CBMNet
CROSSING BIOLOGICAL MEMBRANES NETWORK, A BBSRC NIBB

The University Of Sheffield.
THE UNIVERSITY of York
BBSRC bioscience for the future

Introduction to Industrial Biotechnology

Lecture 5

Consolidated bioprocessing

Learning outcomes

- Understand the costs of an cellulosic biofuel plant
- Appreciate the concept of ‘consolidated bioprocessing’.
- Consider how proteins are secreted at high yields
- Critically analysis an ambitious attempt at consolidated bioprocessing (the journal club).

A working cellulosic ethanol plant

- **ImbiCon** are based in Denmark and have a large operational cellulosic biomass biofuel plant producing bioethanol (http://www.youtube.com/watch?v=ohktkpuLQPg)
- Partner with **Genencor** who provide the **enzymes** that they need at scale.
- After a chemical or physical pretreatment to break open the lignin, a battery of cellulases and hemicellulases are needed to release the sugars. This is a significant component of the overall costs of the process (nearly as much as the feedstock) as they are added at high concentrations, over **100 g/l** solid.

The cost of enzymes

- The cost of enzymes is not new & is a major costs for 1st generation biofuels.
- Huge market for enzymes like **amylase** used to break down starch.
- Lucrative market, as the enzymes are only used once!

- Massive industry for companies like Novozymes and DSM.

- **How do <u>they</u> makes these enzymes?**

- As they are making a 'high value product' they use more defined growth media & do standard batch and fed-batch fermentations (see Molecular Biotechnology).

- Generally use fungi (*Aspergillus oryzae*) or *Bacillus subtilis* (and other species) to make their primary enzymes as these are good at secreting certain proteins **naturally**. NOTE: mainly NOT recombinant protein expression.

- Certain strains of *Bacillus* can secrete large quantities (**20-25 g/L**) of extracellular enzymes.

The future? Consolidated bioprocessing

- An idea to integrate into a **single organism** the ability to:
  1. degrade cellulosic material directly in the timescale of an industrial fermentation and use the released sugars (including pentoses) to convert these various chemicals into a biofuel.

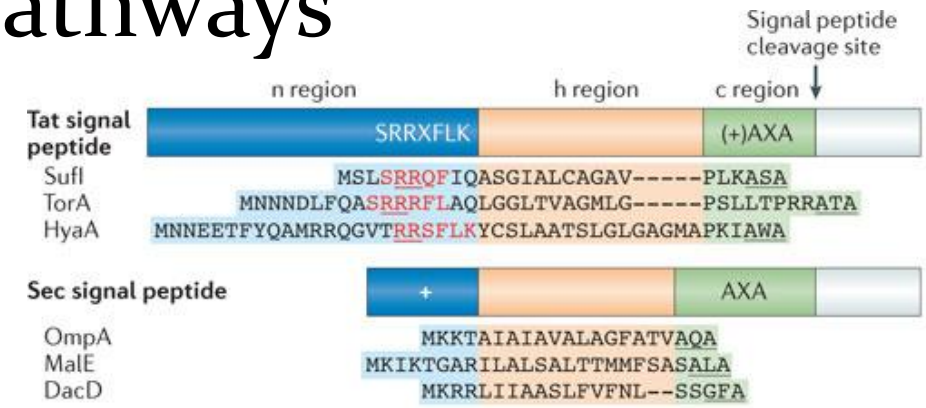
- To do this, either need to take a bug that is:
  1. Good at making a biofuel and add the ability to secrete large amounts of cellulolytic enzymes and degrade cellulosic sugars.
  2. Good at secreting celluloytic enzymes and add biofuels production pathways.
  3. Take a model organism which is easy to engineer and add whatever phenotypes you need to put both of these characteristics in (see this week's paper).

