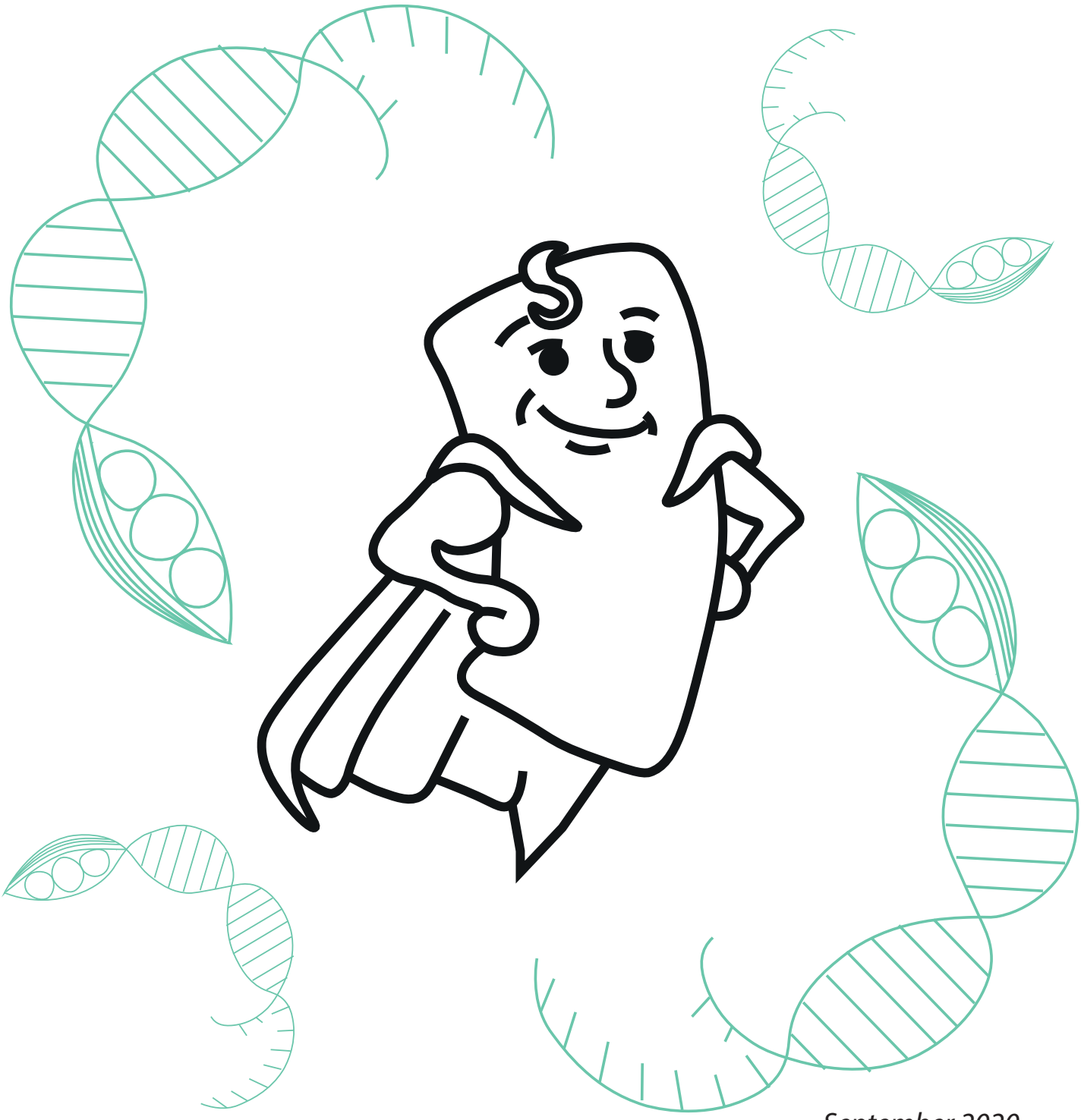
*iGEM Team Brno a.k.a. Generation Mendel presents:*

# Handbook
# How to handle *Bacillus subtilis*

*Everything you have to know before starting in the wet lab*



*September 2020*

# Introduction

This handbook was written by iGEM Team Brno a.k.a. Generation Mendel in September of 2020. We decided to create this easy manual for anyone who is working with B*acillus subtilis* for the first time and are having issues dealing with this bacterium (like we did at the start). We spent many hours working with this organism and we have quite a few useful hacks we'd like to share. We hope that this text will spare you many hours of unsuccessful experiments. Good luck with your project with *B. subtilis*!

We'd also like to thank iGEM team UofUppsala for their help. They sent us great documents detailing their troubleshooting and shared with us (and now also with you) their protocols and workflows. So many thanks to our Swedish friends!

First, we will shortly introduce this microorganism to you from many different perspectives. Then we will discuss the suitability of *B. subtilis* for synthetic biology and afterwards we will share some lab hacks.

## In our Handbook you can find:



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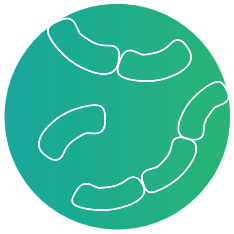
# Biology and genetics of *Bacillus subtilis*



## Microbiology of *Bacillus subtilis*

*B. subtilis* is a model organism for Gram-positive bacteria. Its rod-shaped cells are usually 4-10 µm long and their diameter varies between 0.25 and 1 µm. Their colonies are circular, slightly raised, opaque, smooth and off-white. In an adverse environment, it is able to produce endospores and thus survive till its conditions get more favourable. It can be found in soil and in the gastrointestinal tract of some mammals, including humans [1].

*B. subtilis* is widely used in biotechnologies and molecular biology. Due to the plethora of information and genetic tools available, it is considered to be the Gram-positive equivalent of *Escherichia coli*. *B. subtilis* is also recognized as safe (GRAS) [2].

