MycoSynVac

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Inducible expression system

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Version	Contributors	Comments
1.0	IC	IC report version 1
2.0	IC	IC report version 2: new summary added

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1. Summary

The main aim of MycoSynVac is to obtain a universal *Mycoplasma* chassis that can be deployed as single- or multi-vaccine in a range of animal hosts. The chassis design is based on a *Mycoplasma pneumoniae* cell that will be engineered to be free of its virulence determinants and optimized for fast growth in a serum-free medium.

Work package 3 (WP3) aims to design synthetic gene switches to allow chassis to be grown in bioreactors, maximising production and increasing biosafety.

In this report, we present a new developed L-arabinose inducible system for the expression of antigens in the chassis, which will contribute to reduce metabolic costs during the *in vitro* growth phase of the chassis to maximise production of vaccine.

We show that the designed L-arabinose inducible system, based on the AraR system from *Bacillus subtilis*, is completely functional in *M. pneumoniae*. The AraR protein from *B. subtilis*, which is well expressed in *Mycoplasma*, represses the new synthetic L-arabinose inducible promoters designed for *M. pneumoniae*. And these synthetic promoters can be activated with addition of L-arabinose to the media, showing good dose-respond behaviour. Finally, our results suggest that *M. pneumoniae* have a system(s) (although unknown) to import L-arabinose to the cells, as the L-arabinose inducible system is completely functional in *M. pneumoniae* in the absence of the L-arabinose permease AraE from *B. subtilis*.

2. Introduction: Design of an L-arabinose inducible system for *Mycoplasma pneumoniae*

One of the main objectives included in WP3 is to reduce metabolic costs during the *in vitro* growth phase of *Mycoplasma* to maximise production of vaccine. In order to achieve this aim, we have designed an inducible system, which will prevent the expression of antigens until late growth phase of the culture. Delivering an inducer to the growth medium will activate expression of these antigens. In the case we designed here, we have chosen an aldopentose (L-arabinose) as inducer. As a safety feature, we note that L-arabinose is all but absent *in vivo*, for example in lung tissue, preventing unwanted induction *in vivo*.

There are two types of inducible systems: activation systems or repression systems. However, the only inducible system reported to be functional in *Mollicutes* is the tetracycline-inducible system from *Bacillus subtilis*[1,2], which is a repressor system. Contrary to what occurs with activation systems [e.g. AraC/pBAD from *Escherichia coli*[3]], there is no need of crosstalk between an RNA-polymerase and a protein of the induction system. Taking this into account, we have based our design on the repression system AraR from *Bacillus subtilis*[4–6].

In the L-arabinose system from *Bacillus subtilis*, AraR acts as a repressor protein that binds to specific operator sequences located on the promoter region. In the absence of L-arabinose, binding of AraR to the promoter region prevents transcription of the downstream genes. The presence of L-arabinose induces a conformational change in AraR such that recognition and binding to DNA is no longer possible, thus allowing expression of the regulated genes.

In *Mycoplasma pneumoniae*, our inducible system is designed to function as a reversible switch. Genes coding for different antigens are placed under control of an L-arabinose inducible promoter. In this way, expression of the selected antigens introduced in the vaccine strain is repressed by the constitutive expression of AraR protein. The strain also constitutively expresses AraE protein from *B. subtilis*, which is an L-arabinose permease that will allow import of this monosaccharide into the cells[7,8]. Expression of antigens is thus be triggered by addition of L-arabinose to the growth medium.

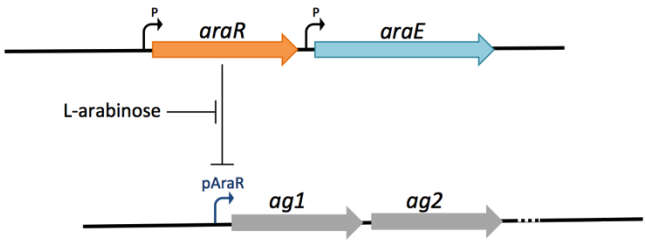


Figure 1. L-arabinose inducible system designed for *M. pneumoniae*. In this system there is a constitutive expression of AraR and AraE proteins from *B. subtilis* through a *M. pneumoniae* strong promoter (P). Expression of the selected antigens (*ag1*, *ag2*,...) is repressed by AraR until L-arabinose is added into the growth medium.

