

**Final report:** Efflux pump collaborative project; CRADA #7829

## Title: Use and engineering of efflux pumps for the export of olefins in microbes

### A/ Generality

Project started in *July 2011* and ran out of funds in *November 2013 (29 months)*.

**Total personnel:** Florence Mingardon (post doc: 90% for 29 months), Camille Clement (student: 100% for 12 months), Angelique Chanal (project coordinator: 30% for 3 months, 10% for 23 months), Edison Huff (student: 100% for 3 months).

**JBEI personnel:** Aindrila Mukhopadhyay (principal investigator: 10% for 29 months), Kathleen Hirano (technician: 100% for 6 months), Melissa Nhan (technician: 50% for 8 months), Eric Luning (technician: 20% for 1 month).

**Budget:** \$773,711 for 29 months (\$312,969 Funds-in and \$460,742 In-kind).

#### **Accomplishments:**

- 1 patent in preparation (ROI #2013-181)
- 1 article in preparation (Mingardon F., Clement C., Hirano K., Nhan M., Luning E. G., Chanal A., Mukhopadhyay A. 2014. Uses of *E. coli* AcrB wild type and variants to improve the tolerance and production of olefins). Targeted journal: *Biotechnology and Bioengineering*

#### **Goals:**

The scope of the project is to investigate efflux pump systems in engineered host microorganisms, such as *E. coli*, and develop a pump engineered to export a target compound. To initiate the project in coordination with other TOTAL driven projects, the first target compound to be studied was 1-hexene. However we were investigating other chemicals as Styrene. The main goal of the project was to generate a set of optimized efflux pump systems for microorganisms (*E. coli* and *Streptomyces* or other host) engineered to contain biosynthetic pathways to export large titers of target compounds that are toxic (or accumulate and push back biosynthesis) to the host cell. An optimized microbial host will utilize specific and efficient cell wall located pumps to extrude harmful target compounds and enable greater production of these compounds.

**Table 1: milestones by Quarters.** Y means accomplished. X means not applicable. N means didn't work. O: on going.

Task/Milestone/ Quarters	1	2	3	4	5	6	7	8	9	10
Develop methods to examine 1-hexene impact on <i>E. coli</i>	Y									
Use existing library of solvent resistant efflux pumps and examine their effect during 1-hexene exposure in <i>E. coli</i> using competition assays.		Y								

Coordinate with TOTAL-JBEI hexene project to develop strategies to co-express pumps.			X	X							
Use best pump candidates strains to couple with 1-hexene production strains and devise strategies for controlling pump expression.				X							
Review of results to date				Y							
Develop methods to examine 1-hexene impact on <i>Streptomyces sp</i> (or other host)		Y									
Generate a library of export pumps in <i>Streptomyces sp</i> (or other hosts) and examine their efficiency during 1-hexene exposure				X	X	X					
Conduct assays with additional target compounds to select winners for a range of target compounds; conduct high throughput assays to select the best pumps to 1-hexene.			Y	Y							
Target changed to Styrene and other chemicals				Y	Y						
Select best candidates and mount a directed evolution project to alter / improve specificity towards substrate compounds; Conducted for 1-hexene.				Y	Y	Y	Y	Y			
Conducted for styrene					Y	Y	Y	Y	N		
Participate in reviewing data to assess which pumps provide resistance in the presence of which compounds				Y	Y	Y	Y	Y	Y		
Provide matched efflux pump to target compound in one microbial host that is metabolically engineered to produce target compound. Target changed to Styrene.						Y	Y	N	N		
Publish/patent results from Styrene and hexene pump study.									O	O	
Study the possibility of transferring RND pump to other host.									Y/N	Y/N	
Investigating 1-hexanol toxicity.									Y		
Investigating other olefin toxicity.									Y	Y	
Investigating the impact of engineered pump on other toxic compounds.									Y/N	Y/N	

## **B/ Results**

### **1/ Introduction/overview**

Some olefins are precursors for the production of bioplastics (specifically styrene and representative alpha-olefins). Their biological production is an attractive alternative to their production from fossil based sources. However most of these chemicals are toxic for the hosts producing them. Efflux pumps are involved in tolerance to some chemicals, by capturing them and pumping them out of the cell they will confer a better survival and could improve their production. In this context we decided to investigate the use of efflux pumps to improve tolerance and production of chemicals.

