Genetic mapping of *Bacillus subtilis* biofilm formation genes

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Contents

Abstract

Biofilms are defined as bacterial cells that form populations by adherence to each other, and/or to surfaces or interfaces that are enclosed by a matrix consisting of extracellular polymeric substances (EPS). The composition and syntheses of biofilms are controlled by various environmental factors (e.g. growth phase and growth media) and vary between different bacterial subspecies. With scanning confocal laser microscopy it was showed that a biofilm consists of micro-colonies separated by water channels that can deliver nutrients to the inner cell population.

Biofilm formation includes various intertwined genetic regulation systems (e.g. quorum sensing and sporulation) for forming a biofilm. In this thesis the genetic regulation of biofilm formation has been reviewed from the past decade. A genetic map has been evolved from this literature study, shown in figure 1. The first major regulators were found in 2001, spo0A(Spo0A) and spo0H(σ^H) that positively regulates the antirepressor of sinR, SinI and negatively regulates abrB(AbrB). SinR acts as a molecular switch between biofilm formation and swarming motility. SinR is also involved in a negative feedback loop with slrR and slrA that can activate TasA formation, a major protein in *B. subtilis* its EPS-matrix. A galactose pathway was also described to be involved in biofilm formation.

Many differences between strains in *B. subtilis* were described in various papers when creating this genetic map, for example most laboratory strains do not have the ability to form biofilms. Indeed, this is likely to happen, however wild type *B. subtilis* biofilm forming strains should be used more often when we want to reveal the functions and regulations of the large quantity of genes simply because of its intertwined genetic systems that already are involved in biofilm formation.

Introduction.

Bacterial cells that form populations by adherence to each other, and/or to surfaces or interfaces that are enclosed by a matrix consisting of extracellular polymeric substances (EPS), are defined as biofilms[1]. Extracellular polymeric substances, –consisting of polysaccharides, proteins, nucleic acids and lipids– gives the biofilm mechanical stability and allows adhesion of the biofilm to surfaces[2]. The composition and syntheses is controlled by various environmental factors, growth phase, growth media, temperature, limitation of oxygen, nitrogen, and cation deficiency and vary between different bacterial subspecies[3]. In 1991 –by Lawrence *et al.*[4]– a new technique, scanning confocal laser microscopy (SCLM), was introduced to observe biofilms. This technique could provide a three-dimensional image of biofilms from living organisms. Shortly after this time water channels, that could deliver nutrients, in biofilms were confirmed[5] and answered the question at that time how cells in the center of a biofilm got their nutrients. Images from the SCLM showed that a biofilm consists of micro-colonies separated by water channels that deliver nutrients [6].

The formation of biofilms.
The formation of a biofilm starts with the interaction of planktonic cells with a surface due to environmental signals [7]. This is the first of the five stages described in Stoodley *et al.*2002. In the second stage EPS is produced to promote the attachment to a surface. The process in stage three is described as the early development of biofilm architecture, and in stage four the maturation of biofilm architecture. The last stage is a mature biofilm that disperses single cells (dispersal cells) from the biofilm (figure 1)[8]. Two types of dispersal cells are described, active and passive dispersal cells. Active dispersal cells are cells that disperse form the biofilm to form new microcolonies [9] and another function could be, that those cells are 'stealth swimmers' recently described in Houry *et al.*[10, 11] . Passive dispersal cells, are cells that disperse from the biofilm due to sloughing and erosion[9]. To determine in which stage the cells find themselves they would have to know the cell density of their population. This process was first described by the authors of Fuqua *et al.*in 1994.