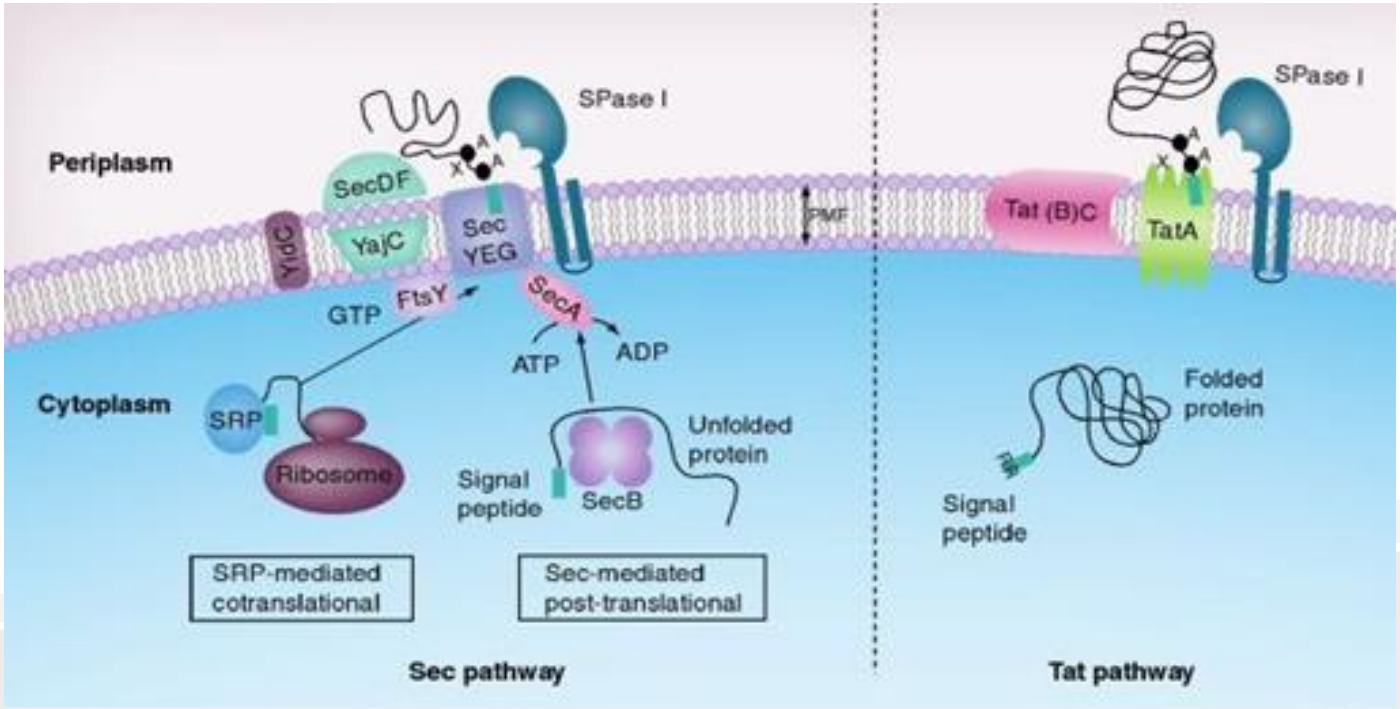
The biggest challenge is creating the capacity for **very high** levels of **protein secretion (>100 g/l)** and activity to degrade the feedstock in a short time period and to **use multiple sugars at once**.

