThermoFisher Scientific®, Waltham, MA, USA) PCR amplification of the entire pVV16 vector (minus the insert) and the inserts (obtained from the chromosome of *M. tuberculosis*), which are verified on a gel, mixed together at various ratios, digested with *DpnI* (New England Biolabs® Inc., Ipswich, MA, USA) to remove parental DNA templates, and transformed directly into *E. coli*. Briefly, PCR reactions were performed using 100ng of chromosomal DNA (for insert amplification), or 10ng of vector DNA, 0.2mM dNTP, 100ng forward and reverse primers and Phusion High Fidelity DNA Polymerase (ThermoFisher Scientific®,

Waltham, MA, USA); denaturing and extension cycles were performed according to Phusion HF DNA Polymerase manufacturer instructions. Annealing temperatures were calculated using Phusion HF DNA Polymerase calculator. The genes *Rv1255c, Rv1257c* were cloned into pVV16 under the control of the groEL promoter. The promoter of *Rv1258c* (Tap*Pro*) was obtained from the chromosome of *M. tuberculosis* by amplifying a 500bp fragment immediately upstream of *Rv1258c*. The primers used in this work and the constructs obtained are summarized in Table 1. All the constructs were also transformed in *M. smegmatis* and *M. bovis* BCG.

Western Blotting

Cultures of *M. smegmatis* mc^2 containing pVV1255 and a wild type strain were used to extract protein for western blotting. Bacterial pellets were sonicated in 2ml lysis buffer (20Mm KPO4; pH 7,4; 0,5mM MgCl2; 0,5mM CaCl2; 1X Inibidor de Protease). Twenty five µL of each sample was loaded on a 10% poliacrilamide gel, and transferred onto an Immbilon-P Polyvyniidene Fluoride (PVDF) transfer membrane (45µm) (©EMD Millipore Corporation, Billerica, MA, USA). The rabbit polyclonal Anti-6xHIS tag® antibody conjugated with HRP (Abcam®, Cambridge, MA, USA) and the CN/DAB substrate (ThermoFisher Scientific®, Waltham, MA, USA) were used to develop the western blot.

RNA extraction and Quantitative Real Time RT-PCR (qRT-PCR)

Mycobacterial RNA was isolated from broth cultures of three *M. bovis* BCG strains containing different constructs: 1) BCGpVVmCh, used as a control for the experiment; 2) BCGpVV1255c and 3) BCGpVV1257c. Pellets from cultures in log phase were washed in GTC buffer followed by a wash with PBS-Tween 80 0.1%, and then treated with 5mg/mL Lysozyme for 15 minutes at room temperature. After

this step 0.75mL of TRIzol was added and the mixture was transferred to screw-cap tubes containing ~0.5mL of 0.1mm glass beads, tubes were placed in the MiniBeadBeater (Biospec Products Inc., Bartlesville, OK, USA) and beat for 2min at maximum speed. Chloroform (0.2mL) was then added to the tubes, which were inverted for mixing. Mixture was centrifuged for 15 minutes at 13,300rpm at room temperature. The upper aqueous phase was removed, placed in a RNAse free tube and 0.5mL of 100% RNAse free ethanol was added. The following procedures were done using the Qiagen RNAeasy Miniprep kit (Qiagen®, Germantown, MD, USA) according to manufacturer instructions.

For the qRT-PCR, 100ng off RNA was used per reaction which was performed using iTaq and iScript SYBR Green reagents (Bio-Rad®, Hercules, CA, USA). Forward and reverse primers targeting the Tap gene were used for these experiments (Table 2). The data was normalized using expression levels obtained for the constitutive gene *sigA*. All reactions were performed in triplicate and the $2^{-\Delta\Delta CT}$ method was utilized to calculate relative gene expression.