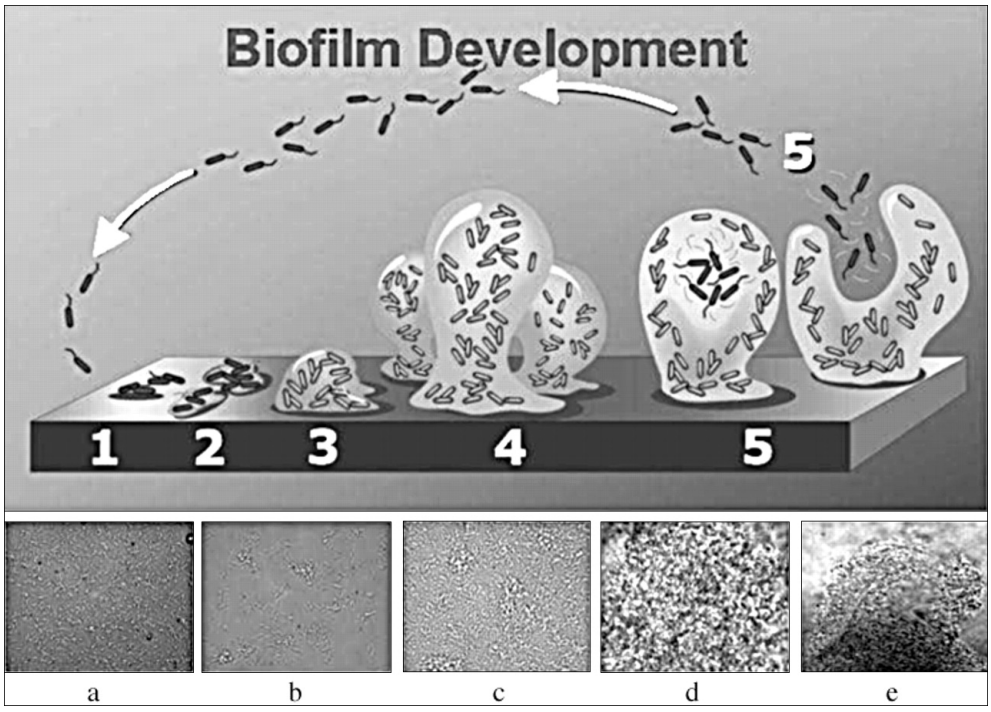

Figure1. Adapted from Stoodley *et al.* 2002[8]. Here the 5-step process of biofilm formation is shown. The upper panel gives a modeled interpretation of biofilm formation and the lower panel shows photographs of a *P. aeruginosa* biofilm growing under continuous flow conditions corresponding to the stages above. Stage 1 shows the attachment of cells to a surface and stage 2 shows EPS production to promote attachment to the surface. In stage 3 the early development of a biofilm is shown and in stage 4 the maturation of the biofilm is shown with its architectural structures. In the final stage a mature biofilm is shown that disperses single cells from the biofilm.

The authors describe that certain genetic processes, such as biofilm formation, in a bacterial cell are only efficient when there is a sufficient large population of bacteria. This type of process is called quorum sensing[12]. In 1998 Davies *et al.* showed that cell to cell communication in *Pseudomonas aeruginosa* is required to proliferate into multicellular complexes. The lasI gene, involved in quorum sensing, regulates signaling compound, N-(3-oxododecanoyl)-L-homoserine lactone. To test if this gene is necessary for *P. aeruginosa* to form a biofilm, a lasI mutant was made. There was found that the mutant formed a thin sheet of densely packed cells without any water channels, in contrast to the wild type (WT) that formed a 5 times thicker biofilm with water channels[13].

In a gram positive bacterium, *Bacillus subtilis* quorum sensing is regulated in a different way. In 1994 a pheromone precursor ComX (competence factor) was identified together with ComQ that are required for production of the pheromone. The competence pheromone is a ~10 amino acids long oligopeptide and affects competence. The pheromone was also identified as a possible cell-density signal (quorum sensing signal)[14]. A new quorum sensing mechanism was described by López *et al.*in 2009. A compound called surfactin was found to cause intracellular potassium leakage from out of the cell. The authors suggest that KinC (membrane histidine kinase) activation is related to the loss of potassium ions. It could be that surfactin can act as a quorum sensing signal that indirectly activates KinC. Possibly*, B. subtilis* could sense the cell density by sensing a change in cell state at chemical level (e.g. concentration of potassium)[15]. This quorum-sensing could have influence on what kind of EPS would be constructed at specific time points.