Protein secretion

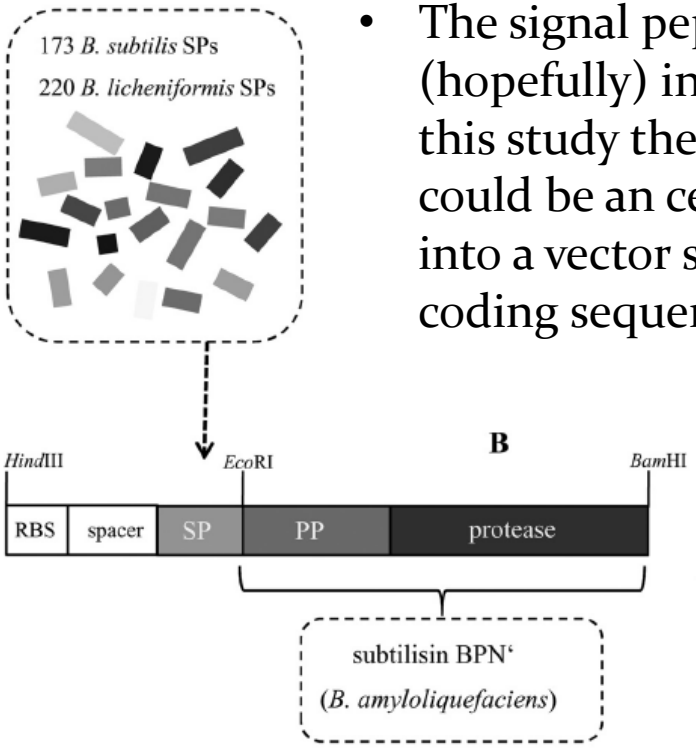
The Sec and Tat pathways

- Proteins are secreted across the cytoplasmic membrane by either the Sec or Tat pathways.
- A signal peptide can be added to direct any soluble protein for secretion.



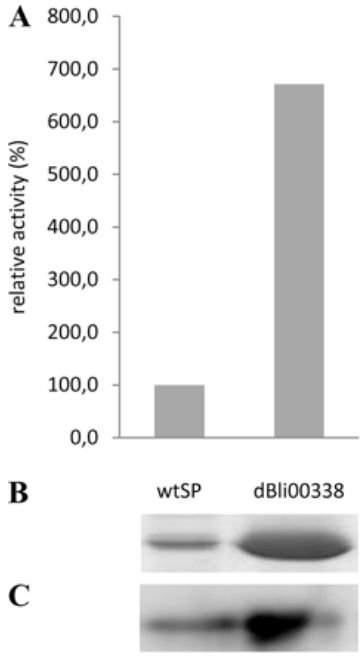


Empirical screening to improve secretion



- The signal peptide can be mutated to (hopefully) increase the level of secretion. In this study the gene encoding a protease (this could be an cellulase or hemicellulase) is put into a vector system whereby the signal peptide coding sequence can easily be changed

- Used nearly 400 different natural signal peptides and found one that gave nearly 700% greater activity!



- The number of copies of the secretion apparatus itself can be increased.
- For Gram +ve's the cytoplasmic membrane is the only barrier to secretion.