RT-PCR

Reverse transcriptase PCR was performed using RNA previously isolated from *M. tuberculosis* H37Rv in order to characterize the genomic disposition of the genes *Rv1258c, RV1257c, Rv1256c* and *Rv1255c.* RT-PCR was carried out using iTaq reagent (Bio-Rad®, Hercules, CA, USA) to obtain cDNA; PCR reactions were performed using Phusion High Fidelity DNA Polymerase (ThermoFisher Scientific®, Waltham, MA, USA), 100ng of cDNA and primers targeting three intergenic regions between: (i) *Rv1258c* and *Rv1257c,* (ii) *Rv1257c* and *Rv1256,* (iii) *Rv1256c* and *Rv1255c* (Table 2). Controls were performed using the same primers however;

reverse transcriptase was not added to the reaction. A schematic view of the genes and position of the primers is shown in Figure 5.

Promoter Induction Assays

In order to assess the responsiveness of the Tap promoter to different conditions (streptomycin and glycine exposure) the *M. bovis* BCG strain containing pVVTapPro was cultured in black clear bottom 96 well plates. For the experiments with BCG broth cultures were grown cell culture flasks containing 7H9 broth with 250mg/L Hygromycin. Cultures were then diluted in 7H9 broth without antibiotics, 0.18ml of culture at an optical density (600nm) of 0.1 was added to each well and 0.02mL of drugs to final concentrations of: Streptomycin (1mg/L, 0.9mg/L, 0.2 mg/L, 0.04mg/L, 0.008 mg/L); Glycine (200mM, 40mM, 8mM and 1.6mM). Cultures were treated for 4 to 5 days; fluorescence as well as the OD_{600} was measured every day. The fluorescence measurements were taken using wave lengths of 580nm and 615nm for excitation and emission respectively. Fluorescence values were normalized using OD_{600} values and plotted on graphs. All treatments and controls were performed in triplicate on the 96 well plates.

Analise Estatística

The data from these experiments was analyzed using Repeated-measures two-way ANOVA.

RESULTS

Overexpressing *Rv1257c* in *M. bovis BCG* causes down regulation of Tap

In order to verify the role of *Rv1257c* and *Rv1255c* in the transcriptional regulation of Tap, these genes were cloned into a shuttle vector under the control of

187 the constitutive Hsp60 promoter, in frame with a C-terminal His tag. The constructs
188 obtained were sequenced to confirm.

189 Broth cultures of *M. bovis* BCG strains containing these constructs were used to
190 extract RNA for qRT-PCR of the Tap gene. In the strain containing pVV*1255c*, no
191 significant difference (fold change of approximately -1) was observed in the
192 expression of Tap in comparison with the control (*M. bovis* BCG containing
193 pVVmCherry). Nevertheless, a two-fold down regulation of the Tap gene was
194 observed in the strain expressing *Rv1257c* when compared to the control (Figure 1).

195 Since there was no significant difference in expression of Tap in the *M. bovis*
196 BCG containing pVV1255, a western blot was performed to confirm that the Rv1255c
197 protein was being expressed. The plasmid pVV1255c was transformed into *M.*
198 *smegmatis*, protein extracts were obtained from this strain and the expression of the
199 His tagged protein was shown by Western Blotting (Figure 1).

200 Induction of the Tap promoter

201 A fluorescent read-out was utilized for the characterization of Tap inducers. A
202 construct containing the 500bp region immediately in front of the Tap gene was
203 cloned in front of a red fluorescent protein. The *M. bovis* BCG strain containing the
204 pVVmCh:TapPro construct was cultured in the presence of potential inducers of Tap
205 transcription. Sequencing data of this construct confirmed that Tap promoter had
206 been correctly cloned and the Hsp60 promoter had been deleted. Also, strains of *E.*
207 *coli, M. smegmatis* and *M. bovis* BCG containing this construct produced a red
208 fluorescent signal. This demonstrates that: the cloned region contains at least the
209 essential elements of a promoter required to drive transcription; the Tap promoter is

able to drive transcription not only in mycobacteria but also in the very different genetic background of *E. coli.*

The *M. bovis* BCG strain containing the TapPro construct was cultured in the presence of streptomycin and glycine at different concentrations and several fluorescence readings were taken over time to verify the induction of the promoter. A two-fold increase in fluorescence was observed over time for the BCG TapPro strain in the presence of 1.6mM and 8mM Glycine. Increased fluorescence was also observed with higher concentrations (200 and 40mM) at 24 hours but not on the later time points, probably due to toxicity associated with prolonged exposure to these concentrations (Figure 2). This induction profile was observed in two separate experiments.

