iGEM 2016

How To

Bacillus subtilis

A Collaboration

Bonn – Freiburg

This manual should serve as an assistance while starting to work with the versatile bacterium *Bacillus subtilis*. The iGEM team 2016 Bonn and Freiburg both struggled with some experiments concerning *Bacillus subtilis*, so we decided to create a manual for future teams and some additional tips and protocols which may help with future work. At this point, we also like to thank the different persons who helped us working with *Bacillus subtilis*: BGSC, Munich iGEM 2012, department of Pharmaceutical Biotechnology of the Greifswald University (Germany), Guido de Boer.

What is *Bacillus subtilis*?

Bacillus subtilis has a rod shaped appearance and belongs to the family of the gram-positive bacteria.

Apart from being widely present in nature, it is also a part of the microbial gut flora. The US Food and Drug Administration (FDA) classifies *Bacillus subtilis* as a GRAS organism. That means it is generally recognized as safe and can be used problem-free in S1 laboratories. *Bacillus subtilis* colonies have an irregular, large size with undulate margin. They have a white and dull colour and a dry texture. (figure 1)

Figure 1. Colonies of *Bacillus subtilis.*
The figure shows the structure of colonies formed by *Bacillus subtilis.* They have an irregular, large size with undulate margin. Their colour is white and dull and they have a dry texture.

The prefered medium of *Bacillus subtilis* is LB medium. The growth follows the typical four phases of bacterial growth (figure 2) and with reaching the exponential growth phase, *Bacillus subtilis* has a doubling time of 30 minutes under ideal conditions. This can be calculated with the slope of the exponential growth phase. In our calculation, the doubling time is 28 minutes.

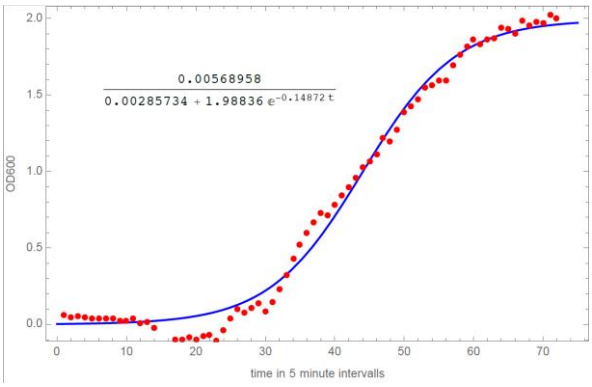

Figure 2. Growth curve of the *Bacillus subtilis* strain WT168.
A sample of *Bacillus subtilis* WT168 was diluted to an OD_{600} of 0.1/ml in LB. The OD_{600} as measured in a plate reader (CLARIOstar, BMG labtech) for 6 hours. The graph represents a typical growth curve of a bacterium.

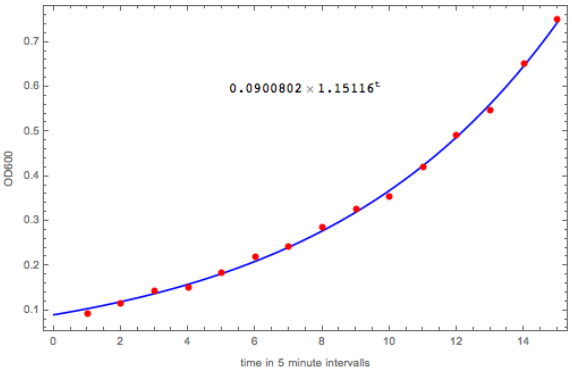

Figure 3. Exponential growth phase of *Bacillus subtilis.*
The graph shows the exponential growth phase of *Bacillus subtilis*. This phase can be used to calculate the doubling time of bacteria. In this case, the doubling time was around 28mins.