We focused our study on olefins (mainly 1-hexene and styrene). When the project started, another collaborative project (Total-JBEI) consisting in producing 1-hexene in organisms was initiated, thus the initial target for efflux pump project was 1-hexene. The first chosen host for these studies was *E. coli* (primarily because the first experiments for 1-hexene production had been initiated in *E. coli*) though subsequently, other organisms were also tested (*Streptomyces sp.*, *Bacillus subtilis* and *Saccharomyces cerevisiae*). However due to the difficulties in producing 1-hexene in *Streptomyces* and also in *E. coli*, the 2 projects (tolerance and 1-hexene production) have progressed independent of each

other. Further, due to the absence of a strain producing the alpha-olefin, the second chemical compound chosen for its capability to be produced by *E. coli* and which could be an ideal molecule to study efflux pump system was styrene. Nevertheless due to the highly mutagenic properties of styrene, the challenges in 1-hexene production and the difficulty in transferring these pumps into other organisms, the entire project was suspended.

## **2/ Summary of results obtained in the first 8 quarters**

### **2-a/ 1-hexene**

When the project started, no initial work regarding toxicity of alpha-olefins has been done at JBEI and/or published by other labs. So our first experiments consisted in developing assays to for 1-hexene exposure in microbes and determining the pumps involved in its secretion. We developed 2 tests: i) one test in liquid cultures which gave us more quantitative data and that could be used for various organisms but challenging to use in high through put, and ii) a saturated atmosphere assay which was more specific to *E. coli* (and mainly *E. coli* K12) but which could be used to simultaneously examine a large number of different *E. coli* strains (i.e. a library).

We showed that 1-hexene is highly toxic for microorganisms. Specifically, *E. coli*, *S. cerevisiae* and *Streptomyces sp.* are sensitive to 50 to 80 mg/L of 1-hexene in liquid culture, and *Bacillus subtilis* to 100 to 200 mg/L of 1-hexene. Due to the difficulty (during this time period) in the production of 1-hexene by any gram-positive bacteria, we decided to focus on *E. coli*.

In *E. coli* more than 30 pumps have been shown as playing a role in the transport of toxic compounds. We obtained 30 gene-deletion mutants from the JBEI collection of KEIO mutants (Baba et al, 2006); each mutant is deleted in a given pump. Among the tested candidates only three of them showed a phenotype different than the wild type strain: *acrA* mutant, *acrB* mutant and *tehA* mutant.

#### **2-a-1/ TehA**

The *tehA* gene in *E. coli* encodes for a pump belonging to the multi-drug resistance category. It has been putatively annotated as a potassium-tellurite, ethidium and proflavin transport in *E. coli* (Turner et al, 1997). However homologs of this pump in plants have been involved in anion transport. While its function and mechanism in bacteria are not completely characterized, our data suggested that the absence of this membrane protein had an impact on 1-hexene tolerance in *E. coli*. We observed this phenomenon with other compounds such hexane, pinene, styrene. We decided to investigate in detail the role and the function of this pump and composed a record of invention (ROI Berkeley Lab internal tracking number is CIB-3204)

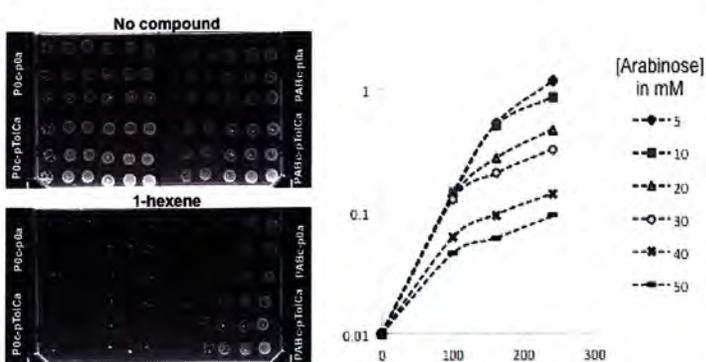
However during the course of the project, the phenotype observed of the *tehA* mutant became less reproducible. As TehA could be a pump involved in the anion transport, our first assumption was that the media batch changed and resulting in a change in the salt and/or the pH. We investigated the effect of the *tehA* deletion at different pH and salt conditions and tested different media. Despite extensive efforts we were unable to obtain conditions that reproducibly result in the phenotype where the *tehA* deletion results in a positive impact. In parallel, we observed that the  $\Delta$ *tehA* strain (*tehA* KO mutant) forms smaller colonies on an agar plate and suspected that the absence of TehA might actually be linked to a stressed strain (secondary effect involving other resistance mechanisms) and not to the function of this pump.

Therefore, we suspended this project and canceled the ROI.

## 2-a-2/ AcrAB-TolC

The two proteins AcrA and AcrB in a complex with another protein TolC form a pump that is well known in playing a key role in solvent tolerance in *E. coli*. The deletion of *acrA* and *acrB* leads to a strain more sensitive to 1-hexene.

We also observed that the over-expression of AcrAB leads to a strain resistant to higher 1-hexene quantity that wild type (Fig 1).