Induction of the promoter was also observed with the streptomycin treatment. Nevertheless, concentrations of approximately 1mg/L were required in order to observe small changes in the fluorescence of the treated cultures in comparison to untreated controls. There was no difference between the untreated and cultures treated with low concentrations (0.2mg/L, 0.04mg/L and 0.008mg/L) of streptomycin (Figure 3).

Mapping of the Tap operon

Reverse transcriptase PCR of inter genic regions between Rv1255c and *Rv1256c, Rv1256c* and *Rv1257c, Rv1257c* and *Rv1258c,* was performed on RNA extracted from *M. tuberculosis* H37Rv. Amplification of all three fragments was observed in two separate experiments (Figure 4).

DISCUSSION

Although transcriptional regulator WhiB7 has been described as responsible for regulating Tap expression, other genes that could be affecting the expression levels of this efflux pump, as well as the conditions in which this gene is regulated are still poorly understood.

In this study we were able to further elucidate conditions and genes involved in the regulation of Tap expression. The qRT-PCR data generated in this work suggests that the putative oxidoreductase coded by the *Rv1257c* gene probably has effect on Tap expression. A two-fold down regulation of Tap was observed when *Rv1257c* was overexpressed in *M. bovis* BCG. Sequencing data has shown that *Rv1257c* encodes for putative oxidoreductase(13). Therefore it is highly unlikely that this protein is directly binding the Tap promoter to regulate its expression. A more plausible hypothesis is that the activity of this enzyme could be causing intracellular shifts sensed by the WhiB7 transcriptional regulator which, in turn controls the expression of Tap. The RT-PCR of the region between *Rv1257c* and *Rv1258c* on the *M. tuberculosis* showed that these two genes are linked in one transcript, as part of an operon. This suggests that they are regulated together.

The utilization of a reporter strain demonstrated that the Tap promoter is driving higher expression of its downstream gene in the presence of streptomycin. After prolonged periods of exposure (24h), small levels of induction were observed with concentrations as low as 1 and 0.9mg/L streptomycin. Interestingly, the transcriptional regulator WhiB7 is also induced in the presence of streptomycin and is known to activate the expression of Tap(14, 17).

The use of low concentrations of streptomycin provided small levels of Tap promoter induction. It is possible that higher concentrations of this drug, such as

257 those used previously (7.5mg/L), would have a more dramatic effect on the Tap
258 promoter, as was observed for WhiB7[21]. Low concentrations of this drug (0.2mg/L,
259 0.04mg/L and 0.008mg/L) did not cause detectable induction of the Tap promoter in
260 this work.

261 Kanamycin is another aminoglycoside inducing WhiB7(17), also previous
262 studies have shown that aminoglycoside antibiotics such as this one are substrates
263 of Tap(7, 18). Nevertheless, this aminoglycoside was not utilized as an inducer of
264 Tap in this study since the Tap promoter reporter strain contains a kanamycin
265 resistance gene coded in the pVVmCh:TapPro plasmid, thus making this strain
266 resistant to kanamycin.

267 Another interesting finding was that the Tap promoter is induced in the
268 presence of several concentrations of glycine. The induction reached more than two-
269 fold with 1.6mM glycine relative to the untreated control. Ramon-Garcia and
270 collaborators obtained gene expression profiles of a Tap deletion strain of *M. bovis*
271 BCG. Based on that data these authors suggest that this efflux pump could have a
272 detoxifying role for the bacteria. The mechanism for this is by extruding toxic
273 precursors of the peptidoglycan layer such as glucosamine-6-phosphate, which are
274 accumulated during stationary phase(10). Glycine treatment has been previously
275 demonstrated to cause inhibition of peptidoglycan synthesis and accumulation of
276 peptidoglycan precursors(19). Considering these previous findings, the glycine
277 induction data from this study is in agreement with the hypothesis that Tap has a
278 detoxifying role for the bacteria. Furthermore, amino sugars are known substrates of
279 this protein, which is also in agreement with this proposition.