## Phylogenesis and nature habitat of *Bacillus subtilis*

*B. subtilis* belongs to the genus *Bacillus*, which includes other widely known species, such as *B. cereus, B. licheniformis* (used for the production of antibiotics) and *B. thuringiensis* (used for production of specific insecticides). Most members of this genus are non-pathogenic or low pathogenic, with the exception for *B. anthracis*. The family *Bacillaceae* is related to *Staphylococcaceae*, as these two families belong to the class *Bacilli*. *Bacilli*, together with *Clostridia*, forms a group of low GC Gram-positive bacteria, called *Firmicutes* [3].

Its complete taxonomy can be found here: *http://lifemap.univ-lyon1.fr/explore.html*

As a member of Gram-positive bacteria, *B. subtilis* lacks outer membrane, which enables extracellular protein production. This makes *B. subtilis* rather useful in biotechnology [4]. Without the protective outer membrane, Gram-positive bacteria need other mechanisms to protect themselves against adverse effects from the environment. For example, their cell wall modifications protect them against lysozymes [5].

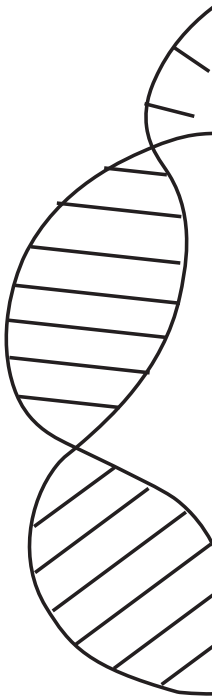
*B. subtilis* can be commonly found in upper layers of soil and gastrointestinal tract of some mammals, including humans. *B. subtilis* is a strictly aerobic organism and its optimal growth temperature ranges from 30 °C to 37 °C, with a minimal temperature of 18 °C and a maximum of 43 °C [6].

The formation of endospores is typical for the genus *Bacillus*. In cases of nutrient starvation, the cells form internal compartments, which then develop into endospores. These endospores have an inner and outer membrane and can thus resist extreme physical and chemical conditions. The dormant cells become vegetative again through the process called germination. This is typically triggered by signals like amino acids and peptidoglycan muropeptides originating from growing cells [7].

*B. subtilis* can form biofilms under certain conditions. Biofilm is a structure which helps bacteria adhere to surfaces, communicate and protect themselves. Biofilm is formed mainly at the air-liquid interface. The depletion of dissolved oxygen [8] and adsorption [9]

also contribute to biofilm formation. Biofilm is beneficial for the survival of the colonies, as it can help protect them from protozoal predators, changing climatic conditions, nutrient depletion or unfavourable pH [8,10].

The presence of proteases, especially subtilisin and neutral protease (nPro), which can affect production of recombinant proteins may be problematic for industrial use of *B. subtilis*. There are however different strains available, with deletions in the genes coding these enzymes. An example of such strain is the widely known *B. subtilis* WB800, which has 8 main extracellular proteases deleted. These enzymes also play a crucial role in biofilm formation [11].



## Strains

In our project, we worked with *B. subtilis* BSB1 wt-derivative 168 trp+ strain. We were however unable to demonstrate successful protein expression due to lack of time.

Our Swedish colleagues designed their project and realized that they would need to protect their secreted proteins. *B. subtilis* naturally produces a considerable amount of proteases. So, they contacted the Bacillus Genetic Stock Center, and managed to get a hold of KO7 - a *B. subtilis* strain with deletions in 7 naturally occurring protease genes. With less proteases secreted into the extracellular media, the half-life of their secreted protein would increase, and their system would thus become more efficient. Unfortunately, they did not have the time to work with this strain.

About this strains you can read more here: *http://www.bgsc.org/new.php?page=3*

## Genetics of *Bacillus subtilis*

*Bacillus subtilis* was the first Gram-positive bacterium with known genome sequence - it was sequenced in 1997 [12]. Approximately 4000 genes are organised into ~1500 operons. Just over half of the genome is required for cell processes, intermediary metabolism and macromolecular synthesis, while a significant proportion of the remaining genome is used for growth and survival in the environment [13].

The most common technique of introducing isolated DNA into *B. subtilis* is natural transformation. Imported DNA can be very efficiently recombined with its homologous sequences on the chromosome. Expressing the gene of interest directly from a plasmid is possible, although the success rate is usually lower than in the case of chromosomal integration. The reason is simple: vectors are converted into a single-stranded form and randomly fragmented during entry. This is not an issue for integration plasmids, since they do not have to remain fully functional and self-replicating. The relevant genes are 'rescued' by integration at the targeted insertion site. AmyE site, coding α-amylase, is often used for the integration, as its success can be then proven by the AmyE test [13].

When designing your project, use vectors with sites for ectopic integration into the chromosome!

## Vectors

Plasmids used in the CYANOTRAP project are synthetic vectors designed for ectopic integration into the chromosome of *B. subtilis*. We used plasmids pDG1664 and pDG3661 (Fig. 1). They are "shuttle vectors" which means that they can be used in *E. coli* and *B. subtilis* with different selection systems. The origin of replication is recognized only in *E. coli* and the plasmids cannot replicate in *B. subtilis*. As a consequence, these plasmids can be propagated as episomes in *E. coli*, but must be integrated into the *B. subtilis* chromosome immediately after transformation to provide antibiotic resistance. The integration takes place through a double recombination event.