The EPS matrix that encloses the microcolonies of a biofilm, contains extracellular products that are either produced by the cell or derivates from lysed cells within the biofilm. The exact structure of any biofilm will probably be different from each environment it is established in. The main structural parts of EPS are exopolysaccharides but vary greatly in their composition and differ in their chemical and physical properties [16]. In 2008 EPS studies were done in *Escherichia coli*. Two forms of EPS were described, free and bound EPS. Free and bound EPS were analyzed with Fourier Transformation Infrared (FTIR) Spectroscopy. The results suggest that bound EPS contains mainly proteins and carbohydrates and that free EPS contains besides proteins and carbohydrates, nucleic acids (extracellular DNA, eDNA)[3]. Extracellular DNA is necessary to form biofilms in *P. aeruginosa*[17].

In *B. subtilis* TasA was identified as a major protein in the extracellular matrix and is also required for the architecture of the matrix. Also YqxM was found to play an important role in de localization of TasA[18]. The TasA protein was shown to form amyloid fibers[19] and a year later the function YqxM (further described as TapA) was described. TapA, TasA anchoring/assembly protein, has two functions, at first it anchors the TasA fibers to the cell wall and secondly it assemble TasA into fibres[20]. In 2012 another extracellular protein was discovered, BslA (formerly YuaB). BslA was shown to be responsible for the hydrophobic layer on the surface of biofilms and formed polymers *in vitro*[21].

This thesis focuses on molecular genetics in *B. subtilis* associated with biofilm formation. *B. subtilis* develops in three genetically intertwined and heterogeneous phenotypes: spore formation, swarming motility, and biofilm formation. Because of the intertwined genetically aspects of biofilm formation, it is highly interesting to study the molecular genetics associated with biofilm formation. During this literature study a genetic map (figure 2) has evolved and placed at the beginning of this thesis to keep track of the many processes described in the past decade about biofilm formation.