In order to map the Tap operon, RT-PCR was performed to amplify the inter genic regions of the genes hypothesized to be part of this operon. The results obtained in this study suggest that the four genes (*Rv1255c, Rv1256c, Rv1257c* and *Rv1258c*) are linked in a single transcript, and thus are all part of one operon. Nevertheless, further experiments are required to confirm this hypothesis, such as northern blot analysis and sequencing of the transcripts.

On the other hand, bioinformatics analysis performed in the TB Database suggests that these four genes are in two separate operons, one containing *Rv1256c* and *Rv1255c,* and another containing *Rv1257c* and *Rv1258c* (Figure 5). ChipSeq data also available in the TB Database predicts a binding site for the putative transcriptional regulator *Rv1255c* in front of *Rv1256c* but not in front of the Tap gene (Figure 5). Furthermore, unpublished microarray data obtained with *M. tuberculosis* RNA by Rohde and coworkers suggests that *Rv1255c* and *Rv1256c* are being regulated in synchrony, but differently from *Rv1257c* and *Rv1258c.* Based on these data, an alternative hypothesis could be that the *Rv1256c* and *Rv1257c* genes are not linked, and the fragment amplified in this study is actually due to the presence of a leader sequence of *Rv1256c* which overlaps with the five prime end of the *Rv1257c* gene.

In conclusion, the observed induction the Tap promoter in the presence of streptomycin reinforces the relevance of this protein for inducible drug tolerance *M. tuberculosis* strains. This, in turn, strengthens the need for therapeutic strategies which use efflux inhibitors, in order to reduce treatment time. Moreover, the findings from this study suggest an important physiological role for Tap, further corroborating the hypothesis that this efflux pump is extruding toxic precursors of peptidoglycan synthesis. In this study we have also suggested a role for the *Rv1257c* gene in

indirectly causing down regulation of the Tap gene. Nonetheless, a more comprehensive understanding of the Tap operon is still required. This knowledge would aid in the development of inhibitors for this efflux pump as well as other inducible drug tolerance mechanisms, which would dramatically reduce treatment time for tuberculosis.

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382 **Tables**

383

384 **Table 1.** Summary of constructs used in this study.

Insert	Vector	Primers Sequence	Final Construct
Rv1255c (Xbp)	pVV16	5’ ggatcctgatggcgggtaccga 3’ 5’ aagctttgactcgggtccagggtg 3’	pVV1255c
Rv1257c (Xbp)	pVV16	5’ gaggaatcacttccatatgaataccgatgtgctggctgg 3’ 5’gtggtggtggtgaagcttgatcgccgagccgggat 3’	pVV1257c
Tap*Pro*[1] (Xbp)	PVVmCh	5’ tagaggatcgtcggcaggacattcgtccggtccg 3’ 5’ tcgcccttgctgaccatgaatatcgcggctgaatctag 3’	pVVmCh:TapPro

385 [1] Gene promoter

386

387 **Table 2.** Summary of primers used for qRT and RT-PCR

Primer Pair	Primer Sequence	Descritpion of fragment
Tap F and Tap R	5’ cgtgctgttcccgaaatact 3’ 5’ ggatagccaacacggcatac 3’	Tap gene
SigA F and SigAR	5’cgacgctgaaccagacct 3’ 5’ ttcgaggtcttcgtggtctt 3’	SigA gene
Rv1258c -57c F and R	5’ ctgcatgccacgtttctc 3’ 5’ gatgattgccagcggtt 3’	250bp region between *Rv1258c* and *Rv1257c*
Rv1257c -56c F and R	5’gtacggcgaaatcatggac 3’ 5’gcgagctggaattcgtga 3’	250bp region between *Rv1256c* and *Rv1257c*
Rv1256c -55c F and R	5’ caacatcttgaccttcagcca 3’ 5’ gtaccgatacagtgttgcgc 3’	250bp region between *Rv1256c* and *Rv1255c*