**Figure 1:** Over expression of pump in absence or presence of 1-hexene. The pictures on the left represent the impact of over producing TolC, AcrAB or both in *E. coli* K12 wild type, in presence (bottom) or absence (top) of 1-hexene. This experiment has been done in presence of 5 mM of arabinose. The graph on the right represents the impact of overproducing AcrAB on *E. coli* K12 growth in liquid culture in absence of)

The figure 1 shows that in a wild type strain (naturally producing the 3 proteins), the overproduction of AcrAB above the native level improves the tolerance, while the overexpression of only TolC has no impact. TolC is possibly expressed to greater levels relative to AcrAB and increasing its quantity above its native level has no impact on the tolerance phenotype. However if the 3 proteins (AcrAB-TolC) are overexpressed in the wild type strain the tolerance to 1-hexene of the resulting strain is better than the strain only overproducing AcrAB. Thus increasing the quantity of this efflux pump improved *E. coli* tolerance towards compounds such as 1-hexene. However, as showed in the graph in the figure 1, increasing the quantity of these secreted proteins is limited by the toxicity linked to their over-expression. Thus to further improve the tolerance to 1-hexene, more efficient pump(s) needed, to be either found in other organisms or generated by engineering.

To identify a pump that may provide better tolerance to 1-hexene than the native *E. coli* AcrAB-TolC system, we used an existing library of **heterologous efflux pumps** in *E. coli*. We observed that some pumps from other organisms were, in the tested conditions, as efficient as the *E. coli* native AcrAB-TolC system, but none were better. We thus decided to focus our efforts on improving the native system of *E. coli* by directed evolution.

We chose to modify AcrB by **random mutagenesis** and we selected and analyzed the variants that give an advantage to the strain when 1-hexene is present. We identified several mutations (A279T, Q584R, F617L, L822P, F927S and F1033Y) that resulted in improved tolerance to 1-hexene, in comparison to the wild type AcrB. To determine if the mutations can be additive, we combined selected mutations into a single *acrB* gene generating 8 new variants with 2, 3 or 6 beneficial mutations. Most of the strains containing the AcrB with two beneficial mutations were found to be more tolerant to 1-hexene relative to AcrB with one mutation (Fig 2). However the combination of 3 or more mutations in the same protein did not result in additional advantage.

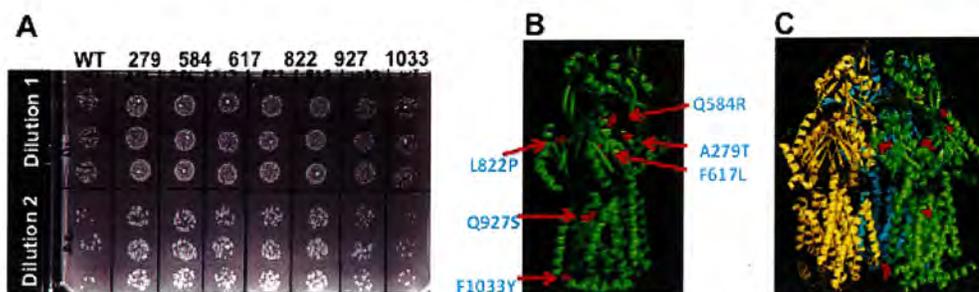


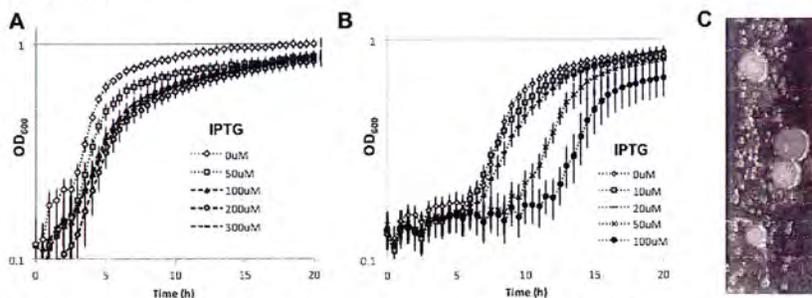
Figure 2:

**Impact of AcrB mutations on tolerance to 1-hexene.** **A:** Survival test of *E. coli* strain producing either the AcrB wild type or variants in 1-hexene saturated atmosphere was performed. Two dilutions of cultures (Dilution 1:  $10^5$  cells and Dilution 2:  $10^4$  cells) were spotted in triplicate on an agar plate. **B:** Localization of the 6 beneficial mutations is represented in red on the structure of a single monomer of AcrB or **C:** on the structure of an AcrB trimer.