Bacillus subtilis is also able to form endospores under distress (see sporulation protocol), which is one of the most efficient adaptations to lack of nutrients. These endospores are a highly stable form of the bacterium which are resistant to heat, UV-light and pressure. Under normal conditions they are able to re-enter their normal life cycle.

Helpful tips to get started with *Bacillus subtilis*

Bacillus subtilis has many different morphological stages you can differentiate under the microscope, for example it can build long threads as well as smaller motile cell. The different stages can be seen in figure 4.

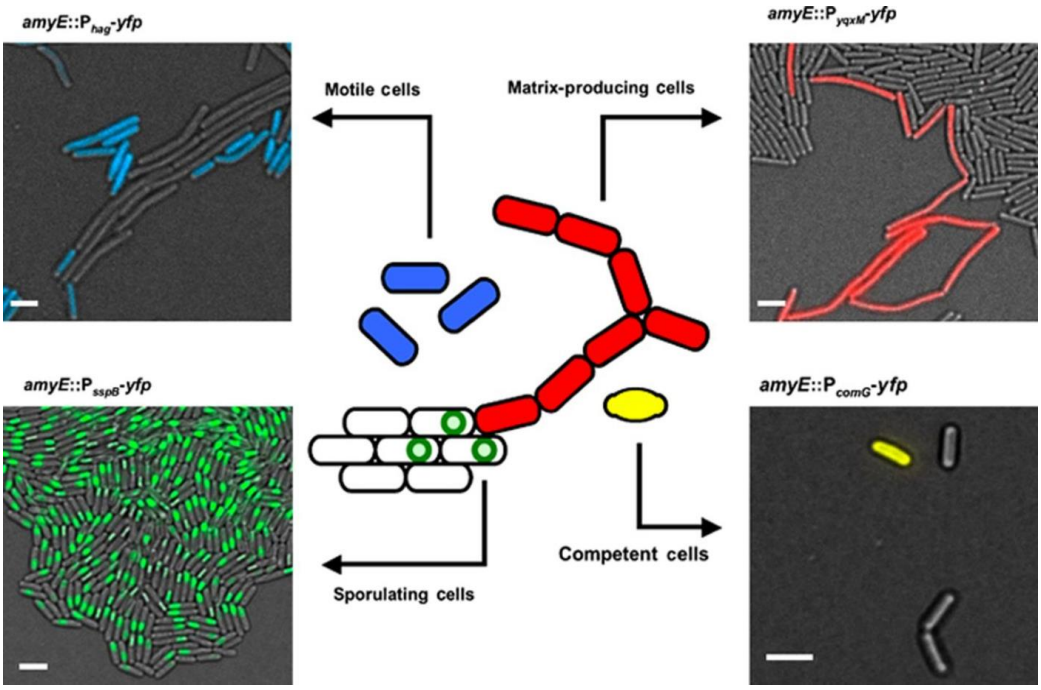

Figure 4. Morphological stages of *Bacillus subtilis.*
The graphic shows the different morphological stages of *Bacillus subtilis.*[1]

An important aspect for the transformation is the amount of DNA added to the competent cells. Where not enough DNA can be a problem, too much DNA could also be toxic for the cells. Through experiments we validated that 600 ng of DNA brings the best results for our strains.[2] When you have trouble with transformation, always consider trying it with different DNA concentrations.

Competent cells

Competent #1 - Chemo-Competent *Bacillus subtilis*

1. Inoculate a culture of *Bacillus subtilis* in 4 ml of LB-Medium
2. Let them grow overnight at 37°C, 200 rpm
3. Measure the OD_{600} of your overnight culture and dilute it in MNGE Medium to an OD_{600} of around 0.1-0.2/ml in 10 ml LB
4. Let the cells grow to an OD_{600} of 1.0-1.3/ml (37°C, 200 rpm)
5. Cells should be competent now (very motile)
6. Aliquot 400 µl of the samples in different tubes (each tube stands for one transformation)
7. You can either use the competent cells directly or add glycerol to dilute to a final concentration of 10% and freeze them in -80°C
   (Protocol from iGEM team munich 2012)