388

Figures and legends

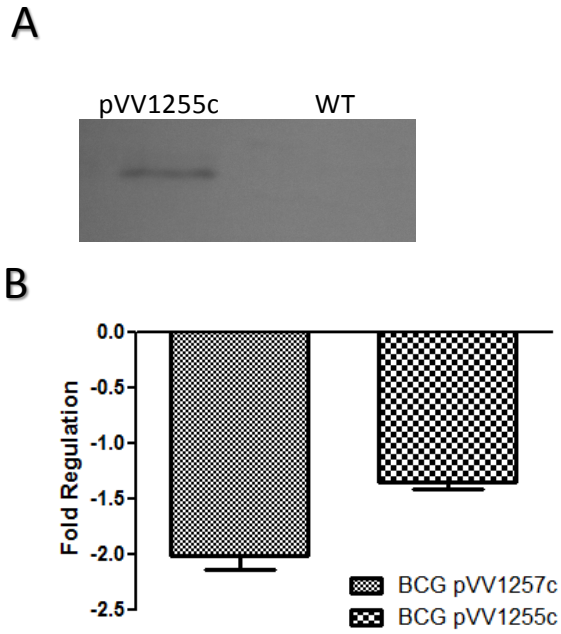

Figure 1. Regulation of the Tap gene by *Rv1257c*. A) Image of the western blot performed with protein extracts from *M. smegmatis* expressing Rv1255c and a wild type strain. B) Fold regulation of the Tap gene in *M. bovis* BCG strains containing pVV1255c and pVV1257c relative to the control strain (*M. bovis* BCG containing pVVmCherry).COLOCAR VALOR ESPECIFICO NO TEXTO NAO APROXIMADO DE FOLD CHANGE

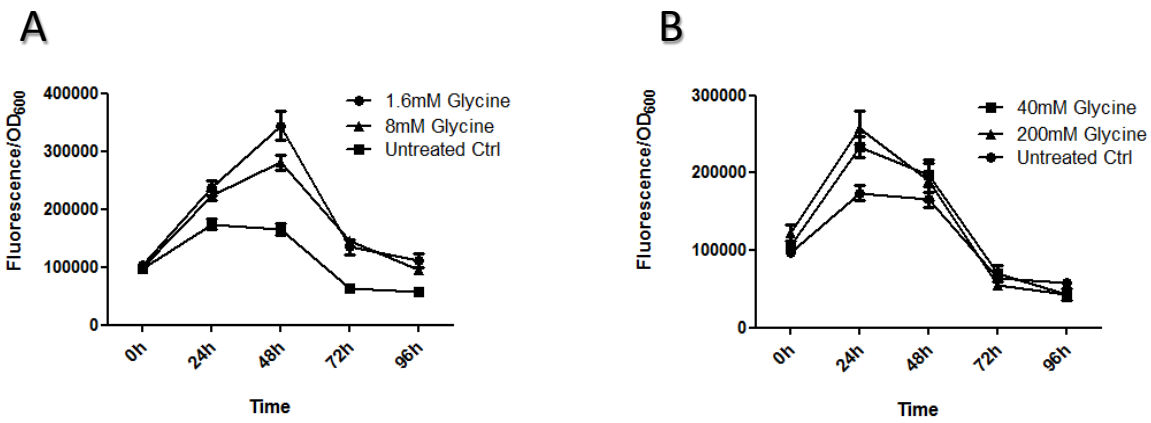

Figure 2. Fluorescence values normalized with $OD_{600.}$ Measurements taken every 24h for a total of 96h. A). Cultures with low concentrations of glycine in comparison to untreated control. B) Cultures with high concentrations of glycine and untreated control.