In order to implement this system in *M. pneumoniae*, we studied the expression of AraR and AraE proteins as well as developed synthetic L-arabinose inducible promoters for *M. pneumoniae*.

3. Results and discussion

3.1. Heterologous expression of AraR and AraE proteins in *M. pneumoniae*

Key elements for the system are the repressor protein AraR and the L-arabinose permease AraE. Genes coding for these proteins were isolated from genomic DNA from *B. subtilis*. In both cases, PCR was also used to include a *Mycoplasma* promoter (p438) at the 5'-end of the ORF. A short sequence coding for a tag was also included at 3'-end (*araR-flag* tag and *araE-V5* tag). Tags were added to help protein detection in later analyses, but in the future we will consider removing these tags.

Cassettes generated for expression of each protein were cloned together into a minitransposon vector MTn*cat* (Figure 2A). The resulting minitransposon was electroporated into *M. pneumoniae*, and chloramphenicol was used to select transformed cells. Thereby, a minitransposon with *araR-flag* and *araE-V5* cassettes was randomly integrated into the genome generating a non-clonal strain, named RE[1]. The strain was confirmed by PCR analysis by detection of the inserted genes into the genome (Figure 2B).

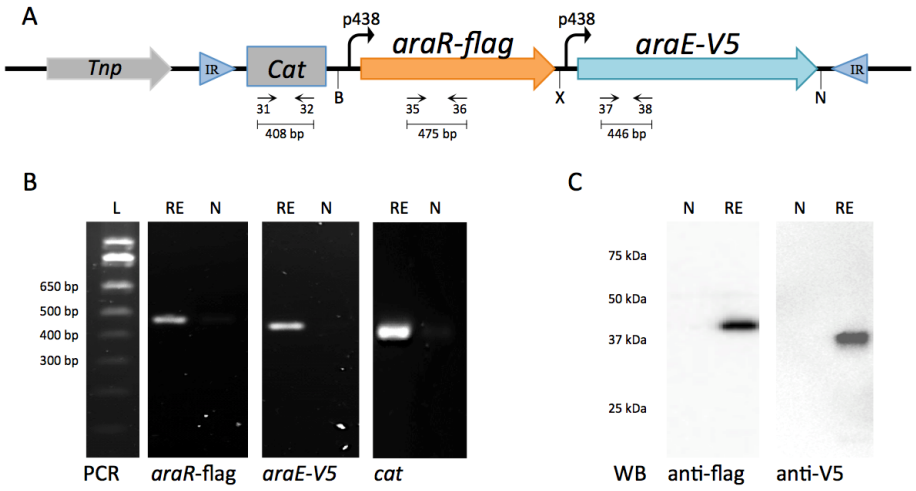


Figure 2. AraR and AraE proteins. (A) Schematic representation of relevant DNA segments of the MTn*cat* used to insert *araR* and *araE* cassettes into *M. pneumoniae* genome. *Tnp*, Transposase from Tn4001[9]. IR, inverted repeats, flanking the region that are later transfered to the genome. *araR-flag* and *araE-V5* genes coding for AraR-flag and AraE-V5 proteins, respectively. p438, constitutive promoter. BamHI (B); XbaI (X); and NotI (N) restriction sites. *Cat*, chloramphenicol selection cassette. **(B) PCR detection of inserted genes on the genome of RE mutant strain generated.** *araR-flag* gene (primers 35-36); *araE-V5* gene (primers 37-38); and *cat* gene (primers 31-32). Arrows with numbers in (A) indicate the position of primers used on PCR analysis and lines with blunt ends show size of the expected PCR product. L: ladder. **(C) Western Blot analysis using anti-flag (Sigma F1804) and anti-V5 antibodies (Sigma V8012).** 10 μg of total protein extract analysed from each sample. RE, RE mutant. N, negative control (*M. pneumoniae* M129 strain).

[1] Non-clonal strains are always maintained under antibiotic pressure.

Expression of AraR-flag and AraE-V5 proteins in RE strain was assessed by western blot. A single band could be detected when analysed either AraR-flag or AraE-V5 proteins (Figure 2C). AraR-flag has the expected size (42 kDa). However, AraE-V5 presented a smaller size than the expected 52 kDa. Notably, no other bands indicating degradation of AraR-V5 were detected. These results indicate that AraR-flag protein from *B. subtilis* can be expressed well in *M. pneumoniae* and this raises the question of whether the AraE-V5 will be functional in *M. pneumoniae* (see section 2.4).