## 2-b/ Styrene

We found that the AcrAB-ToIC *E. coli* pump was also involved in tolerance during styrene exposure. A pathway for the production of styrene in *E. coli* has been reported in the literature (McKenna et al, 2011) and showed that styrene can be produced at a toxic level (300mg/L). Having previously shown that AcrB can be modified and for improved tolerance towards a toxic compound, and having also shown that AcrB is involved in styrene transport, we decided to apply the directed evolution strategy to look for AcrB variants that will give improved advantage when styrene is added in the medium or produced endogenously. We wanted to examine both scenarios to see if the mutations found when styrene is added in the media could also work when styrene is produced by the cell and vice versa. This concept could validate the fact that looking for improved proteins when compounds are added in the media can also work when the compound is produce by the cell.

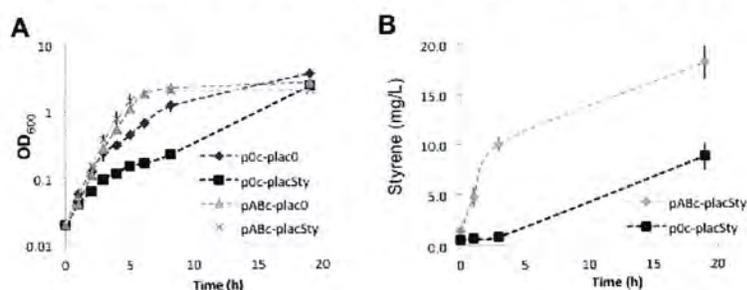
To test this hypothesis, we reconstituted the pathway allowing the production of styrene and cloned the 2 main genes involved in its production in operon under the control of either a weak, or a strong promoter. With the strong promoter we determined in liquid culture that the production of styrene had a strong impact on growth. However we observed high standard deviation between replicates (Fig 3B). When streaking the strain on agar plates, we observed colonies with atypical growth (Fig 3C). We hypothesized that the irregular growth of individual colonies was responsible for the high variation in growth between liquid culture replicates. The DNA sequence of the plasmids, extracted from cells with atypical growth, revealed the presence of insertional sequences (IS) in the promoter or coding sequence of the gene encoding the 2 main enzymes of this pathway, thus inactivating styrene production.



**Figure 3: Impact of pump over-production on growth during styrene production.** Growth of *E. coli* K12  $\Delta$ acrAB containing pABc and either the pIacSty (styrene genes under the control of a weak promoter) **(A)** or the pIacSty (styrene genes under the control of a strong promoter) **(B)** in LB

supplemented with various concentration of IPTG, at 37 °C was monitored using an Omnilog system. Bars represent standard deviation between 8 replicates. **C:** Atypical growth and shape of colonies is seen in *E. coli* K12  $\Delta$ acrAB containing pIacSty grown on LB-agar plates supplemented with 100  $\mu$ M of IPTG.

We then pursued our experiments with the “styrene” genes under the control of a weaker promoter to decrease the styrene production and to avoid such strong variations and genetic modifications (Fig 3A). Using this system we could observe that AcrAB-TolC was also playing an important role in olefin production and was essential to reach higher level of chemicals (Fig 4).



**Figure 4: Impact of production of styrene on growth and production. A:** Growth of *E. coli* NST74  $\Delta$ acrAB producing the pump (pABc) or not (p0c) and producing styrene (p<sub>lac</sub>Sty) or not (p<sub>lac</sub>0). **B:** Styrene concentration produced by *E. coli* NST74  $\Delta$ acrAB producing the pump (pABc) or not (p0c). Bars represent standard deviation between duplicates.

We then decided to generate a mutant pump library and to screen for pump variants that could improve the tolerance when styrene is produced by the microorganism, or when styrene is added to the medium. We developed several screens, some with growth competition assays, and some with growth comparisons to look for pumps providing an advantage. However we could not find any pump variant that could provided improved growth advantage. Strains with improved growth were all the result of genomic or plasmid modifications and not due to an improved pump. Styrene was highly mutagenic and the strain stability was difficult to maintain. To overcome this problem, we engineered some strains to improve their stability and to decrease the mutagenic effect of styrene (we deleted recombinases, low fidelity polymerases, we worked on a minimal strain that does not contain IS) but we were not successful. We concluded that styrene is too mutagenic to have reproducible and reliable results and decided to suspend this project.

### 3/ Last results obtained in the last 2 quarters and not presented during the last SC

In the last quarter, we focused our work on finishing the last experiments that could allow us to draft a publication and a patent. The remaining questions of this work were:

- what is the role of the mutations found to improve 1-hexene tolerance?
- are they effective with other compounds and more specifically with other alpha-olefins?
- and do they also function in other organisms? We focus our last months trying to answer these questions and writing a manuscript.

#### **3-a/ What is the role of the mutations found to improve 1-hexene tolerance?**

To determine if the mutations were linked to a higher quantity of protein in the membrane or to a better stability, or due to a more efficient pump, we decided to compare the quantity of wild type AcrB to the quantity of its variants. We first decided to use the mass spec facility at JBEI to perform this experiment. However the results obtained were too fluctuating between replicates and no conclusion could be made using this method (the deviation was mainly due to membrane preparation and no reproducible results could be obtained). We thus decided to compare their relative quantity using western blot technique.