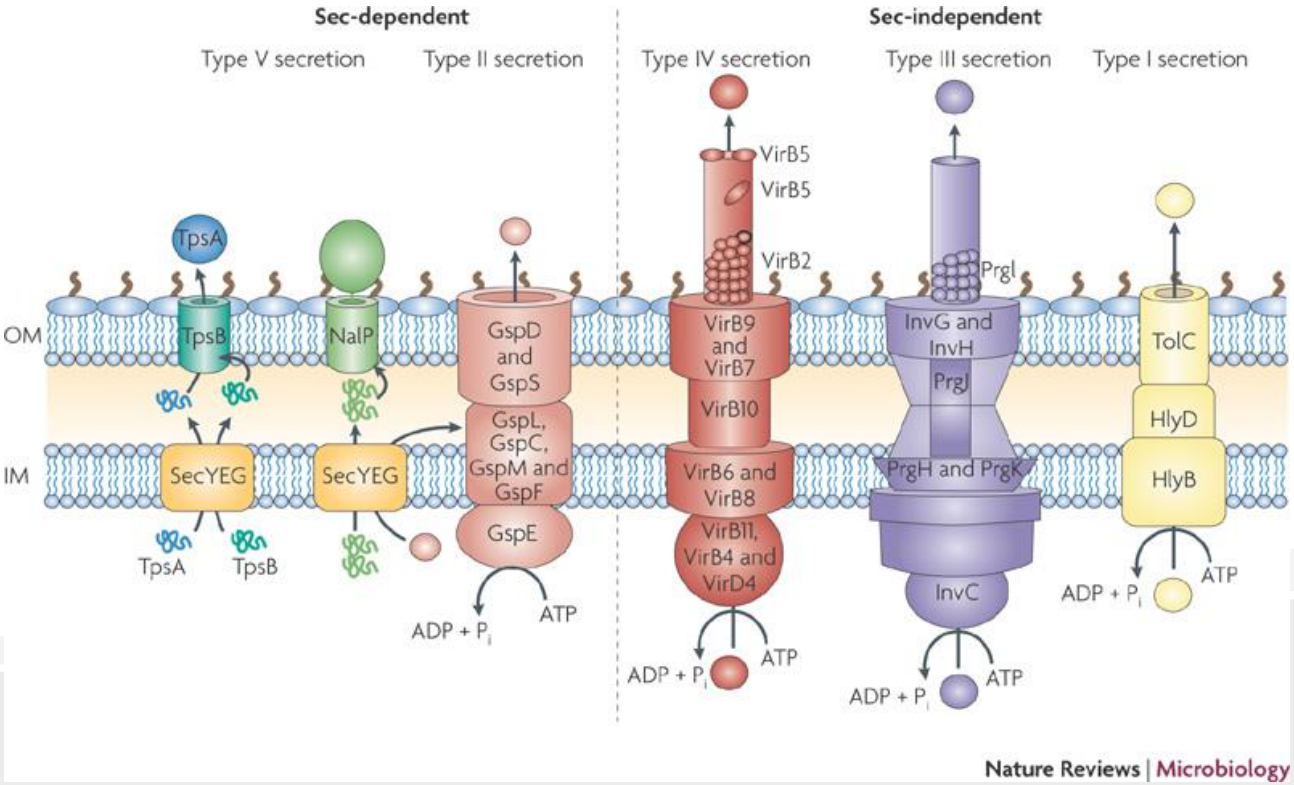
Secretion in Gram –ve's

The problem of the outer membrane

In Gram –ve bacteria there is a second barrier to cross to enable secretion.

2 main routes that could be useful for consolidated bioprocessing.

1) Use a secretion systems from a pathogen, e.g. Type I-VII
2) Make a fusion to a protein that is secreted



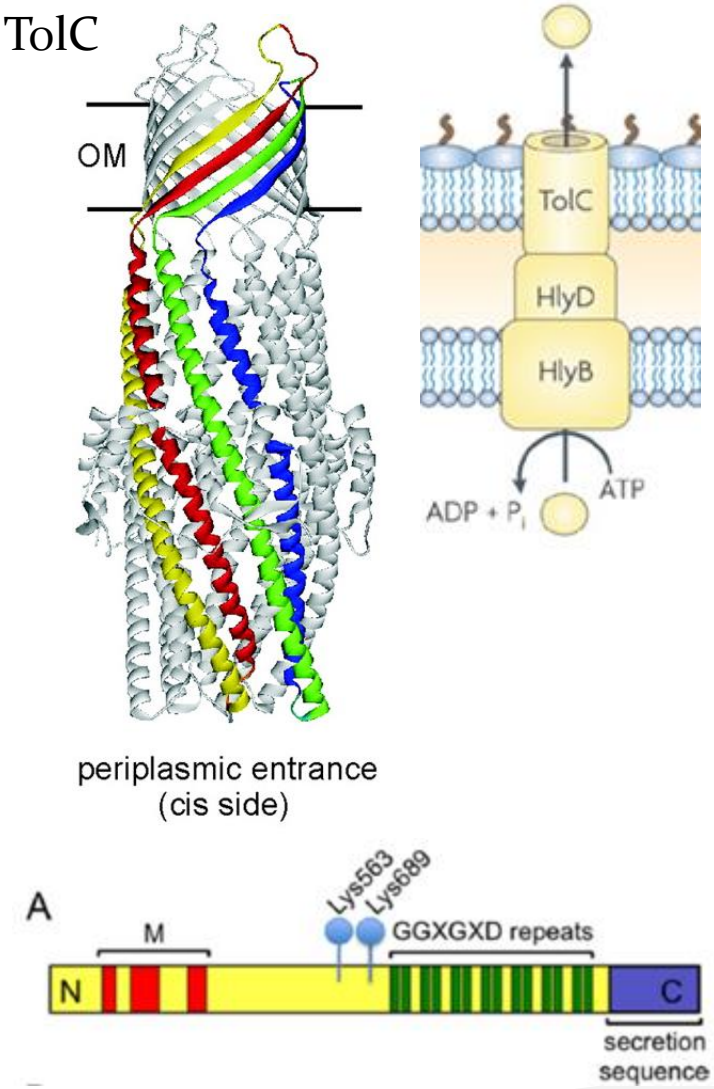
Type I secretion

The protein is moved through a complex that spans both membranes with no periplasmic stage.