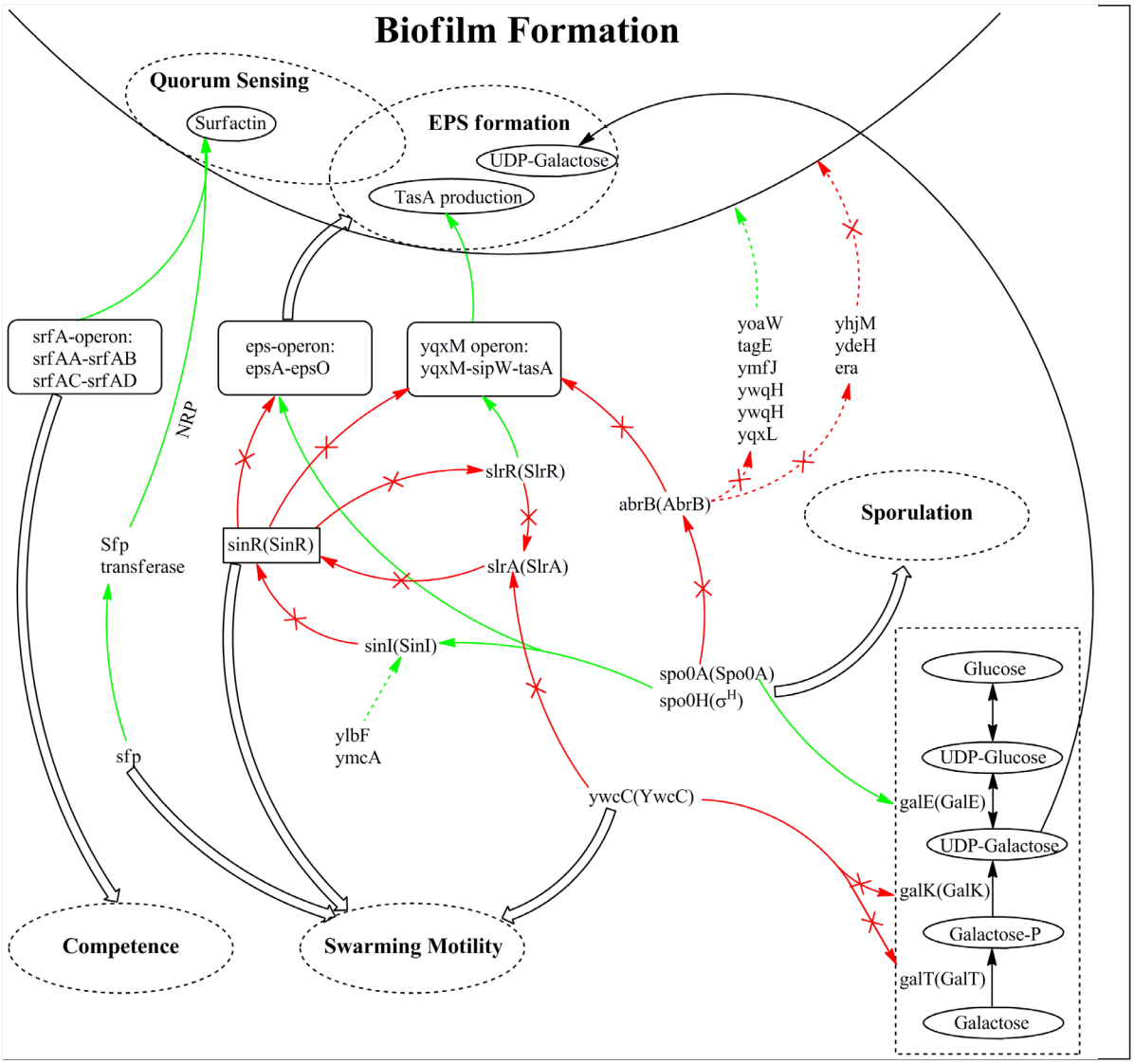

Figure2. The genetic map (described in the text) is shown with green arrows (promoting), red arrows (repressing) and hollow arrows (resulting). On the right-hands side the galactose pathway is shown, both galK and galT are repressed by ywcC. The master regulator spo0A(Spo0A) is promoting galE, sinI, the eps-operon and repressing abrB that represses the yqxM operon. The other master regulator sinR(SinR) represses the eps and yqxM-operon and is involved in a negative feedback loop with SlrA and SlrR that promotes the yqxM operon. The sfp gene is involved in producing surfactin (together with the srfA-operon) and in swarming motility. The srfA-operon is not described in the text, for further details see Nakano et al. 1991[36], Cosmina et al. 1993[37] and Hamoen et al. 2003[38].

Various biofilm formation genes.

The authors of Branda *et al.* decided in 2001 to investigate pellicle formation by wild type isolates of *B. subtilis* because of the fact that domesticated *B. subtilis* (lab strains) lost the ability of multicellularity as in the formation of pellicles.

In an experiment –in a liquid standing culture– with comparison between lab- and wild type strains forming pellicles, the lab strain formed a thin, fragile, and smooth pellicle when the wild type strain formed a thick pellicle with a vein-like structure on its surface. Almost the similar was observed on agar plates. A wild type colony or pellicles in liquid at high magnification level showed aerial projected tongue-like structures that were composed from long chains of cells bundled together. In these aerial projected tongue-like structures, it was shown by means of fusing lacZ to sporulation genes (sspE and spoIID) in the genome from *B. subtilis*, and growing the cells on agar plates containing chromogenic galactoside X-gal that the blue dye accumulated particularly at the tips of the aerial projected tongue-like structures. This result suggests that sporulation takes place at those aerial projected structures in wild type colonies and pellicles.