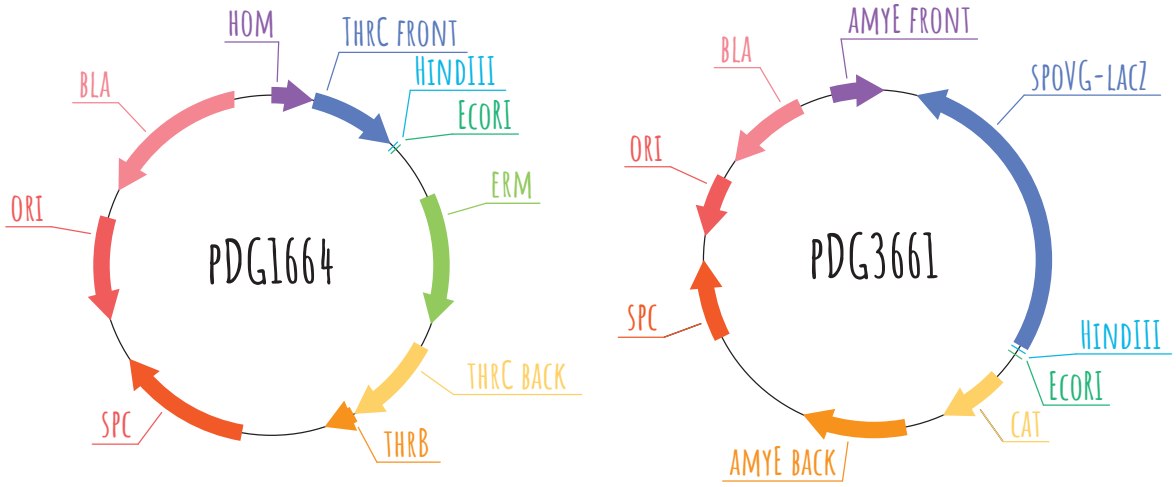



Figure 1. Scheme of pDG vectors

GeneBank identificators:
pDG1664: U46201.1
pDG3661: AY618310.1

Team UofUppsala chose two integration schutte vectors: plasmids pBS1C and pBS2E (although the protocol optimization experiments were done only with pBS1C). Unlike us, they linearized the plasmid by ScaI and PvuI restriction enzyme prior to transformation, as it facilitates DNA intake by *B. subtilis*. They performed integration into the AmyE locus and LacA locus by plasmids pBS1C and pBS2E, respectively. They selected transformed cells with ampicillin and chloramphenicol and also with erythromycin and lincomycin. Their plasmids contain marker *mRFP* for special Red-White screening in *E. coli*. They had better results when working with pBS1C. Team from Uppsala also performed Double transformation with these vectors.

pBS1C: Addgene: #55168 [14]
pBS2E: Addgene #55169 [14]

# *Bacillus subtilis* in synthetic biology

When searching for tools to modify a microorganism, you will most likely come across tools targeting *E. coli* [15]. No wonder, *E. coli* is a gram-negative model organism of synthetic biology. Nowadays, we are in the golden age of this field of science and as scientists take more and more interest in gram-positive organisms (such as *B. subtilis*) a large number of tools have been also recently designed for them.

We will now try to provide interesting and perhaps convincing information that could be useful when deciding which chassis is most suitable for your project.

*B. subtilis* could be called a workhorse for biotechnology. It is an organic factory which in its wild-form can produce large amounts of macromolecular compounds. The yield ranges from grams to tens of grams per litre of medium. One of its biggest advantages is its N-terminal signal peptides, which allow for its proteins to be secreted directly into the medium. Compounds naturally produced by *B. subtilis* (and also *B. licheniformis*) are completely non-toxic, which is also a big advantage [16]. Several studies confirm that *B. subtilis* allows the formation of disulfide-bonds [17].

Today, *B.subtilis* is most likely the best-studied gram-positive bacteria. Since the 1980s, its transcriptome and proteome has been studied thoroughly [16]. It is also one of the organisms used in the studies attempting to minimize the length of the genome. In these studies, they have managed to delete 36.5% of its genome - which corresponds to 1605 genes. Shorter genomes tend to be more suitable for use in biotechnology and synthetic biology. Unfortunately, the generation time appears to have doubled as a result [17]. Solving this issue is currently a priority so that this modified bacterium may soon be applicable for many different purposes. Nowaday, you can find *B. subtilis* strains with deleted protease genes, without a part of the quorum sensing system and much more.

A fairly wide range of pre-prepared tools for *B. subtilis* is also available. There are many synthetic or native promoters that increase the production of heterologous proteins [17]. There is also a RBS library and BioBrick boxes designed specifically for *B. subtilis*. Very interesting and useful tool is a shuttle vector which could be used in three organisms (*B. subtilis, E. coli, S. cerevisiae*). This vector - pEBS (5.8 kb) - was used to characterize and construct Broad-Spectrum Promoters [18]. Finally, there are three Crispr Cas9 tools available. These can be used for large chromosomal deletions, point mutations, single and double gene mutations, gene insertions and to suppress gene expression. More information can be found in the cited review [17].

Of course, even *B. subtilis* has its own weaknesses. The biggest one are issues with heterologous expression of eukaryotic proteins, as its transport through the cell membrane is complicated [19]. Its secretion of proteases into growth medium, instability of plasmids or its sporulation are also not favourable. Most of these problems can however be, and some already are, solved by modifying *B. subtilis* strains or plasmids [19, 20]

# Working with *Bacillus subtilis* in the wet lab

## Rule No.1 : You can NOT store *B. subtilis* cultures for future use in the fridge.

It seems quite trivial, but it can really be a bother sometimes. You can store *E. coli* in the fridge for a long time and it will remain useful for months. Storing *B. subtilis* in the cold is not really the issue. When you take the cells out however, they will often lyse. This can be dealt with quite easily! We will give you a hint in the section below.

## How to cultivate?

*B. subtilis* is very easy to cultivate (if you remember The Rule No.1). We use the same cultivation conditions as for *E. coli*. The overnight cultures grow with no issues at 37 °C and 200 rpm. We usually use 50 ml falcons with 10 ml of LB medium. The larger volume of the flask is better for propper shaking. It is better to lay the flask on its side for better movement of the culture. Although *B. subtilis* grows well also in lower temperature, the laboratory routine is to cultivate it in 37 °C (in fluid medium and also on plates) - colonies are larger and it takes less time. If you want to slow down the growth, you can leave plates at room temperature (e.g. for the weekend), but make sure that the UV light is not used to disinfect your laboratory overnight. Let the colonies grow upside down on the plate.