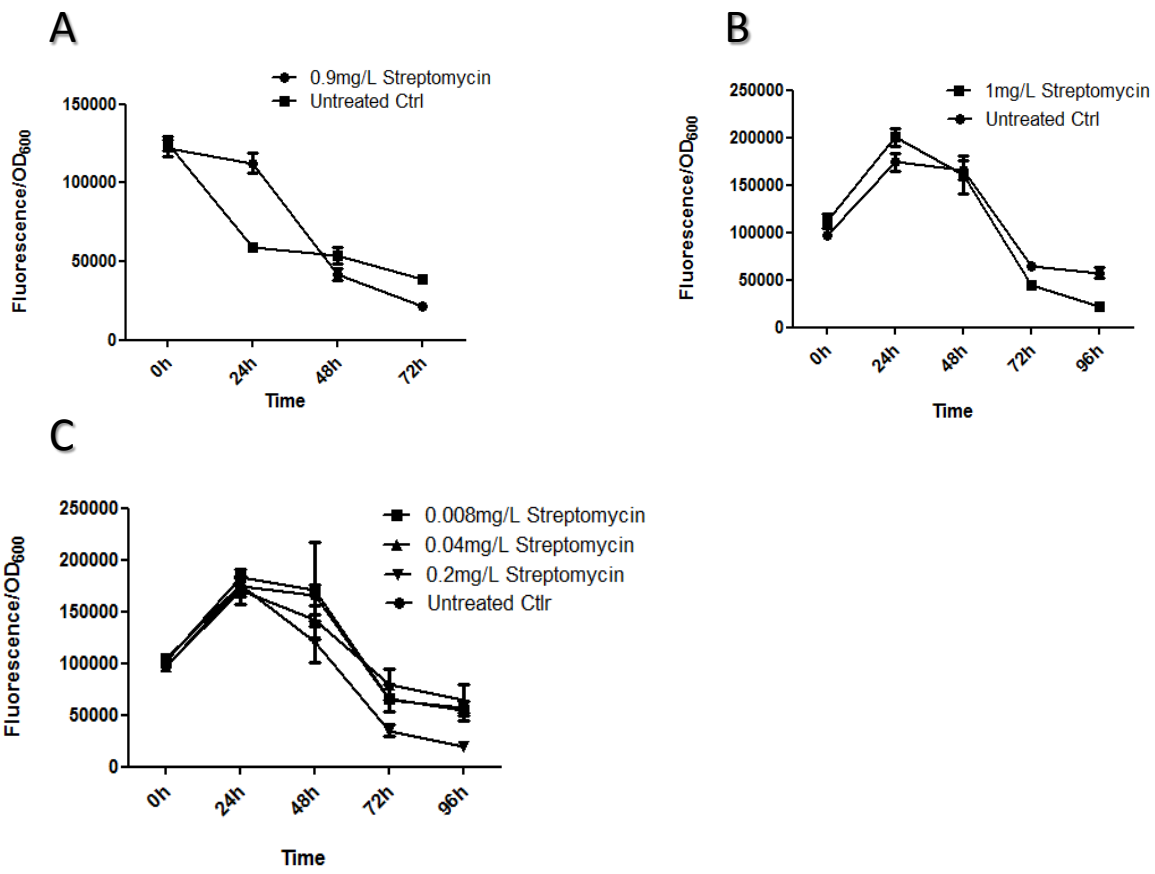

Figure 3. Fluorescence values normalized with OD_{600}. Measurements taken every 24h for a total of 72h or 96h. A & B). Cultures from two separate experiments with high concentrations of Streptomycin in comparison to untreated controls. C) Cultures with low concentrations of streptomycin and untreated control.

Figure 4. 1% Agarose gel of RT-PCR. Lane1 – Inter genic region between *Rv1258c* and *Rv1257c*; Lane 2 – Inter genic region between *Rv1257c* and *Rv1256c*; Lane 3 – Inter genic region between *Rv1256c* and *Rv1255c*; Lane 4 – No RT control of lane 1; Lane 5 – No RT control of lane 2; Lane 6 – No RT control of lane 3.