The regulators of early-sporulation genes spo0A and spo0H were found to serve also as key-regulators for forming multicellular structures. Because those structures, such as biofilms, are held together with EPS, the authors found two genes, yveQ and yveR, that are under regulation of the two translated spo0 genes, Spo0A and σ^H. Mutations in the yveQ and yveR genes resulted in thick but weak pellicles that eventually became disrupted also the surfaces of the pellicles and colonies were smooth and lacked aerial projected structures. This suggests that yveQ and yveR are important for producing EPS and therefore necessary to produce the architecture of *B. subtilis* biofilm[22]. In another study, Ren *et al.*2004, the authors did similar experiments and confirmed the results found by Branda *et al.*2001. In conclusion the yve-operon is important to maintain the biofilm and is necessary for optimizing the EPS synthesis[23]. In 2005 the yve-operon was renamed as epsA-O operon where yveQ and yveR are named epsG and epsH respectively[24].

Surfactin, known for the aerial hyphae in fungi and streptomycetes, also plays a role in *B. subtilis* aerial projected structures. Mutants lacking surfactin formed pellicles with aerial projected structures that eventually merged together resulting in pellicles and colonies without aerial structures[22].

In the same year, Hamon and Lazazzera confirmed that spo0A is required for biofilm formation. Interestingly, another gene, abrB, was found to be involved in the regulation of biofilm formation by spo0A. The authors found that a spo0A mutant did not form biofilms, but a spo0A and abrB double mutant did form a biofilm (1.5-fold less compared to the wild type). This result suggested that in a wild type, Spo0A inhibits abrB translation, and can form a biofilm. A spo0A mutant on the other hand cannot inhibit the abrB gene, thus abrB represses the biofilm formation[25].
In a study focused on AbrB-regulated genes in biofilm formation, 39 genes were identified being repressed by AbrB. To determine which genes are really involved in biofilm formation, 23 out of the 39 genes were disrupted and were tested for their ability to form biofilms. It appeared to be that 9 out of 23 genes were significant involved in biofilm formation. Six mutants down regulated biofilm formation and three mutants up regulated the biofilm formation. Two operons, yqxM and yoaW, were further examined because mutating those operons led to higher than twofold, 5.6-fold and 2.2-fold respectively, reduction of biofilm formation compared to the wild type. The reduction of biofilm formation in the yoaW operon was determined to be caused by the loss of YoaW because yoaW is flanked by two terminator sequences. However, this is not the same for the yqxM operon, the yqxM gene is in front of two other genes, sipW and tasA. Mutations in yqxM, sipW, and tasA were made and only the sipW mutant reduced biofilm formation significantly[26].

In a study done by Branda *et al.*in 2004 a different approach was used to find new genes involved in biofilm formation. The *B. subtilis* Functional Analysis (BFA) was used to find important genes that are involved in multicellular communities. Six genes were identified; yhxB, sipW (confirming the results from Hamon *et al.* 2004), ecsB, yqeK, ylbF, and ymcA. A mutation in the yveR (epsH) gene resulted the same phenotype as a mutation in the yhxB gene indicating that yhxB is involved in EPS synthesis. The results of the sipW gene confirm the results found in Hamon *et al.* earlier (described above). The ABC-transporter Ecs where the transmembrane subunit is translated by ecsB is likely to be involved in forming multicellular communities. The ecsB mutants showed flat pellicles with weak cell-cell interactions. Deletion of yqeK resulted in pellicles that initially were thin an flat and eventually became thicker, however the pellicle contained cracks in the surface. This suggests that yqeK is involved in formation of multicellular communities. Both genes ylbF and ymcA disrupted pellicle formation, suggesting that they also are involved in multicellular communities, and show a high similarity in amino acid sequence(48-67%). However, it is not likely that those genes are paralogs of each other, both genes still have different domain structures[27].

SinR/SinI system.