## How to store Bacillus subtilis?

The best way of storing *B. subtilis*, as well as *E. coli*, cultures is as glycerol stocks. Use LB agar to densely spread one colony of *B. subtilis* (use sterile microbiological loop) on the plate. Following day, harvest the plates and transport the bacteria into 2 ml of liquid LB medium with 20% glycerol (sterile). These glycerol stocks are then stored at -80 °C. You could also use HMFM for preparing stocks. If you need to use a sample from the stock, put it out of the freezer, open it on ice in a flow box and simply scoop up a small amount of frozen culture in the media or on the top of the agar plate with a sterile loop. Work quickly and do not let the stocks melt. Afterwards, you can close the stock and return it to -80 °C.

## How to prepare competent cells?

The preparation of naturally competent cells is necessary for the successful transformation of *B. subtilis*. This can only be done in certain media and in the post-exponential phase of the culture's growth. These competent cells are able to efficiently take up exogenous high-molecular-weight DNA. In the competent culture, not every cell is in its competent state. There should however be enough to generate a sufficient amount of transformants. Their preparation includes a two-step process using glucose minimal salt-based media with Spizizen salts.

This experiment requires the preparation of two different media - SPI & SPII - and an overnight culture of *B. subtilis*. On the next day, the culture has to be inoculated into the SPI medium in duplicate - one for measurements and one for later use. After inoculation, the $OD_{600}$ of the culture must be measured. The cultivation must continue until the values indicate the departure from the exponential rate (T0 - Fig.2). This is very important. This usually takes about 5 hours and 30 min. As the values deviate from

the exponential curve, culture is inoculated into the SPII medium and cultivated under the same conditions for 90 min. Afterwards, the culture is placed on ice to stop its growth and centrifuged. Pellet is resuspended in a fraction of the supernatant and glycerol. Aliquots can then be made and stored at -80°C. You can find the complete protocol in the section Protocols (PROTOCOL 1A) in the end of our Handbook.

It is also possible to prepare electrocompetent cells from *B. subtilis* and perform electroporation. Team UofUppsala initially tried this approach and due to a very low transformation rate, they ended up using an approach similar to ours.

Team from Uppsala used a different protocol, kindly provided by Dr. Isabel Sá-Nogueira from Microbial Genetics Lab from the New University of Lisbon. In this protocol, transformation of the freshly prepared competent cells is performed straight away. Closely measuring the $OD_{600}$ is also required for this method, but only one SP medium is used. After the curve exits exponential growth phase (T0 - Fig. 2) and after 1 h of incubation under the same conditions, 1 µg of DNA should be added to 500 µl of competent cells. Transformed cells should then be incubated for one hour. See more detail in the PROTOCOL 1B.

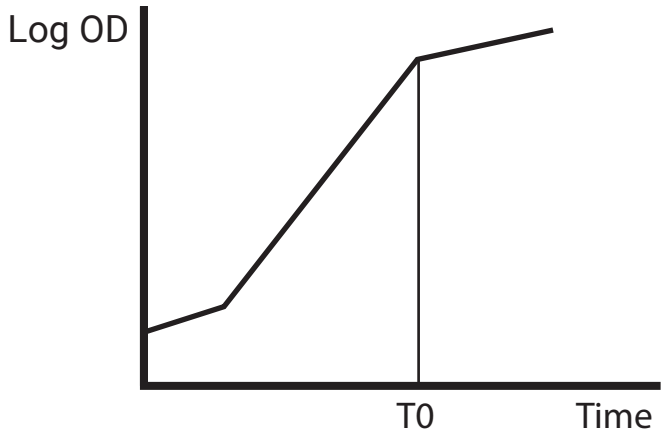



Figure 2. Expected growth curve of a *B. subtilis* colony inoculated in SP Medium. T0 indicates the time point at which the culture exists exponential growth.

Team from Uppsala changed this protocol a little and created a simplified version:
Their most efficient version of the protocol involves a 5h30 incubation in SP medium (with vector DNA added at the beginning), followed by a 1h30 incubation in LB medium. The incubation in LB ensures that the proteins providing antibiotic resistance are being produced before they are plated in media containing these antibiotics. This heavily increases the survival of the cells – after the addition of LB cultivation, the transformation rate improved from 5 to 161 colonies. Since the DNA is always incubated at the very beginning of the protocol, there is less space for variation between trials, which leads to more consistent results.

## How to perform a successful transformation?

As stated above, the best way to transform *B. subtilis* is chromosomal integration. *B. subtilis* upregulates the expression of the DNA recombination machinery when under stress inducing conditions (competency state).

Transformation of *B. subtilis* is quite easy and efficient - if you know how to do it properly. When using naturally competent cells, heat shock is not required. So in this case

the proper preparation of competent cells is the decisive step. The transformation protocol is rather easy. You are most likely transforming *B. subtilis* with plasmid vectors containing your heterologous DNA sequence. Do not purify ligation reactions - use the reaction mixture right away. Our transformation workflow is detailed in PROTOCOL 2.

The naturally competent *B. subtilis* cells are preserved in the form of glycerol stocks. We add 1 µg of plasmid to 100 µl of natural competent cells. Positive and negative controls should also be prepared. The positive control should contain empty supercoiled plasmid carrying antibiotic resistance (of course). You could use the competent cells without plasmid as the negative control. These controls confirm that the transformation was performed well and that the antibiotic was working on non-resistant cells. The tubes containing competent cells and plasmids are incubated on a shaker at 200 rpm and 37 °C for 1 hour (no less, no more). Afterwards, the competent cells are planted on agar plates with a medium containing chloramphenicol (or other antibiotic of your choice). The plate is left for a few minutes in the flowbox. leave the plates to grow (upside down) overnight at 37 °C. Results can be evaluated on the next day.

We also recommend preparing vital control for the competent cells, especially when using a new batch of competent cells. Add a few µl of competent cells to 100 ul of LB, incubate as described above and plant them on LB agar without antibiotics. This control ensures that your cells are still alive after indication of competence.