**Figure 5: Impact of beneficial mutations on quantity of AcrB variants.** **A:** Total fraction of proteins from a culture of strains producing either the wild type, either the variant F617L, either the variant 4 (A279T, Q584R) either the variant 6 (A279T, Q584R, F617L) were analyzed by western blot using anti-His antibody and a chromogenic reagent. **B:** Soluble fraction of total proteins from a culture of strains producing either the wild type, either the variant F617L, either the variant 6 (A279T, Q584R, F617L) were analyzed by western blot using anti-His antibody and a chemiluminescent reagent.

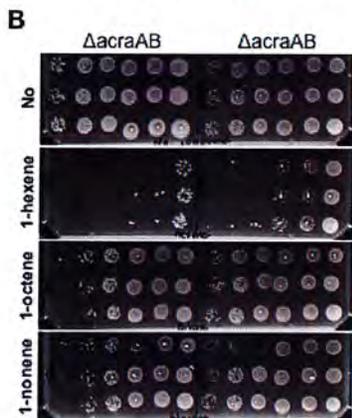
We first compared the total quantity of AcrB wild type and AcrB variants produced, and found it very similar (Fig 5 A). However we found that increasing the number of mutations resulted in the decrease in the levels of the protein in the soluble fraction (Fig 5 B). Taken together, our data suggest that the mutations found are disruptive and destabilizing for the protein. This further suggests that the improvements in tolerance are likely due to improvements in the efficiency or mode of action of the pump, rather than increased protein production or stability.

Several crystallographic and site-directed mutagenesis studies of AcrB have determined the role of domains and of several amino acids in the protein. These data allow us to hypothesize about the effect of some beneficial mutations. The tolerance improvement of the F927S and F1033Y mutation is unclear since the mutation is not associated with known functional domains. However, the mutations A279T, Q584R, F617L and L822P were localized in key positions known to be important for pump function. The amino acid L822 is positioned between two  $\beta$ -sheets, at the “ceiling” of the vestibule of the pump, suggested to be a highly probable substrate entrance point, and the mutation of a leucine to a proline at this location possibly altered the flexibility and/or the opening of the vestibule facilitating 1-hexene entrance into the pore. The amino acid A279 is located in the binding pocket in which residues Glu273, Asn274, Asp276, Ile277, play an important roles. Why the introduction of a polar amino acid at this position would improve the efflux of hydrophobic compound is unclear, but every substrate must eventually leave the binding site in order for its efflux to occur, so the substitution A279T (hydrophobic to polar residue) may help substrate release to the gate and the funnel. Q584 is located in a position potentially involved in trimer assembly. The AcrB subunit is reported to fold independently, and then assemble into a trimer. It has been shown that the P223 from one AcrB interacts with Q584 from another AcrB polypeptide, and is required for the assembly and stability of the trimer. The mutation Q584R could thus impact trimer assembly or/and stability. Finally, the amino acid F617 has been shown to be located in the switch loop of the hydrophobic binding pocket. Reported crystal structures suggest that this amino acid could directly interact with the pump’s various substrates. Mutating this amino acid F617 to an alanine has been reported to have a direct impact on substrate uptake and was responsible for a substantial decrease in transport of novobiocin, but had a minor effect on the transport of oxacillin and various other macrolides. Our results suggest that a mutation to a leucine at this position may improve transport of 1-hexene.

### 3-b/ are they effective with other compounds and more specifically with other alpha-olefins?

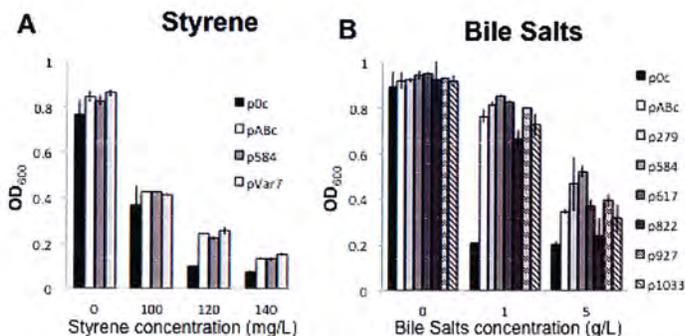
In order to determine if the mutations were specific to 1-hexene or could work with various alpha-olefins, we selected 2 other compounds: 1-octene and 1-nonene. 1-heptene was not selected for its high price.

We first determined that the pump was involved in the transport of these compounds (Fig 6). But we also observed that the compounds and especially 1-octene was not toxic for the wild type cell. Effectively *E. coli* (encoding the pump) can grow on plate between the agar and a thick overlay of 1-octene, to levels similar to the control, i.e. with no substrate was present (Fig 6). Due to the non-toxicity of tested longer chain alpha-olefins we didn't analyze our variants.