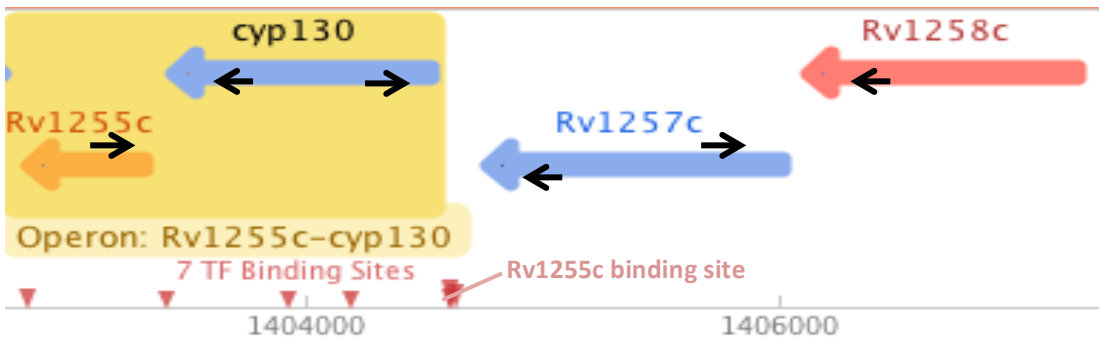

Figure 5. Schematic representation of the four genes putatively in the Tap operon on the *M. tuberculosis* chromosome. Black arrows indicate approximate positions of the primers used for the RT-PCR of the regions between the genes. Image taken from TB Database FAZER FIGURA NO APE

CONCLUSÕES GERAIS

Neste trabalho partiu-se da hipótese, baseada em estudo por Silva e colaboradores, de que um dos seguintes genes: *Rv1257c, Rv1256c* ou *Rv1255c*; presentes em *M. tuberculosis e* deletados em *M. bovis* BCG estariam inibindo a expressão do gene *Rv1258c,* codificador da bomba de efluxo Tap. A partir dos resultados do qRT-PCR foi possível concluir que o gene *Rv1257c* esta indiretamente inibindo a expressão do gene *Rv1258c.*

A utilização de cepas expressando proteina fluorescente vermelha, sob o controle do promotor do gene Tap, foi essencial no sentido de alcançar uma compreensão mais clara a respeito de condições que ativam a expressão deste gene. O uso desta metodologia permitiu observar indução do gene Tap na presença de estreptomicina. Este resultado reforça a importância desta bomba de efluxo na resistência induzida, observada em *M. tuberculosis.* Desta forma, faz-se necessário o desenvolvimento de estratégias terapêuticas que utilizem inibidores de bombas de efluxo em conjunto com os antibióticos, no sentido de reduzir o tempo de tratamento da tuberculose.

Considerando que a glicina inibe a síntese do peptidoglicano, causando acúmulo de seus precurssores (Hammes et al., 1973), foi possível inferir que a proteina Tap realiza o efluxo de precurssores tóxicos do peptidoglicano. Este resultado corrobora a hipótese proposta por Ramón-Garcia e colaboradores que observaram inibição de genes relacionados a síntese do peptidoglicano em *M. bovis* BCG, com o gene Tap removido do cromossomo (Ramon-Garcia et al., 2012).

Este estudo reforça a necessidade de se obter um conhecimento mais aprofundado a respeito do operon da proteína Tap e sua regulação, de maneira a auxiliar no desenvolvimento de inibidores dessa bomba de efluxo e possivelmente de outros mecanismos de resistência induzida. Neste trabalho os experimentos

foram desenvolvidos utilizando o *M. bovis* BCG, no entanto pretende-se repetir todos os procedimentos em *M. tuberculosis*. Além disso serão realizados experimentos com outras condições que supostamente induzam a expressão da Tap, como infecção de macróphagos, além de outros antimicrobianos. Observou-se também a necessidade de um metodo mais eficiente para normalizar os valores de fluorescencia. Dessa forma, pretende-se utilizar a proteína fluorescente verde sob o controle de um promotor constitutivo na cepa contendo a contrução pVVmCh:TapPro, para realizar esta normalização.

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