Competent #2 - Electro-Competent *Bacillus subtilis*

1. Inoculate a liquid culture of *Bacillus subtilis* and let it grow overnight
2. Vortex the culture gently and give 500 µl each in 3x 20 ml competency medium
3. Grow the bacteria in the flasks at 37°C 250 rpm shaking till you reach an OD_{600} between 0.5 and 0.7/ml
4. Add 1 ml of a 20% glycine solution to the first 1.25 ml to the second and 1,5 ml to the third flask to reach total glycine concentrations of 1%, 1.25% and 1.5%
5. Keep shaking for 1 h (because of the glycine the optical density should not change significantly)
6. Cool down the cells on ice for 15 mins (if not done already, transfer cells in 50 ml Falcon tubes)
7. Centrifuge at 8500 rpm for 10 min at 4°C for getting bacteria pellets

8. Pour off the supernatant and wash the cells three times with ice-cold washing-buffer (20 ml/10 ml/5 ml). Centrifuge the cells down at 8500 rpm for 10 min at 4°C between each washing procedure and decant the supernatant
9. Resuspend the cells in 1 ml ice cold washing-buffer (all three different cultures are supposed to get in it!)
10. Make aliquots (recommended: 120 µl → enough for two transformations)
11. Freeze in liquid nitrogen and store at -80°C

(Protocol submitted by department of Pharmaceutical Biotechnology of the Greifswald University)

Results:

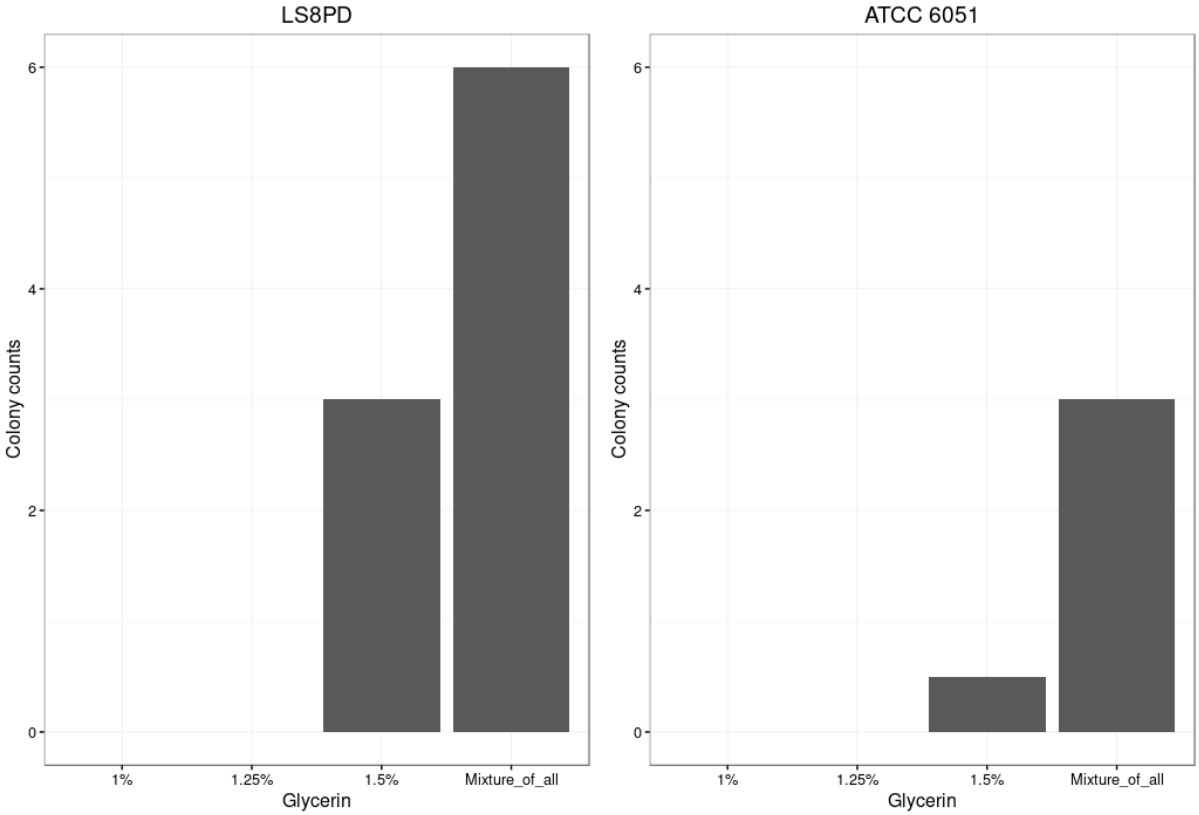

Figure 5. Electroporation efficiency for *Bacillus subtilis* regarding glycine concentration.
The electroporation worked best with a higher glycine concentration. Glycine works as a weakening agent, making the cell wall looser by replacing the alanine in the cell wall [3]. The highest colony amount was spotted by using a mixture of all three glycine concentrations (1%,1.25% and 1.5%) for the preparation of electrocompetent cells.

Transformation of *Bacillus subtilis*

Transformation #1

Using fresh, competent *B. subtilis* cells:

1. Add 600 ng of DNA and incubate at 37°C (200 x g) for 1 h
2. Add 100 µl of expression mix to each sample
3. Incubate at 37°C for another hour
4. Plate 400 µl the samples on selective agar

Note: if you use the frozen aliquots you can also just add the DNA to the sample, don't centrifuge the competent cells!

(Protocol from iGEM team Munich 2012)

Transformation #2 - Electroporation

Electroporation is a transformation method that relies on sending an electrical current through the cell and creating holes or pores in the cell membrane. Through those pores, the plasmid will enter the cell before they are getting closed.
For doing this transformation, special equipment is needed:

1. Electrocompetent cells
2. Competency Medium
3. Plasmid you want to transform
4. Electroporator
5. Electroporation cuvettes

Procedure:

1. Mix electrocompetent cells with the plasmid and acquire a total volume of 60 µl with a final DNA concentration of 10 ng/µl
2. Place the cell-plasmid-suspension, the electroporation-cuvettes and competency-medium on ice for 10 min (you need 1 ml competency-medium & 1 electroporation cuvette per transformation, but it is recommend to take more)
3. Pipet the cold cell-plasmid-suspension in the prechilled electroporation-cuvette and tap the cuvette multiple times (this way you get rid of bubbles and spread your suspension equally)

4. Make sure that the electroporation-cuvette is dry (take care that you don't touch the metal sides anymore!)
5. Electroporate at 2100 Volt (the electroporator will give out a "time-constant". A time-constant from 3.0 to 5.5 is a positive indicator although the transformation could also be successful at a lower time-constant)
6. Flush the electroporated mixture out of the electroporation-cuvette with 1 ml of competency-medium
7. Let the cells grow for 3 h at 37°C 300 rpm shaking
8. Pellet cells by centrifugation at RT 5 min and decant the supernatant (do not throw away)
9. Resuspend the pellet in 100 µl of the supernatant
10. Plate on selective agar

(Protocol from Greifswald)