3.2. Synthetic L-arabinose inducible promoters for *M. pneumoniae*

L-arabinose dependent promoters from *B. subtilis* are well characterised[5,6]. Based on the structure information of those promoters, several sequence candidates were designed for a synthetic L-arabinose inducible promoter (pAraR) for *M. pneumoniae*. The sequences were first analysed *in silico* using an algorithm to predict their probability to be recognised as a promoter in *M. pneumoniae*[10]. In this way, 4 sequences were predicted to be functional promoters (Figure 3).

Promoter	Score Pribnow	Probability
p438	2.23	0.860
pAraR-12	2.23	0.885
pAraR-13	2.21	0.740
pAraR-14	4.43	0.670
pAraR-15	4.43	0.615

Figure 3. Results from promoter prediction analysis. p438, constitutive promoter functional in *M. pneumoniae*. pAraR11-pAraR15, synthetic L-arabinose promoters designed. Score Pribnow indicates the quality of the Pribnow box on the promoter (lower values correspond to better promoters). The result of the analysis is shown as a probability. A sequence with a probability >0.6 is recognised as a functional promoter in *M. pneumoniae*.

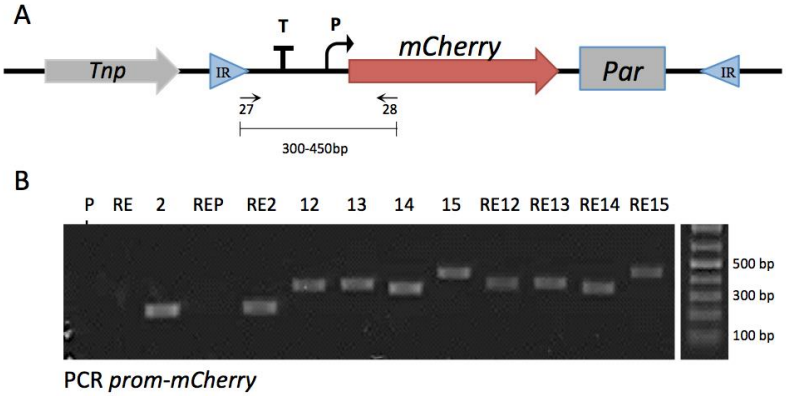
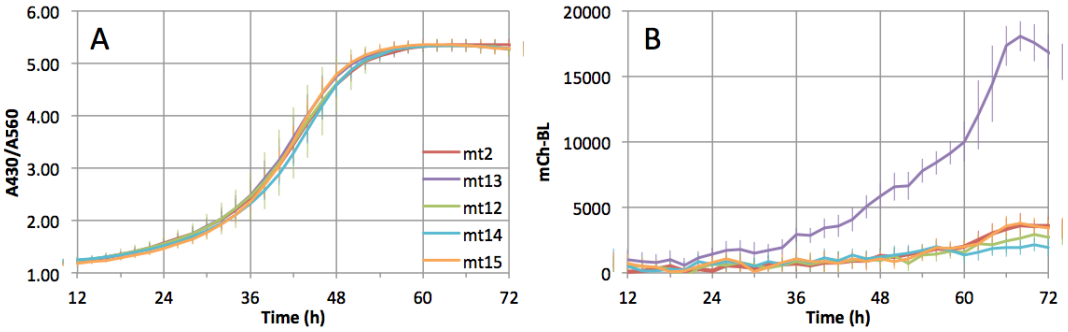


Figure 4. (A) Schematic representation of relevant DNA segments of the MTn*par-mCh* platform. *Tnp*: transposase from Tn4001. Included between two inverted repeats (IR): a terminator sequence (T), a promoter region (P), the *mCherry* ORF and a puromycin selection cassette (*par*). **(B) PCR detection of inserted genes on the genome of pAraR mutant strains generated.** *prom-mCherry* gene (primers 27-28). Arrows with numbers in (A) indicate the position of primers used on PCR

analysis and lines with blunt ends show size of the expected PCR product. L: ladder.