### UofUppsala also have some tips for successful transformation

Transformed *B. subtilis* colonies may take longer than one day to show up on the plates.

We suspect that too much condensation on the plates may delay growth: certain plates had a specific pattern where colonies in the center were smaller and took longer to grow than those closer to the edges of the plate. So, make sure to keep your plates at room temperature around 2h before usage. If necessary, remove the excess condensation and let them dry near the flame for a couple minutes.

If your transformation efficiency is low, try to linearize plasmid with one restriction enzyme beforehand. We recommend incubating the DNA for longer periods of time, since it requires less enzyme consumption per digestion (these values are usually mentioned in the specifications sheet of the respective restriction enzyme).

### UofUppsala has also some tips for double transformation

Our Swedish colleges transformed both plasmids into the same culture and plated them in LB agar supplemented with erythromycin, lincomycin and chloramphenicol (concentrations are in the table below).

### And here are some tips for this experiment

The easiest approach would be to transform two plasmids at the same time. However, due to the inherent low efficiency of natural competence transformation, this method often fails.

A two-step approach might be preferable: *B. subtilis* should first be transformed with one of the plasmids; after an overnight incubation a few colonies should be picked, used for colonyPCR, and then transformed with the second plasmid.

If 2-step double transformation fails, perform a growth curve of a few of the colonies obtained after the first transformation: the presence of an insert in the chromosome will increase the amount of time needed for the colony to reach the stationary phase.

If the transformation yields no results, swapping the order in which the vectors are transformed may solve the issue.

## Which antibiotics could be used for selection of transformants?

Be careful! *B. subtilis* is NOT sensitive to ampicillin. Do not try to select it with this antibiotics. It is not working (our own experience). Vectors that are suitable for *B. subtilis* and also contain the *bla* gene are most likely shuttle vectors and are also used with *E. coli*. The choice of antibiotic depends on the selection system available - very often this is determined by the gene of resistance in the sequence of the vector. In our cause, we worked with pDG vectros which code the resistance for erythromycin, spectinomycin and chloramphenicol. If you want to use erythromycin, it is better to use it in combination with lincomycin (a.k.a MLS selection) - there are always fewer colonies of the negative control after transformation.

Table 1. For plasmids pDG1664 and pDG3661 we used this antibiotics

| ATB | Usage for: | Vector | Stock concentration | Dilution in | Final concentration |
|---|---|---|---|---|---|
| Ampicillin | selection of transformants in *E. coli* | pDG3661<br>pDG1664<br>pBS1C<br>pBS2E | 150 mg/ml | water | 100 µg/ml |
| Erythromycin | selection of transformants in *B.subtilis* , in comb. with lincomycin | pDG1664 | 10 mg/ml | ethanol | 0.5 µg/ml* |
| Lincomycin | selection of transformants in *B.subtilis* , in comb. with erythromycin | pDG1664<br>pBS2E | 25 mg/ml | water | 12.5 µg/ml* |
| Chloramfenicol | selection of transformants in *B.subtilis* | pDG3661<br>pBS1C | 50 mg/ml | ethanol | 5 µg/ml |

* Combination of erythromycin and lincomycin is called MLS selection. The concentration could vary in different protocols. Our Swedish friends use final concentration 1 µg/ml Erythromycin + 25 µg/ml Lincomycin.

## How to isolate chromosomal DNA

In our project, we tried to isolate chromosomal DNA for restriction digestion and also for sequencing. There are many different methods for this step. We tried two and tested their effectiveness. The first method - we called it the physical method - uses extreme temperature changes. The second - chemical method - utilized a lysis buffer containing lysozyme. The chemical method was not really working. We also tested the impact of the age of our cultures, as DNA cannot be isolated from endospores. As expected, fresh overnight culture gave better results than a culture which was stored for a couple of days.

The physical method was sufficient for the preparation of samples for long-rangePCR, restriction digestion and visualization on agarose gel. The physical method was better but not sufficient for preparation of samples for sequencing. The sequencing reactions were "dead". The cause might be a low concentration of isolated DNA. In PROTOCOL 4 you can find our workflow.

## How to perform colonyPCR?

Team from Uppsalal also performed colonyPCR with their samples. They used a similar method of lysis as we did. After transformation, the colonies could be screened using colonyPCR (a portion of the colony of interest is added directly to the PCR mix). That way, the number of colonies sent for sequencing can be narrowed down as you will get an indication of successful transformation. *B.subtilis* is harder to lyse than *E. coli*, requiring additional steps which you can find in PROTOCOL 4.

### And here are some useful tips for colonyPCR with *B. subtilis*:

Have a positive control – using primers for a gene that is present in both wildtype and transformed *B. subtilis*. For example, iGEM Uppsala 2020 used primers for the RNAse III gene as a positive control for w168 transformations.

Few days after inoculation, *B. subtilis* will start to sporulate. These spores are significantly more resistant to lysis, so it is better to perform colonyPCR on fresh colonies.

## How to perform Chromosomal Knockout?

Chromosomal knockouts are an essential part of synthetic biology. They are commonly used for loss of function studies, both in academic research and in the industrial setting. They are also employed in biotechnology and in projects such as iGEM as a means to increase the efficiency of a given process.

*B. subtilis* upregulates the expression of the DNA recombination machinery when grown under stress-inducing conditions. It is possible to exploit this phenomenon by transforming cells with an insert flanked by 2 homology zones. These fragments will guide the transformed DNA to the desired region of the chromosome. The gene targeted by these homology zones will be interrupted by the insert, thus being knocked out (Fig. 3).