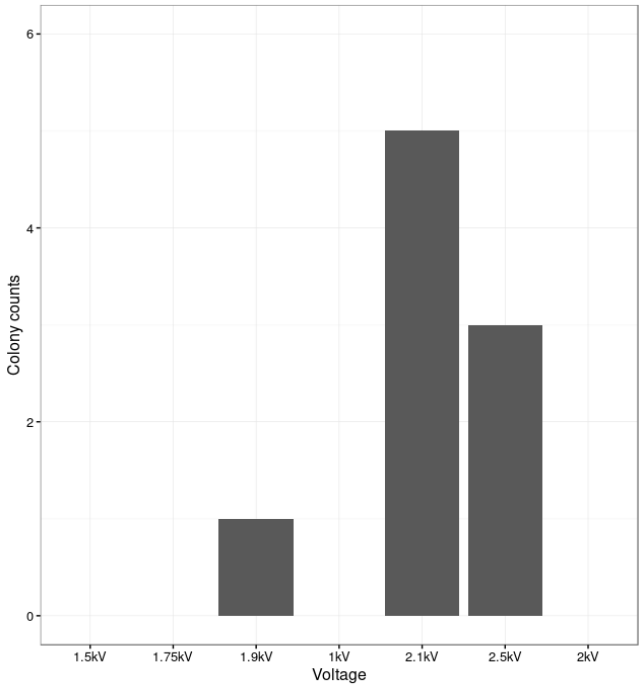

Figure 6. Correlation between electroporation voltage and number of colonies of *Bacillus subtilis*.
In order to find the best electroporation current, electroporation was performed with different voltages. The electroporation did not work at low currents. The bacteria were not able to take up the plasmids. Is the current getting too high, the bacteria get lysed.
The results show the optimal current for the electroporation is at 2.1 kV. At this current the number of successfully transformed bacteria was the highest.

Table 1. Electroporation efficiency for *Bacillus subtilis* regarding DNA-concentration.
The table shows the number of formed *Bacillus subtilis* colonies after the transformation using electroporation with different amounts of DNA-concentrations. It shows that a higher amount of DNA (10 ng/µl) leads to a higher number of colonies (11). (de Boer, 2015)

DNA-concentration (ng/µl)	Number of colonies
10	11
5	3
1	1
0	0

In order to find the optimal DNA-concentration for the transformation the electroporation was performed with different DNA-amounts.
The higher the DNA-concentration the higher the number of successfully transformed bacteria. Therefore, it is recommended to use a higher DNA-concentration. 10 ng/µl is a sufficient amount.

Sporulation

1. Overnight culture

- Inoculate your culture of *Bacillus subtilis* in 4 ml LB-medium
- Let them grow overnight at 37°C, 200 rpm

2. Exponential growth

- Measure the OD_{600} of your overnight culture and dilute it in LB-medium to an OD_{600} off around 0.1-0.2/ml in 10 ml LB
- Let the cells grow to an OD_{600} of 0,8/ml (37°C, 200 rpm)

3. Sporulation

- Centrifuge 10 ml of the cells at 13,000 x g for 1 minute
- Wash the pellet with 1 x PBS
- Re-suspend the pellet in 5 ml DSM (Difco Sporulation Medium)
- Let the cells grow for 24 hours at 37°C (200 rpm)

4. Lysozyme treatment (additional for spore purification)

- Treat the samples with lysozyme (15 mg/ml) at a dilution of 1:6
- Incubate for 1 h at room temperature
- Wash 6 times with 1 x PBS

5. Additional:

→ count spores using a Neubauer improved counting chamber and make aliquots with a defined number of spores per aliquot (e.g. 100 Million spores per 500 µl)

(Note: always use fresh DSM since the $FeSO_4$ is rusting when it is in dilution)

Data from the purification:

In forward and side scatter you can easily distinct the difference between the purified and unpurified samples (figure 5). Purification leads to a better and solid read out in further experiments and applications.