For the analysis of the activity of synthetic promoters in *M. pneumoniae*, we developed a minitransposon platform (MTn*par-mCh*) containing the *mCherry* reporter gene (Figure 4). It should be noted that we have included a terminator sequence (T) upstream from promoter region to prevent expression from upstream transcription activity. Using a Gibson assembly cloning strategy, the promoter sequence (P) can be easily exchanged to test each of the designed synthetic promoter sequences.

The 4 sequences predicted to be functional pAraR promoters were successfully cloned in the minitransposon platform. The resulting constructs were used to generate non-clonal *M. pneumoniae* mutant strains (puromycin used for selection). In this way, 4 strains carrying a MTn*par-mCh* with one of the pAraR synthetic promoters were generated: mt12, mt13, mt14 and mt15 (Figure 5, table). Two control strains one carrying a similar MTn*par-mCh* with a constitutive promoter (p438) and a second with and empty MTn*par* were also obtained, named mt2 and mtP, respectively. PCR analysis was carried out to confirm the presence of promoter-*mCherry* on the genome of each mutant strain (Figure 4B) and the PCR fragments were sequenced to confirm the promoter sequences (Data not shown).



Strain	Promoter tested	*in silico* analysis Promoter probability	*mCherry* expression in *M. pneumoniae*
mtP	non	-	-
mt2	p438	0.860	+
mt12	pAraR-12	0.885	(+)
mt13	pAraR-13	0.740	+++
mt14	pAraR-14	0.670	(+)
mt15	pAraR-15	0.615	+

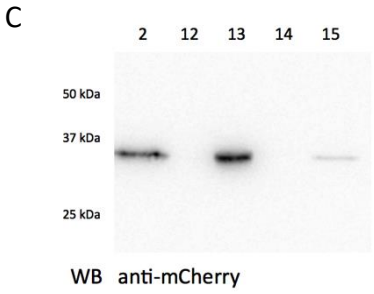


Figure 5. Synthetic pAraR promoters in *M. pneumoniae*. Time course experiment (graphs). *M. pneumoniae* strains: mt2 (Red) as a positive control of mCherry expression; mt12 (green); mt13 (pink); mt14 (blue); and mt15 (orange). **(A) pH curves.** Indirect measure of growth of *M. pneumoniae*. All strains present comparable profiles. **(B) mCherry profile.** mCherry expression in the absence of repressor AraR. Fluorescence values after subtracting background. Represented on the graphs are mean values from 2 independent bio-replicates and error bars corresponding to SD values[11]. **(C) Western Blot analysis using anti-mCherry (BioLegend 677702).** Strains shown: (2) mt2 as a positive control; (12) mt12; (13) mt13; (14) mt14; and (15) mt15. 10 µg of total protein extract analysed from each sample.

In order to analyse the activity of synthetic promoters in *M. pneumoniae*, a time course assay was developed, where growth and mCherry expression are analysed. The medium used for *M. pneumoniae* has phenol red, which is a pH indicator that changes colour from red (pH 7.5) to yellow (pH 5.5). This allows monitoring *M. pneumoniae* growth indirectly by pH changes on the medium (medium colour measured as the ratio between Abs 430nm and Abs 560nm), because this bacterium acidifies the medium while growing. mCherry expression is analysed by fluorescence emission detection. Given the coloured medium, background noise in fluorescence reads is high and variable with pH. This background signal is subtracted to obtain a real profile of the fluorescence emission produced by mCherry. In each experiment a non-mCherry strain (with a similar metabolic load to the strains being analysed) is included to obtain a background profile that can be subtracted from the reads of the mCherry strains.

Time course experiments for each synthetic pAraR promoter were performed in parallel. Strain mtP was used as a negative control to determine the background noise in these experiments. Given that all strains present similar growth profiles (Figure 5A), mCherry profiles are directly comparable (Figure 5B). The results showed that mt15 and mt2 have similar mCherry expression profiles, and mt12 and mt14 are very close. The mt13 is the one that presents higher expression of mCherry, as the profile reaches a maximum 3 times higher than mt2.