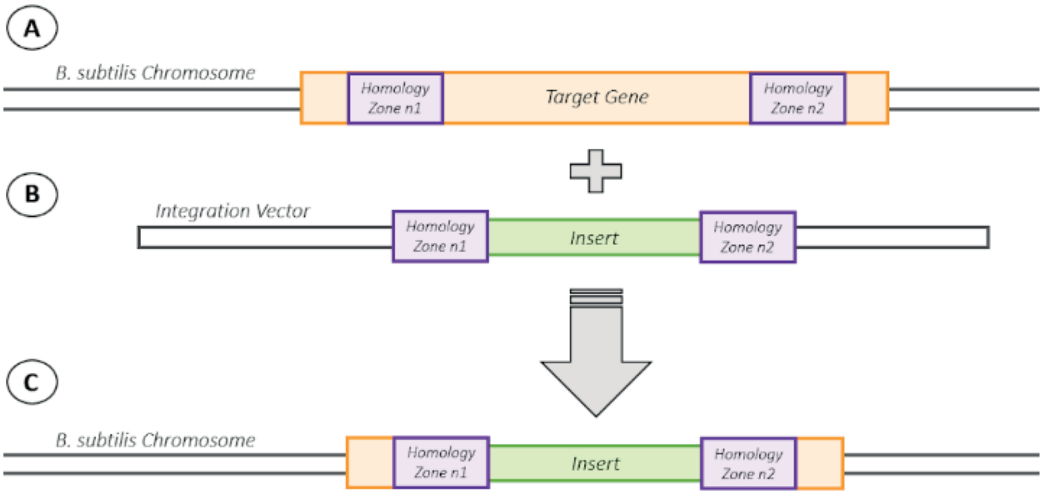



Figure 3. Chromosomal knockout. Plasmidic insert will substitute a portion of a given chromosomal gene, rendering it inactive. A – target gene, uninterrupted and harboring homology zones; B – vector containing the insert and the homology zones; C – target gene, interrupted by the insert.

### The practical use of Chromosomal Knockout is AmyE test or Starch hydrolysis test

AmyE test is a simple method used to verify ectopic integration into the chromosome of *B. subtilis*. The integration site in the chromosome encodes alfa-amylase, which is able to degrade starch in the media. If the integration site is intact, the alfa-amylase is produced, the starch gets digested and a clear halo around the colonies can be observed after iodine treatment. If the integration into chromosome is successful and the sequence encoding alfa-amylase is split by the insert, the enzyme is not produced and starch is not degraded. In our experiment we tried using an AmyE test to confirm the transformation of our *B. subtilis* with our vectors. We compared our samples with *B. subtilis* 168, which was not transformed with any DNA [21].

### Laboratory usage of AmyE test

Two layer agar is used for this method - the first layer is a regular agar containing corresponding antibiotic and the second layer is a thin agar with starch supplement. After the addition of a few crystals of iodine, the starch will turn dark blue. Do not forget to work in a fume hood as iodine fumes are harmful. More information is provided in the PROTOCOL 3.

## How to change homology sequence in plasmid for integration?

In order to perform a targeted knockout of a given gene, the homologous regions must first be extracted from the chromosome through PCR (Fig. 4A). In parallel, the insertion vector that will serve as the backbone needs to have the original homology zones removed through PCR (Fig. 4B). The primers used for these PCRs should have Gibson Assembly adaptors: overhangs of around 20bp, which need to be incorporated into the extremities of both the homology regions and the plasmid (Fig. 4A and 4B). This is done because the last step of this process – Gibson Assembly – requires identical sequences of around 40bp at the edges of the fragments (Fig. 4C). The final product of this assembly is a linearized vector with 2 homology regions for a target gene. This will serve as the backbone for the transformation of the desired insert, which can also be added to the plasmid following the same pipeline.

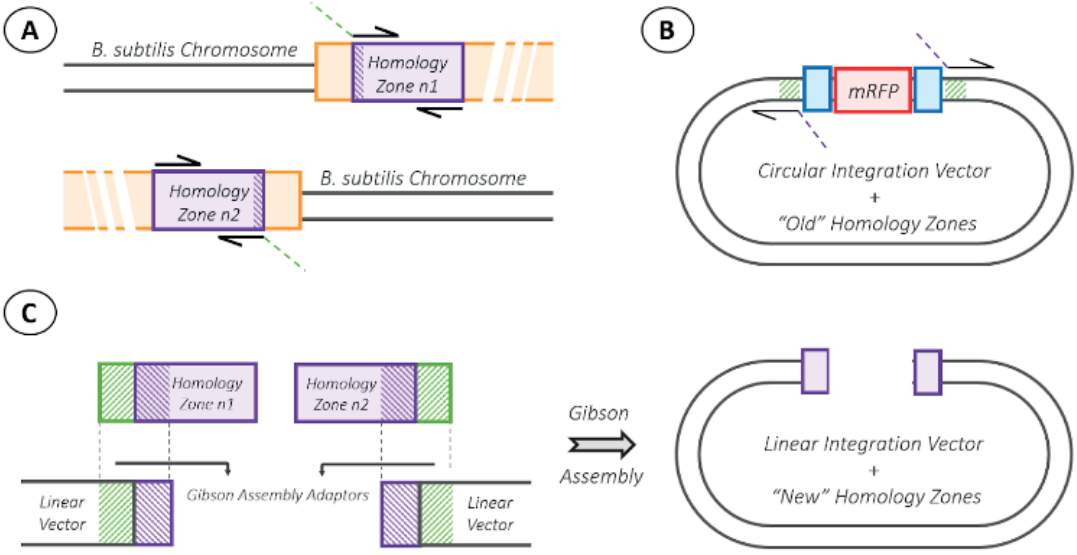



Figure 4. Adding "new" homology zones to an integration vector can be done through Gibson Assembly. A – extraction of the regions from *B. subtilis* chromosome. B – Linearization of the plasmid by removal of both the insert (usually an mRFP) and the "old" homology zones. C – Gibson Assembly adaptors, homologous regions present in the extremities of the fragments, are necessary for the assembly.

# Conclusion and references

Well, we hope that this document will help you out at the beginning of your work with *B.subtilis*. If you need a more detailed description of the experiments, you can find in the Experiment section of our wiki, together with more protocols. If you have some questions or you need our advice, write as an email or contact us through Slack!