**Figure 6: Impact of AcrAB production on growth, in presence of styrene 1-hexene, 1-octene and 1-nonene on cell growth.** A survival test in absence of compound or in presence of 1-hexene, 1-octene, or 1-nonene saturated atmosphere was performed. Six different dilutions of liquid cultures of strain missing the AcrAB pump (left) or producing the AcrAB pump (right) (10 cells to  $10^7$  cells, left to right on the picture) were spotted in triplicate on an agar plates. Plates were then exposed to chemicals and put at room temperature on the bench for 2 days.

However we did tested some of our improved variants in presence of styrene and bile salts (the natural product of the pump). None of the evolved AcrB variants provided a growth benefit in presence of styrene (Fig 7A). Thus, the mutations either provide no advantage against styrene or do not have a measurable advantage. However in presence of bile salts, the mutation Q584R improved the growth of the strain (Fig 7B).

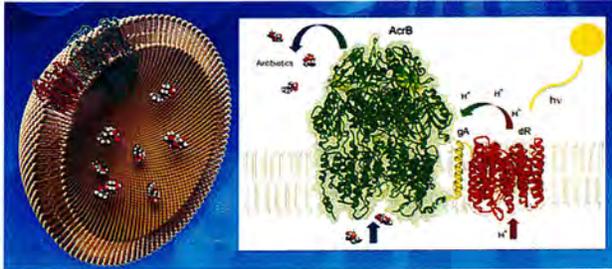


**Figure 7: Impact of beneficial AcrB mutations on growth, in presence of 1-hexene and styrene.** Different concentrations of styrene (A), or bile salts (B) were added to a  $OD_{600}=0.2$  culture of *E. coli* K12  $\Delta acrAB$  containing either the AcrB wild type (pABc) or the AcrB variants. OD densities were monitored after 2 hours of culture at 37 °C. The bars represent standard deviation between duplicates.

### 3-c/ do they also function in other organisms?

Another important test was to determine if we could transfer the AcrB subunit to another organism, specifically *Bacillus subtilis* and *Saccharomyces cerevisiae*. Kapoor. et al, have recently shown that AcrB by itself is functional in a liposome (Fig 8) if coupled with a functional proton transporter (the pump just requires enough  $H^+$  to energize the rotational movement needed to secret compounds).

*Bacillus subtilis* and yeast possess several membrane proteins working with the proton-motive force and both of their cytoplasm is charge negatively. It has been showed in the past that *E. coli* membrane proteins could be cloned in *Bacillus* and yeast and could still be functional. We thus decided to test if *E. coli* AcrB (wild type and variants) could also work in other organisms and especially *B. subtilis* and *S. cerevisiae*.



**Figure 8:** This figure is reproduced from Kapoor V. et al, in 2013, and shows that AcrB (represented in green) can work in a liposome system without AcrA and TolC. The only condition and need is the presence of a  $H^+$  gradient that was generated in this experiment by a rhodopsin (represented in red).

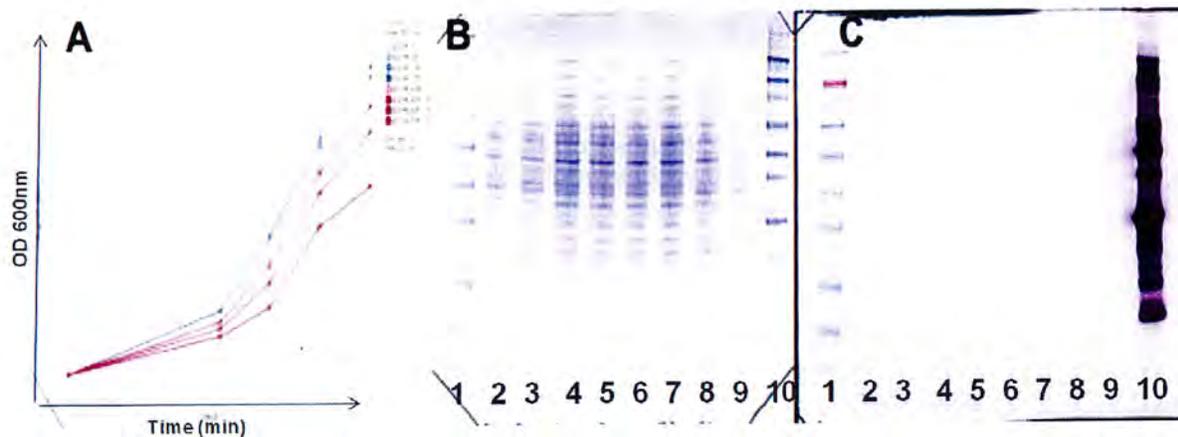
### 3-c-1/ *Bacillus subtilis*

Some *Bacillus* species (*Bacillus cereus*) also produce some proteins homologous to AcrB (Fig 9). The sequence identity is quite low (23% of similarity and 44% of identity) however the length and the pattern of the transmembrane domains is relatively similar. Suggesting that AcrB could possibly be incorporated in the *Bacillus* membrane.