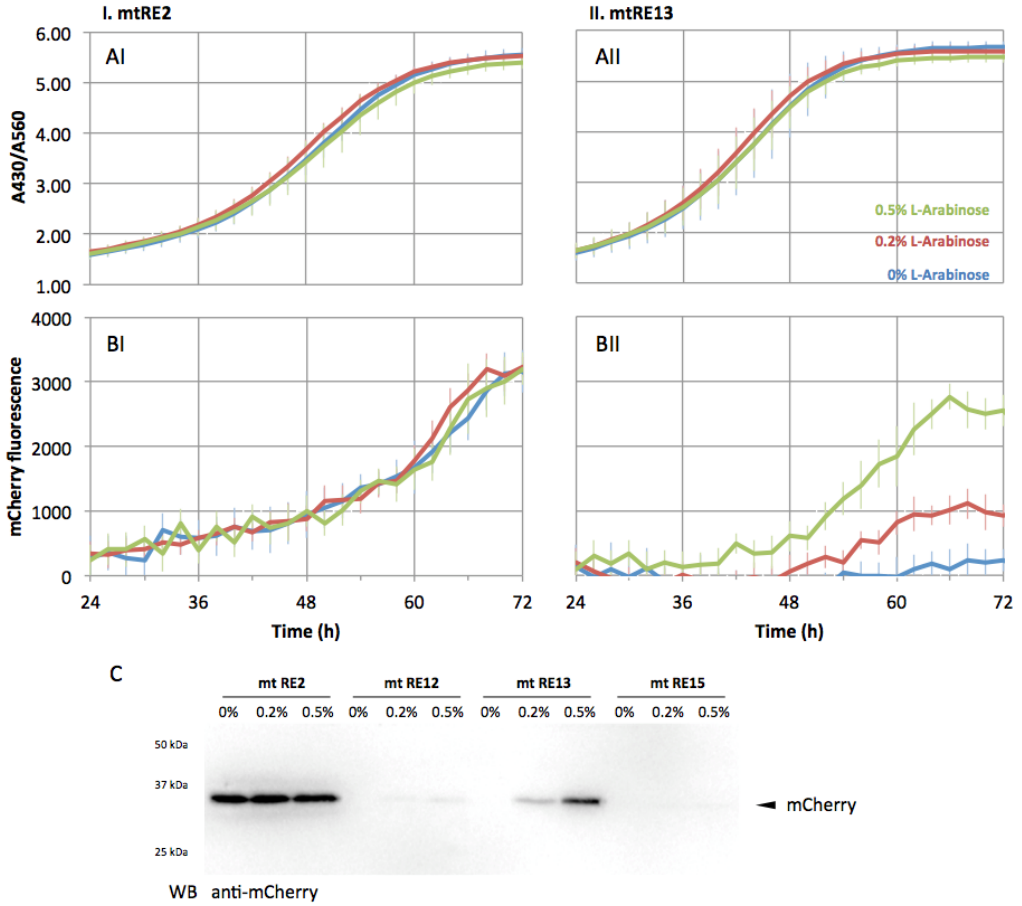
Expression of mCherry was also analysed by western blot (WB) (Figure 5C). Total protein extracts were collected at the late exponential phase of growth. A single band of the expected size for mCherry (30 kDa) could be detected in the control strain mt2 and also in mut13 and mt15, but not in mt12 and mt14. The intensities of these bands also show that mCherry is expressed in higher levels in mt13 compared to the control strain, and is lower in mt15.

Taken together, all these results indicate that synthetic pAraR promoters are functional in *M. pneumoniae*. The strongest promoter analysed is pAraR13. Promoter pAraR15 has a comparable strength to the constitutive promoter p438. And finally, pAraR12 and pAraR14 sequences seem to be weak promoters but, given the WB result, an analysis by RT-qPCR might be used to confirm this.

3.3. Full AraR system in *M. pneumoniae*

In order to study the complete AraR system from *B. subtilis* in *M. pneumoniae*, minitrasposons MTn*par-mCh* with synthetic pAraR promoters 12, 13 and 15 were electroporated into the RE strain, and chloramphenicol and puromycin were used together to select transformed cells. In this way, 3 non-clonal *M. pneumoniae* strains were generated: mtRE12, mtRE13, and mtRE15 (Figure 6, table). Similarly, two control strains were also obtained. Strain mtRE2 was generated with the MTn*par-mCh* carrying the constitutive promoter p438 and strain mtREP was obtained with an empty MTn*par*. As before, PCR analysis was used to confirm the presence of the promoter-*mCherry* cassettes on the genome of these new mutant strains (Figure 4B) and PCR fragments were also sequenced to confirm the promoter sequences (Data not shown).