- TolC is a OM protein that can reach into the periplasm to couple with other proteins
- HlyBD are the ATP-dependent inner membrane components that bind the substrate protein and catalyse their export.

Natural substrate is HlyA. This has a **C-terminal** secretion signal and is exported as an unfolded protein to the extracellular environment.

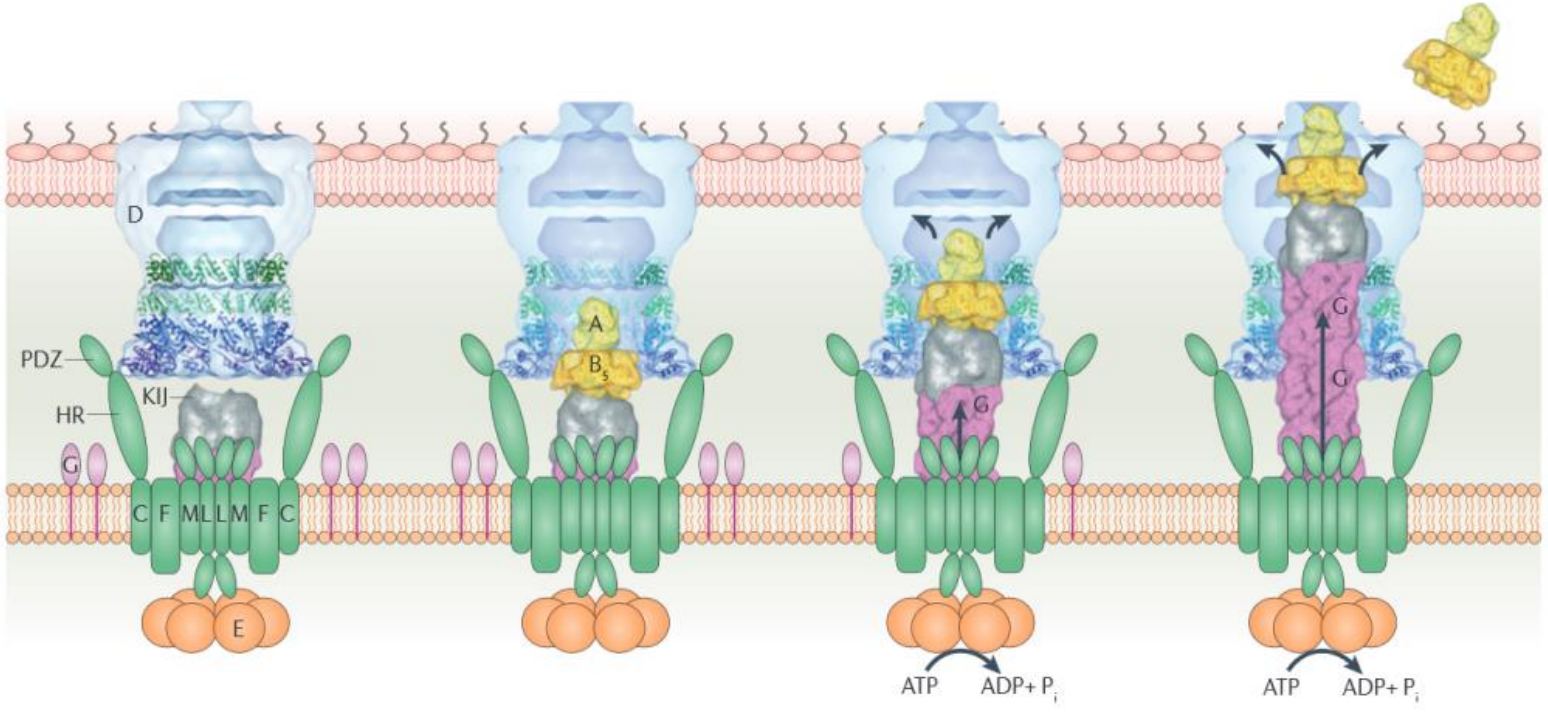
On the outside the GGxGxD motif uses free Ca^{2+} ions to help the protein fold.