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# PROTOCOL 1A: Preparation of competent cells of *Bacillus subtilis*

This protocol includes preparation of two special glucose based minimal salt mediums. The protocol is suitable for preparation of 20 stocks of competent cells (0.5 ml each).

## Material and chemicals:

*Bacillus subtilis* overnight culture (e.g. *Bacillus subtilis* 168) - in LB medium, 37 °C, 200 rpm
87% glycerol - sterile

### Spizizen salts - 1x (for 1 L)

| | |
|---|---|
| $MgSO_4 \times 7H_2O$ | 0.2 g |
| $K_2HPO_4$ | 14 g |
| $KH_2PO_4$ | 6 g |
| Trisodium citrate x $2H_2O$ | 1 g |
| $(NH_4)_2SO_4$ | 2 g |
| $ddH_2O$ | 1 l |

This salt solution is not necessary to be sterile, but if you want to store it for a longer time, it would be better to sterilize it in an autoclave and store it at 4 °C.

### SPI 1x (100 ml) - for one batch of competent cells (20 aliquotes of 0.5 ml)

For medium preparation, it is necessary to keep the order of chemicals for prevention of precipitation. You can sterilize glucose by filtration with 0.2 nm filtr.

| | | |
|---|---|---|
| Spizizen salts | solvent | 100 ml (but calculate with glucose addition!) |
| Casamino acids | 0.02% | 20 mg |
| Yeast extract | 0.1% | 100 mg |
| **now autoclave** | | |
| Glucose - sterile solution | 0.5% (final concentration in media) | (depends in which volume you will sterilize it) |

### SPII 1x (90 ml)

For medium preparation, it is necessary to keep the order of chemicals for prevention of precipitation. You can sterilize glucose, $CaCl_2$ and $MgCl_2$ by filtration with 0.2 nm filters. $CaCl_2$ and $MgCl_2$ could also be sterilized in autoclave but as a separate solution. You could dilute these chemicals in a small amount of $dH_2O$, but be careful about solubility.

| | | |
|---|---|---|
| Spizizen salts | solvent | 90 ml (but calculate with glucose, $CaCl_2$ and $MgCl_2$ addition!) |
| **now autoclave** | | |
| $CaCl_2$ - sterile | 0.5 mM | 2 mg |
| $MgCl_2$- sterile | 2.5 mM | 21.4 mg |
| Glucose - sterile | 0.5% (final concentration in media) | (depends in which volume you will sterilize it) |

## Workflow:

1. This experiment requires preparation of two different media - SPI and SPII. We recommend preparing these media a day before the main experiment and store them in the fridge. The day before you also have to prepare an overnight culture of *Bacillus subtilis* - in 10 ml of LB media, 200 rpm, 37 °C.

2. The next day in the morning the overnight culture has to be inoculated to the prepared medium SPI in duplicate - one for measurement and one for next use. We recommend working in a flowbox to keep everything sterile. Pour 50 ml of SPI medium to sterile Erlenmayer flask and add 0.5 ml of ON culture. Do the same with a second Erlenmayer flask.

3. The culture should be cultivated in a water batch in 37 °C with gentle shaking. Since inoculation you have to measure $OD_{600}$ of the culture. Perform the measurement strictly from the same flask to avoid contamination. You have to cultivate the cells until the values divert from the exponential growth (this is very important). We recommended to do the first measurement after 1 and half hour from inoculation and then measure aftereach 30 minutes. This stage of experiment usually takes around 5 and half hours.

4. In the moment of deviation from the curve of exponential phase of growth of culture, you have to inoculate 0.5 ml from SPI from the second flask from which you did not do a measurement to the 90 ml of SPII medium. Cultivate in the same conditions for 90 minutes.

5. After this time, you have to stop the growth on ice. From now on you have to work on ice. Centrifuge the culture for 5 minutes at 6000 rcf in 4 °C. You have to save 9 ml of supernatant in a sterile falcon and mix it with 1 ml of 87% glycerol. Then resuspend the pellet in this mixture. Now you can make aliquots of 0.5 ml on ice and store them at -80°C.

## Notes:

The cells stay competent for about 1 year, but the competence is going to be lower with longer storage time.

With this protocol you will have around 20 aliquots with 0.5 ml of competent cells. One aliquot is suitable for 5 transformation reactions.

If the lag phase (linear curve) of the culture growth took more than 6 hours - it is better to start the next day again.

After preparation of competent cells the efficiency of transformation is as high as it gets - so use this advantage and do not wait for anything - follow PROTOCOL 2: Transformation of *Bacillus subtilis.*

# PROTOCOL 1B: Competent cells + transfromation from UofUppsala

## Material and chemicals:

Spizizen Salts are distinct only in a few details - they do not use $MgSO_4$ x $7H_2O$ and $(NH_4)_2SO_4$, but the amount of other chemicals corresponds with our recipe

They use only one SP medium with following chemicals

### For 10 ml of SP

| Chemicals | Final concentration: | Addition volume |
|---|---|---|
| Spizizen salts | solvent | 9.215 ml |
| Glucose | 50% (w/v) | 400 µl |
| Casein hydrolyzate | 5% (w/v) | 200 µl |
| Tryptophan | 5 mg/ml | 100 µl |
| Ammonium ferric citrate | 22 mg/ml | 5 µl |
| Potassium glutamate | 40% (w/v) | 50 µl |
| $MgSO_4$ | 1M | 30 µl |

## ISN 1.0 WORKFLOW:

### Day 0

From a single colony inoculate 5 ml of SP medium in a tube and incubate at 37 °C O/N with aeration and 150 rpm.

### Day 1

1. Dilute 1:50 of the O/N culture on an Erlenmeyer flask with 10 ml of fresh SP medium and incubate at 37 °C, 150 rpm.

2. Follow growth by checking $OD_{600}$ every 30 min until the culture exits exponential growth phase (T0). Incubate for further 60 min in the same conditions. (This incubation tends to last a total of around 4h 30min.).