**Figure 9:** In red are represented the hydrophobic domains (transmembrane domains) of AcrB from *B. cereus* (top) and from *E. coli* (bottom). This prediction has been calculated by TMHMM server 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>)

We thus cloned *E. coli* wild type AcrB with its native signal sequence but also with the signal sequence of the *B. subtilis*  $\alpha$ -amylase AmyC or of the *B. cereus* AcrB. The 3 genes, differing only in the signal sequence, were cloned in a shuttle plasmid pDG148 under the control of an IPTG inducible promoter. Each plasmids and the control plasmid (not expressing the pump) were introduced in *B. subtilis* 168. We could determine that by increasing the gene expression of pump by increasing the quantity of IPTG we affected the growth in liquid culture suggesting that the gene was being expressed (Fig 10 A). However no protein could be detected in the total cell protein fraction (even using specific antibodies) (Fig 10 B/C).



**Figure 10: Production of AcrB in *Bacillus subtilis*.** **A/** Growth of *B. subtilis* containing the plasmid with *acrB* and its native signal sequence (in red) or the empty control plasmid (in blue). The different curbs represent the growth of strains in presence of divers concentration of IPTG (10, 50, 100 and 200 μM of IPTG correspond light to darker red/blue curbs respectively). **B/** SDS page gel of total protein from the different *B. subtilis* strains (line 2 and 3: control empty plasmid, line 4 and 5: plasmids containing *acrB* with its native signal sequence, line 6 and 7 withthe *B. cereus amyC* signal sequence and line 8 and 9: with the *B. cereus acrB* signal sequence signal sequence). Line 1 and 10 are ladders. **C/** Western blot using anti-histag on total proteins from different *B. subtilis* cultures (the different lines correspond to the lines as describe in 9/B).

Since a very low (or even undetectable quantity) of AcrB may be sufficient to confer a tolerant phenotype, we decided to test the growth of the different strains in liquid, in presence of bile salts. However no differences could be observed between the strains containing the AcrB genes and the strain containing the control empty plasmid. This result suggests that either the protein is not produced or that the small quantity that could be produced is not functional. Reverse transcriptase PCR experiments confirmed that full length mRNA was indeed being produced by the cell, so the expression/translation didn't seem to be problematic, however the fact that no protein was detected could reflect some traductional problems. The gene was not codon optimized for *B. subtilis*, some rare codons could affect the protein production or the protein might not be properly assembled or/and incorporated to the membrane resulting in its degradation.

At this time we were at the end of the funding cycle and of time to order a synthetic gene, we thus focus our last experiment on *S. cerevisiae*.

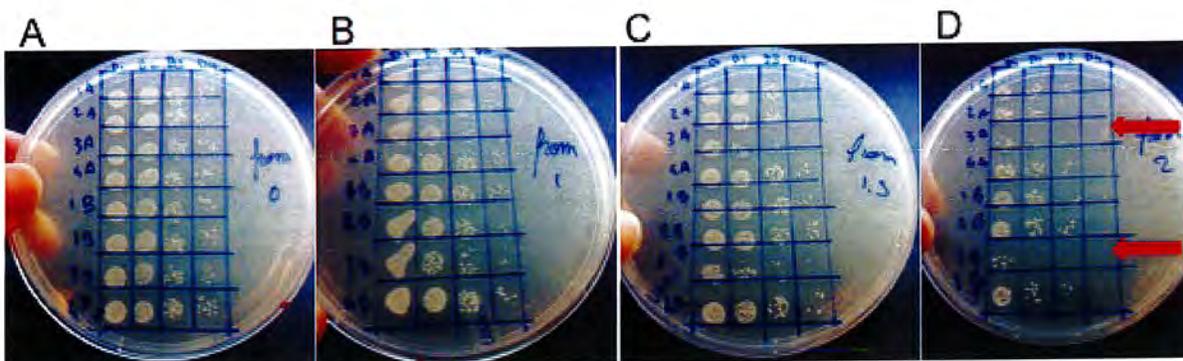
### 3-c-2/ *Saccharomyces cerevisiae*

In order to obtain a "sufficient" production of pump in yeast, we ordered a codon optimized version of *acrB*. The gene was ordered to "gene script" in August 2013 but was delivered only in November, with limited time and funds remaining in this project. We cloned the gene in 2 shuttle *E. coli*-yeast plasmids: one high-copy and one low-copy, under the control of the promoter of a yeast transporter gene (*pdr5*), and introduced them in yeast cells.