E. coli will secrete a range of recombinant proteins just by adding the secretion signal. Yields (best 0.1 g/l) however, are not sufficient for consolidated bioprocessing.

Type II secretion

- A 2-step mechanism, with the protein first secreted via Sec to the periplasm, where the signal peptide is removed and the exoprotein folds & waits for secretion.
- The exoprotein binds to the parts of the T2SS apparatus, which stimulates the ATPase activity of GspE so that pseudopilin subunits are added to the pseudopilus.
- The growing pseudopilus physically ejects the exoprotein out across the OM.

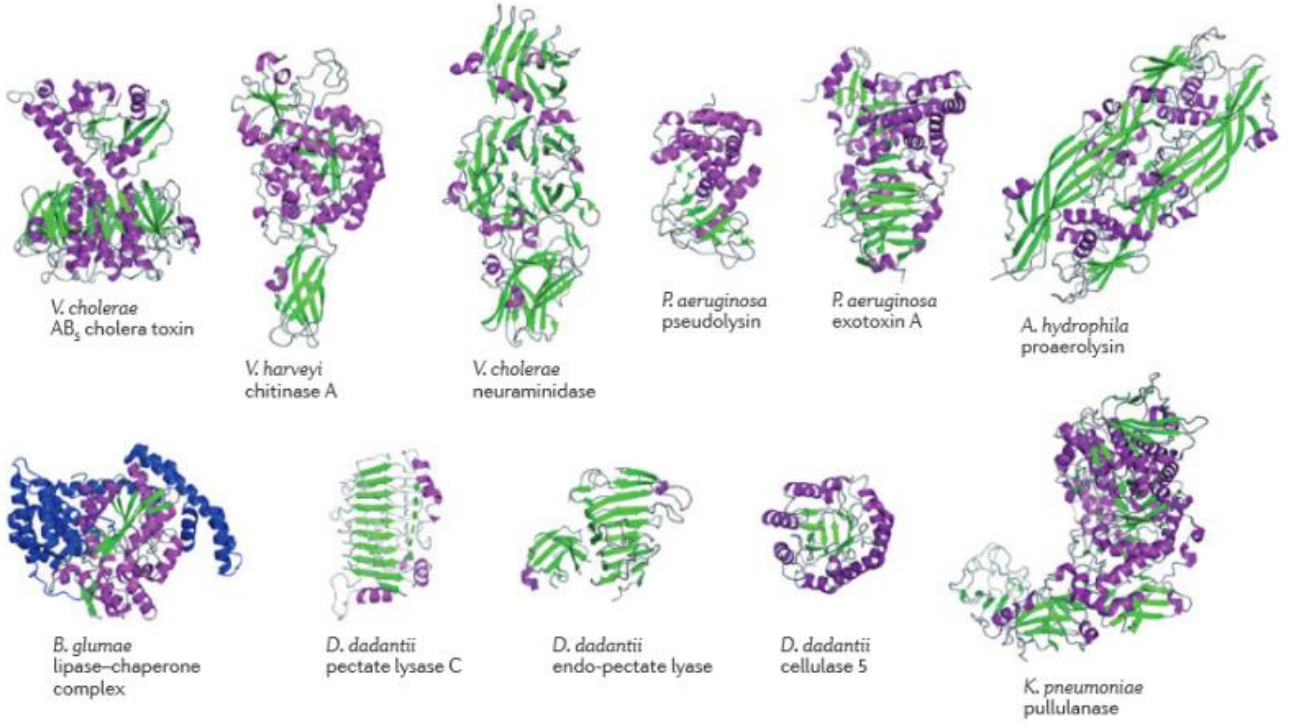


A secretion system with no signal?

A number of Gram-ve bacteria use these systems to secrete degradative enyzmes into their environment, like **sialidase** from *Vibrio cholerae*, **chitinase** from *V. harveyi* and a **cellulase** from *Dickeya dadantii*.

The T2Ss signal has yet to be identified. Many substrates have a significant beta-sheet content, but not all. Makes this difficult to engineer!