3. Add 1ug of DNA to 500 µl of competent cells and incubate at 37 °C,150 rpm, 60 min.

4. Plate the whole culture in desired media.

5. Resuspension in 50 µl recommended.

## ISN 2.0 WORKFLOW:

Development of competence in *Bacillus subtilis* and Transformation - modified protocol form the ISN 1.0 - without OD measurement

### Day 0

From a single colony, inoculate 5 ml of SP medium in a tube and incubate at 37 °C O/N with aeration and 150 rpm.

### Day 1

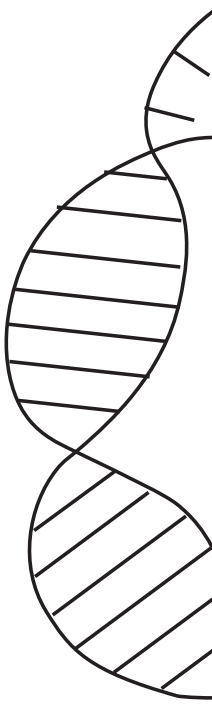
1. Dilute 1:50 of the O/N cultures on a 16×125 mm test tube (or equivalent) with 500µl of fresh SP medium.

2. Add 1ug of DNA and incubate at 37 °C with aeration, 150 rpm, 5h 30min.

3. Centrifuge the culture and resuspend in 1ml of LB medium (pre-heated at 37 °C) (Centrifugation + Resuspension is done in a microtest tube, but the incubation is done in the same tubes as the previous steps).

4. Incubate for 1h 30min at 37 °C with aeration and 150 rpm.

5. Plate the whole culture in desired media.



## PROTOCOL 2: Transformation of Bacillus subtilis

### Material and chemicals:

naturally competent cells *B. subtilis* - glycerol stocks or freshly made, isolated plasmid, plates LB medium and corresponding antibioticum (in this case 5 µg/ml chloramphenicol)

### Workflow:

1. Thaw the naturally competent cells in Thermoblock tempered to 37 °C.

2. Add 100 µl of competent cells to 1 µg of plasmid (in 1.5 ml Eppendorf tubes).

3. Incubate the mixture of natural competent cells and plasmid for 30-60 minutes at 37 °C and 200 rpm.

4. Plant the mixture on LB agar plates with corresponding antibiotic (5 µg/ml chloramphenicol) and leave to grow overnight at 37 °C.

### Notes:

To ensure the test to be conclusive it is necessary to carefully plan and choose suitable positive and negative controls.

The best time of incubation after the transformation reaction was 60 minutes. When the time was shorter (30 minutes) the transformation was not as efficient as 60 minutes incubation. Longer transformation was also not suitable.

## PROTOCOL 3: AmyE test (Starch hydrolysis test)

### Material and chemicals:

Solid 1.6% LB agar plates
Bacterial agar
Starch
$dH_20$

To dissolve starch, you have to bring the solution to the boiling point. Boiling also sterilizes the media - boil at least 15 minutes.

| | |
|---|---|
| Bacterial agar | 1% |
| Starch | 1% |
| $dH_2O$ | 100 ml |
| Boil with mixing until the solution is clear (dark yellow) | |

### Notes:

You can store this solution in a closed flask at room temperature. Boil in the microwave before next use.

Prepare a higher amount of media - you never know how many times you will repeat the test. 100 ml of the media is for preparation of 20 plates.

### Workflow:

1. Weight appropriate amount of agar and starch in a glass flask. Pour in the distilled water and mix properly.

2. Put a magnetic mixing stick inside and put the flask at the magnetic hotplate. Turn the mixing and heating on.

3. Bring to the boil and mix until the solution becomes clear. Do not forget to take out the mixing stick.

4. Let the solution cool down to approximately 45 °C and pour 5 ml of this solution on the top of LB agar which you prepared beforehand (if you want to use antibiotics and you do not have antibiotics in the LBA which were done before, you can also put the antibiotic to this thin starch layer). Let the layer solidify.

5. Steak bacteria onto this special plate. Do not forget to prepare control plates (e.g. *Bacillus subtilis* without integrated gene - count with the fact that these bacteria are not usually resistant to antibiotics, so use a plate without antibiotics). Incubate at 37 °C overnight.

6. The next day in the morning, put a few iodine crystals in the "empty" half of the Petri dish and cover it with the other half that contains the agar and bacteria (agar layer is on the top, the crystals lay straight on the plastic or glass) so the iodine vapor interacts with the starch in the agar.

7. In a few minutes, the starch starts to turn dark blue. Do not forget to take a picture of your plates because the starch will turn lighter after a certain amount of time.

# PROTOCOL 4. Isolation of the genomic DNA of *Bacillus subtilis* and modification of protocol for colonyPCR

## Workflow:

### Isolation of the genomic DNA of *Bacillus subtilis*

1. Prepare an overnight culture.

2. Centrifuge the cells, discard the supernatant and transfer the biomass (amount like if it was a single colony) by microbiological loop into 200 µl of sterile water.

3. Vortex to get a homogenized solution.

4. Boil in a water bath for 10 minutes.

5. Cool in the freezer (ca. for 10 - 15 minutes).

6. Centrifuge 10 minutes on maximum rpm (ca. 13 000 rpm).

7. Add 100 µl of the supernatant into a new 0.5 ml tube.

8. Use 4 µl as a template for PCR reaction (for 40 - 50 µl reaction).

### Modification from Uppsala for colonyPCR

1. With a tip of a pipette pick a portion of the colony and inoculate to the 5 µl of $dH_2O$ in a PCR tube.

2. Incubate at 98 °C for 15 mins.

3. Incubate at -20 °C for 15 mins.

4. Repeat step 1 and 2 twice.

5. Add the lysed cells to the colonyPCR mix.

# iGENERACE MENDEL

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