To determine if the pump was functional, we developed a test in presence of various concentrations of bile salts. The 1-hexene saturated atmosphere assay was not functional with yeast (yeast growth is slower than *E. coli* and the 1-hexene evaporation too fast to see any toxic effect of 1-hexene). The bile salts assay consisted in growing the cells in presence of bile salt and then spotting them on agar plate to evaluate their survival. However it proved to be challenging follow the OD using a

spectrophotometer because the bile salts interacted with the casamino acids required for yeast culture and resulted in a precipitate and interfering with OD reading.

However our first set of results were surprising: with the low copy plasmid it appears that AcrB had a positive impact on growth in presence of bile salt (Fig 11). However with the high copy plasmid the pump didn't have any impact on growth in presence of bile salts and surprisingly the growth of the strains possessing the high copy plasmid, with or without the pump, was similar to growth of the strain expressing the pump on a low copy plasmid (Fig 11).



**Figure 11:** Impact of producing AcrB in yeast when the strain is cultured in presence of bile salts. All the agar plates contain Ura-YPD (rich medium) and no bile salts. The spots are dilutions of cultures that grow in absence (A) of bile salts or in presence of 1 g/L (B) 1.5 g/L (C) or 2 g/L (D) of bile salts. 4 strains (1A and 1B are duplicates of the empty high copy plasmid; 2A and 2B are duplicates of the high copy plasmid carrying *acrB*; 3A and 3B are duplicates of the empty low copy plasmid (red arrow: strain (in duplicate) with a different phenotype) and 4A and 4B are duplicates of the low copy plasmid carrying *acrB*).

To explain these results several additional analyses are required: analysis of gene expression, protein production and protein localization as well as plasmid stability. These experiments were not possible to within the remaining time and funds and the project was suspended.

#### **4/ Conclusion, main issues and bottleneck of the project**

During the 2 years of the project we were able to generate *E. coli* strains with a better tolerance to 1-hexene by increasing the quantity of AcrAB-TolC pump as well as by engineering the AcrB inner membrane protein. We actually proved that AcrB could accept few mutations that are making it more adapted/efficient to 1-hexene. We were able to study the AcrAB-TolC pump and demonstrate its involvement in tolerance to various toxic chemicals as well as its requirement for the high production level of certain olefins (styrene).

However during these 2 years, we were confronted with difficulties associated with working with highly volatile and/or mutagenic olefins. Some were highly toxic (1-hexene), some less toxic (1-octene). It was impossible to predict the effect of chemicals on cells and their level of toxicity. For these reasons we were obliged to find and develop a large number of different assays and we had continually adjust our experimental strategies to the chemicals or the organisms that we were working with. One of the main disadvantages of this inability to use assays without modification was that we had to start from the beginning every time we evaluated a new chemical and/or a new organism. This included the assessment of the toxic concentrations, the optimal co-solvent, the quantity required to saturate the atmosphere, the cell density needed to see a phenotype etc.

Finally, the main bottleneck of this project was to not have a strain able to produce in sufficient quantity a “stable” (non-mutagenic) compound such as the originally planned 1-hexene. For the highest impact of our study we needed to be able to ask the question: can improved and engineered pumps increase production of olefins? Despite tremendous efforts, 1-hexene could not be reliably demonstrated to be microbially produced. Styrene would have been an adequate substitute to test our hypothesis but was highly mutagenic. Per previous studies at JBEI (Dunlop et al 2011) short chain alcohols are not natively addressed by AcrAB-TolC. And finally most of the olefins with demonstrated microbial production (pinene, limonene...) were excluded in the scope of work. However, despite these challenges the project was successful in meeting several goals, generated several interesting results and a huge knowledge, and led to an interdisciplinary collaboration to successfully generate a patent and a scientific manuscript.

## **5/ References**

- (1) Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. 2006. *Mol Syst Biol.* 2:2006.0008.
- (2) Turner RJ, Taylor DE, Weiner JH. Expression of Escherichia coli TehA gives resistance to antiseptics and disinfectants similar to that conferred by multidrug resistance efflux pumps. 1997. *Antimicrob. Agents Chemother.* 41(2):440-4.
- (3) McKenna R, Nielsen DR. 2011. Styrene biosynthesis from glucose by engineered E. coli. *Metab Eng.* 13(5):544-54.
- (4) Kapoor V, Wendell D. 2013. Engineering bacterial efflux pumps for solar-powered bioremediation of surface waters. *Nano Lett.* 13(5):2189-93.
- (5) Dunlop MJ, Dossani ZY, Szmidski HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A. 2011. Engineering microbial biofuel tolerance and export using efflux pumps. *Molecular Systems Biology* 7:487.

## **DISCLAIMER**

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.