The time course assay developed to analyse the activity of promoters, could be also used to study repression and induction of promoters in *M. pneumoniae*. In this case, each strain is grown in parallel in the presence of different concentrations of the corresponding inducer. A non-mCherry strain is included to obtain the background profile for each growth media conditions.



Strain	mtREP	mtRE2	mtRE12	mtRE13	mtRE15	mtR13
AraR	+	+	+	+	+	+
AraE	+	+	+	+	+	-
Promoter tested	non	p438	pAraR-12	pAraR-13	pAraR-15	pAraR-13
mCherry	-	+	+	+	+	+

Figure 6. L-arabinose inducible system. Time course experiment (graphs). *M. pneumoniae* strains tested: (I) mt RE2 carrying a constitutive promoter; and (II) mtRE13 carrying the synthetic pAraR13 inducible promoter. **(A) pH curves.** Indirect measure of growth of *M. pneumoniae*. Comparable growth profiles in all three conditions analysed. **(B) mCherry profile.** Induction of mCherry expression at different L-arabinose concentrations: 0% L-arabinose (blue); 0.2% L-arabinose (red); and 0.5% L-arabinose (green). Fluorescence values after subtracting background. Represented on the graphs are mean values from 3 independent bio-replicates and error bars corresponding to SD values. **(C) Western Blot analysis using anti-mCherry (BioLegend 677702).** Strains shown: mtRE2 as a positive control; mtRE12; mtRE13; and mtRE15. Expression of mCherry

analysed for 0% L-arabinose (-); 0.2% L-arabinose (a); and 0.5% L-arabinose (b). 10 µg of total protein extract analysed from each sample.

Time course experiments were performed in parallel with strains mtRE2 and mtRE13 to analyse the AraR system. Induction of mCherry expression was assessed at different L-arabinose concentrations (0%; 0.2%; and 0.5% of L-arabinose). Strain mtREP was used as a negative control in each L-arabinose concentration. Given that the strains showed similar growth profiles at all three conditions (Figure 6A), mCherry profiles are directly comparable (Figure 6B). The results showed that mtRE2 presents similar mCherry expression profiles independent of AraR expression and L-arabinose concentration, as expected when a constitutive promoter is used to express mCherry. The case of mtRE13 is however different: this mutant presents an extremely low mCherry profile in the absence of L-arabinose, meaning that the presence of AraR is silencing pAraR13 promoter (but still might be a little leaky). Addition of L-arabinose to the medium increases mCherry expression as shown on the profiles obtained when testing concentrations of 0.2% and 0.5% of L-arabinose. Importantly, the induction of mCherry expression is higher when the concentration of L-arabinose is higher, showing that the inducible system functions in *Mycoplasma*, in a dose-dependent manner.

Induction of mCherry in strains carrying the whole AraR system was also analysed by western blot (WB) (Figure 6C). In this case, parallel cultures with different concentrations of inducer (0%; 0.2%; and 0.5% of L-arabinose) where set in parallel for each strain. Total protein extracts were collected at the late exponential phase of growth. Again, strain mtRE2 expresses mCherry protein independent of L-arabinose concentration. In the case of strains carrying the whole AraR system (mtRE12, mtRE13 and mtRE15), no mCherry could be detected when they were grown in the absence of L-arabinose. However, this cannot discard the possibility that some of these pAraR promoters tested might be leaky, as WB appears to be less sensitive than the fluorescence detection assay. Confirming time course experiment results, mtRE13 shows increasing amounts of mCherry with the concentration of L-arabinose. Similar results, although with fainter bands, can be observed for mtRE12 and mtRE15, meaning that promoters included in these two strains have significantly lower strengths than pAraR13, included in strain mtRE13.

Overall, these results indicate that the AraR system from *B. subtilis* is functional in *M. pneumoniae*. Moreover, the system appears to work with promoters with very different strengths. An RT-qPCR might be necessary to determine how leaky these different pAraR promoters may be.

3.4. AraR system in *M. pneumoniae* in absence of AraE

The AraR system is functional in *M. pneumoniae*, but the concentration of L-arabinose used to gain high levels of induction seemed a little high, considering that mutant strains carry only a single copy of the inducible promoter as well as a single copy of the *araR-flag* gene. Moreover, AraE protein did not seem to have the expected size and thus might not be functional.