In 2005 a repressor was found, called SinR, which negatively regulates transcription of the eps-operon (that includes the epsGH (yveQR) genes described above) at multiple sites and functions as a master regulator for biofilm formation. The authors found that mutations in sinR bypassed the requirement of sinI, ylbF and ymcA (described above) in biofilm formation. Both genes sinR and sinI transcribe a DNA binding protein called SinR, and a SinR antagonist respectively, both proteins form a protein complex[24](and references in there).

The phenotype of a sinR mutant shows rigid colony formation in which cells grow as chains consisting of non-motile cells (figure 3). The phenotype of a sinI mutant shows, in contrast to sinR, motile cells that do not form chains and where the colonies are flat, without any architecture (figure 3). A sinR/sinI double mutant shows the same phenotype as a sinR mutant (figure 3). Interestingly the authors found that a sinI mutant in early growth has a biofilm defect, however after prolonged growth time (102h) the cells formed a thick pellicle. This indicated that possibly sinI required a suppressor mutation. Because the phenotype showed the same rigid colony as a sinR mutation, it could be that the suppressor is a spontaneous mutation in the sinR gene that restores biofilm formation. In fact there were various frameshifts found in the sinR gene that disrupted the translation of SinR. The frameshift mutations were neighboring DNA-tracks that are responsible for phase regulation of swrA[28], a swarming motility gene. Thus, the authors hypothesized that the sinR could be involved in swarming motility in *B. subtilis*. Swarming motility in sinR and sinI mutants was examined and showed that the sinR mutant was not motile in contrast to the sinI mutant that was motile (figure 3). When both genes were disrupted the phenotype showed the same as the non-motile sinR mutant (figure 3). This indicates that sinR and sinI are necessary for swarming motility in *B. subtilis* and that it could function as a switch between non-motile biofilm growth and motile swarming growth [24].