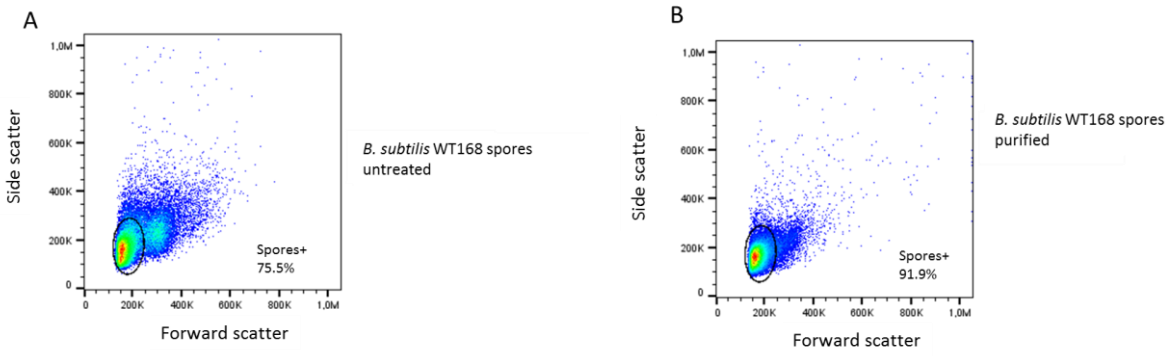

Figure 7. *Bacillus subtilis* spore purification.
(A) The spores of *Bacillus subtilis* WT168 were analyzed using flow cytometry. The set gate for spores shows that 75.5% of the sample consisted of spores. (B) The spores of *Bacillus subtilis* WT168 were treated with lysozyme for 1 h. The flow cytometry analysis shows that after purification the amount of spores is considerably higher with 91.9%.

Antibiotics

Table 2: The concentrations of antibiotics used for *Bacillus subtilis.*
The table shows the minimal concentrations of different antibiotics used for the selection of antibiotic resistant *Bacillus subtilis* cells.

Antibiotic	Stock solution	Dilution for *B. subtilis*
Spectinomycin (Spec)	100 mg/ml	1 to 1.000
Chloramphenicol (Cml)	25 mg/ml	1 to 5.000
Erythromycin (Erm)	10 mg/ml	1 to 1.000
Ampicillin (Amp)	100 mg/ml	1 to 1.000
Kanamycin (Kan)	50 mg/ml	1 to 1.000

Media

DSM – Sporulation Medium

8 g	Nutrient Broth
1 g	KCl
1 ml	$MgSO_4$ (1 M)
1 ml	$MnCl_2$ (10 mM)
1000 ml	Aqua bidest

Autoclave and add:

0.5 ml $CaCl_2$ (1 M)
1 ml $FeSO_4$ (1 M)

10x MN Medium (1 Liter)

136 g	$K_2HPO_4*H_2O$
60 g	KH_2PO_4
10 g	Na-citrat * $2H_2O$

MNGE-Medium (100 ml)

9.2 ml 10x MN-Medium

82.8 ml	Sterile water
10 ml	Glucose (20% filtered)
500 µl	K-Glutamate (40%)
500 µl	Fe[III]-ammonium-citrate (2,2 mg/ml)
1 ml	Tryptophan (5 mg/ml)
300 µl	$MgSO_4$ (1 M)
(1 ml	Threonine (5 mg/ml) only for selective agar after the transformation)

Expression mix 10,5 ml

5 ml	Yeast extract (5%)
2.5 ml	Casamino-acids (CAA)(10%)
2.5 ml	Sterile water
500 µl	Tryptophan (5 mg/ml)

Competency Medium 500 ml:

10 g	LB
45.5 g	Sorbitol

→ autoclave

Washing-Buffer 500 ml :

45.5 g	Sorbitol (total 0.5 M)
45.5 g	Mannitol (total 0.5 M)
50 ml	Glycerol (total 10%)

→ autoclave

References

[1] (Lopez D., Vlamakis H. &Kolter R Generation of multiple cell types in *Bacillus subtilis.* FEMS Microbiology Reviews. Jan 2009)

[2] http://www.askabiologist.org.uk/answers/viewtopic.php?id=9446 – 16.10.2016

[3] Zhi Zhang, Development of an Efficient Electroporation Method for Iturin A-Producing Bacillus subtilis ZK, April 2015

B. subtilis manual

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