In order to determine if AraE is functional and necessary for the AraR system in *M. pneumoniae*, a new minitransposon MTn*cat* with a single cassette for expression of AraR protein was prepared and electroporated

into *M. pneumoniae*, generating the non-clonal strain mtR. This strain was then used in a second round of transformation by electroporation using the MTn*par-mCh* with the pAraR13 promoter, obtaining the non-clonal strain mtR13 (Figure 6: Table).

Time course experiments were set in parallel with strain mtRE13 and mtR13 at 0% and 0.5% L-arabinose. Results showed that, in both strains, mCherry expression is silenced in the absence of L-arabinose (Figure 7). Interestingly, induction of mCherry at 0.5% of L-arabinose is doubled in the strain without AraE protein, indicating that this protein is not necessary for the intake of L-arabinose. The higher levels of mCherry induction shown by mtR13, might be explained by the reduced metabolic load that this strain has compared to mtRE13. Therefore an absence of AraE appears to be preferable for this system to work in *Mycoplasma*.

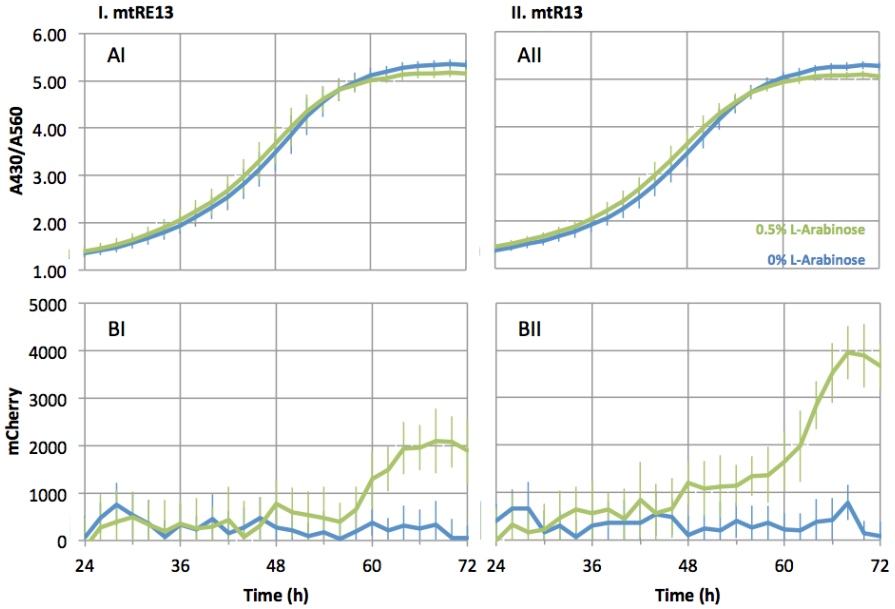


Figure 7. L-arabinose inducible system. Time course experiment (graphs). *M. pneumoniae* strains tested: (I) mtRE13 that has AraR and AraE; and (II) mtR13, which has only araR. Both strains have the same MTn*par-mCh* with the synthetic pAraR13 inducible promoter. **(A) pH curves.** Indirect measure of growth of *M. pneumoniae*. Comparable growth profiles in the two conditions analysed. **(B) mCherry profile.** Induction of mCherry expression at 0% L-arabinose (blue); or 0.5% L-arabinose (green). Fluorescence values after subtracting background. Represented on the graphs are mean values from 3 independent bio-replicates and error bars corresponding to SD values.

4. Conclusions

The AraR repressor inducible system from *Bacillus subtilis* is functional in *Mycoplasma pneumoniae*.

We have shown that pAraR promoters are repressed by AraR protein and can be induced by addition of L-arabinose to the media. Our results also suggest that the AraR system is completely functional in *M. pneumoniae* in the absence of the L-arabinose permease AraE, indicating that the cells have other (although unknown) system(s) to import L-arabinose. Thus, AraE is not necessary.

Finally, time course fluorescence experiments appear to be more sensitive assays than WB for detecting small amounts of mCherry expression.

5. Future directions

An RT-qPCR assay needs to be assessed to determine potential leakiness levels of each inducible promoter.

For the vaccine strain that MycoSynVac is developing, a single platform carrying the whole AraR system needs to be prepared and needs to be based on a different genome integration system, so that the insertion point can be design (WP4).

The next step will be to combine the AraR system with the designs being developed for antigen display in *M. pneumoniae* (WP1).

6. References

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