Also, limited yield at present



Protein fusions for secretion

- Well-known that making gene fusions between a recombinant protein and a carrier protein, like maltose binding protein, results in high levels of protein in the periplasm.
- San Yup Lee's group identified the secreted proteins from *E. coli* using proteomics
- Overexpressed each in turn to see which secreted to high levels.
- Chose **OsmY** as their best protein and showed it would lead to the secretion of a number of proteins, including alpha-amylase.

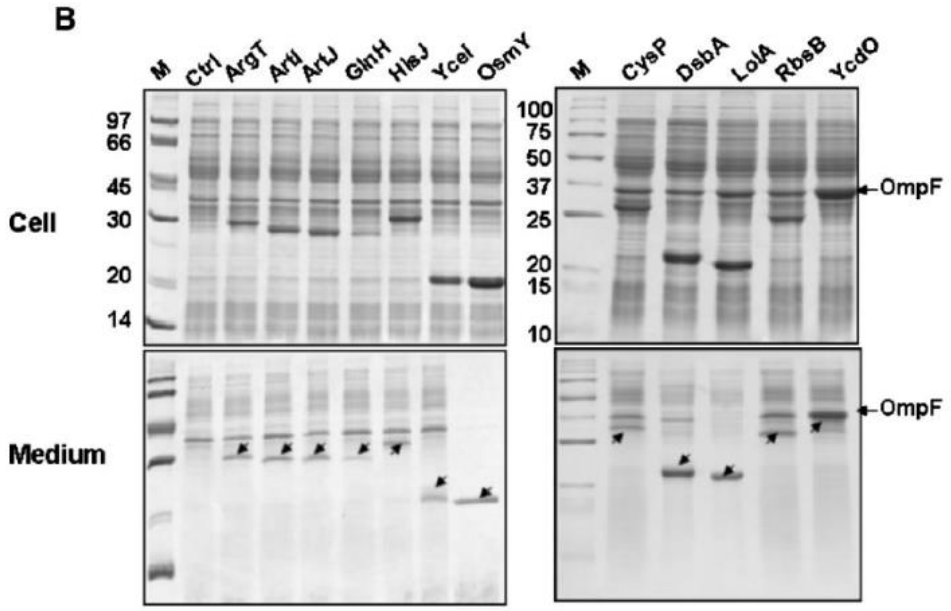


Figure 2. The results of overexpression and excretion studies on 20 potential fusion partners. Recombinant BL21(DE3) cells carrying the plasmids overexpressing these 20 potential fusion partners were cultured in LB medium in flasks at 37°C, and induced with 50 μM IPTG except for OsmY and FkpA (10 μM). Samples were taken at 4 h after induction. **A**: Classification of expression and excretion for 20 potential fusion partners. The percentage of fusion partners in each category is shown in parenthesis. The fusion partners are indicated except for those classified "expression and excretion," which contain all the rest. **B**: Protein profiles in cells and medium for the 12 excreted fusion partners. BL21(DE3) harboring pTrc99A was included as a control (Lane Ctrl). Proteins from cell samples (0.024 mg) were separated on 12% SDS–PAGE gels (**top panels**). Proteins from culture medium samples were separated on 15% SDS–PAGE gels (**bottom panels**). Loaded on the gels were aliquots corresponding to 20 μL of culture medium for OsmY without TCA precipitation, 0.4 mL of culture medium for DsbA and LolA, and 2 mL of culture medium for the others after TCA precipitation. Arrows indicate the respective target proteins in the culture medium. The position of OmpF is also indicated. Molecular size markers are shown in kDa.

Wider reading

- Reed & Chen (2013) Biotechnological applications of bacterial protein secretion: from therapeutics to biofuel production. *Research in Microbiology*. **164:**675-682.
- Desai & Rao (2010) Regulation of arabinose and xylose metabolism in *E. coli*. *Appl. Environ. Microbiol.* **76:**1524-1532.
- Degering *et al*., (2010) Optimization of Protease Secretion in *Bacillus subtilis* and *Bacillus licheniformis* by Screening of Homologous and Heterologous Signal Peptides. *Applied & Environmental Microbiology.* **76:**6370-6376.
- Olson *et al*., (2012) Recent progress in consolidated bioprocessing. *Curr. Opin. Biotech*. **23:**1-10.