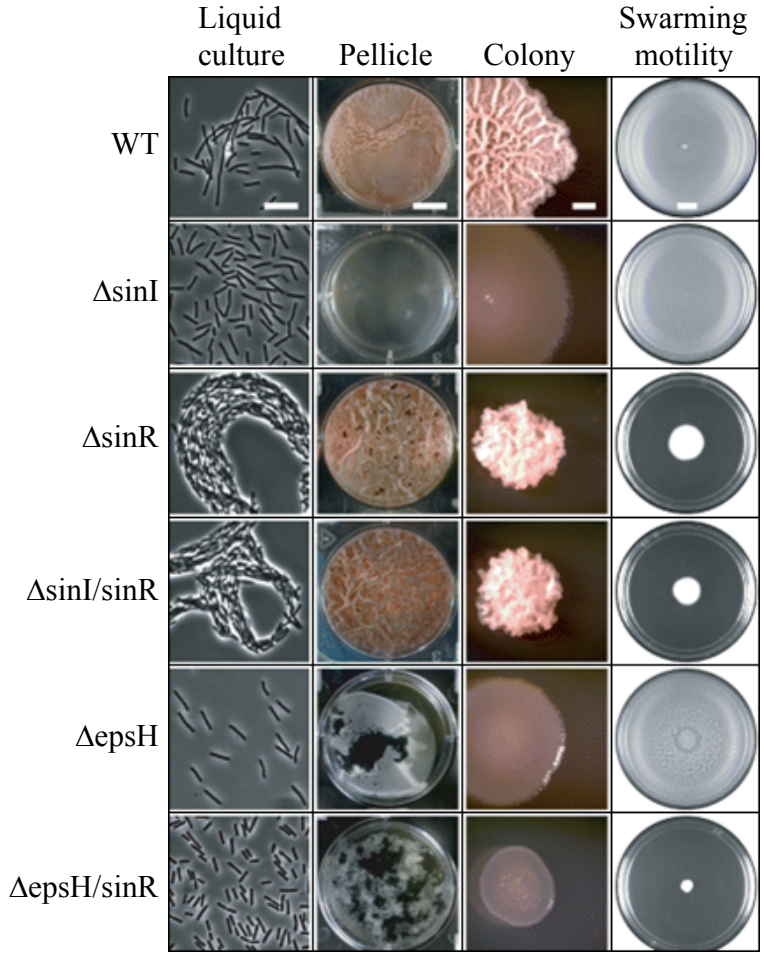

Figure3. Adapted from Kearns *et al.* 2005[24]. This table shows the various phenotypes resulting from various mutations in *B. subtilis*. The wild type (WT) in liquid culture shows some attachment in de middle of the picture. The wild type forms a architectural pellicle or colony, and has swarming motility. The sinI mutant (ΔsinI) in liquid culture shows more dispersed cells compared to the wild type. The sinI mutant shows no architectural pellicle or colony, and has swarming motility. The sinR mutant (ΔsinR) in liquid culture shows extreme clumping compared to the wild type. The sinR mutant shows a pellicle and colony that are robust and thick. No swarming motility is observed. The double sinI/sinR (ΔsinI/sinR) mutant shows the same phenotype as the sinR mutant. The epsH mutant (ΔepsH) shows much more dispersed cells in the liquid culture. The pellicle and colony are thin and have no architecture; however the mutant has swarming motility. In a epsH/sinR double mutant (ΔepsH/sinR) the cells are more close than the epsH mutant in liquid culture. The pellicle is thin and has no architecture the colony is smaller than the epsH mutant but has no architecture. The double mutant has a defect in swarming motility.

Another new found feature of sinR is that it binds to the DNA in the eps-operon. For an already known eps gene, epsH (yveR) described above, it was shown that an epsH mutant disrupts biofilm formation [22]. In a sinR/epsH double mutant (a sinR mutant provokes biofilm formation), epsH overruled the biofilm provoking sinR mutation and no biofilm was formed, however the culture stayed non-motile (figure 3). This indicated that SinR negatively regulates genes in the eps-operon. The authors showed by means of Electrophoretic Mobility Shift Assays (EMSAs) that SinR binds to possible multiple sites at the epsA promotor (P_{epsA}) (begin of eps-operon). When SinI was added to the SinR- P_{epsA} reaction, SinI reduced the binding of SinR to P_{epsA}.

When a mutation was introduced in ylbF or ymcA (described above) no biofilm was formed. Remarkably when a sinR mutation was introduced in a ylbF or ymcA mutant biofilm formation was restored. Indicating that somehow YlbF and YmcA represses sinR, promotes SinI or directly acts on the eps-operon[24].

In 2006 another operon was described to be negatively regulated by SinR, the yqxM-operon (yqxM-sipW-tasA). Before AbrB was described to inhibit the transcription of sipW[26], now sinR also plays repressing role[29]. A lacZ gene was fused to the promoter region of the yqxM-operon, when a mutation in sinR was introduced the lacZ expression increased in 5-fold compared to the wild type. When a mutation of sinI was introduced the lacZ expression decreased over a 100-fold compared to the wild type. Whit EMSA the authors found that SinR binds at least at two different sites in the promoter region of the yqxM-operon[29].

The stoichiometry of SinR/SinI was studied by Chai *et al.* in 2008. At first immunoblot experiments with antibodies against SinR and SinI, were performed. The authors found approximately 50 molecules/cell SinI and approximately 900 molecules/cell SinR, this gave a ratio of 1:18 SinI to SinR. However this SinI/SinR ratio is too low to repress SinR to form a biofilm. Another approach, fusing GFP to SinR and SinI, revealed that approximately 2% of the total cells from a biofilm produce SinI. The authors concluded that in those SinI producing cells the ratio of SinI to SinR would be, 2,8:1 ($^{1}/_{18}$ divided by 0,02) [30]. The fact that only 2% of the cells in a biofilm produce SinI and thereby repressing SinR that leads to activating the eps-operon and the yqxM-operon, suggests that only a subpopulation of the biofilm produces the EPS matrix for the whole multicellular biofilm.

Extended evidence on the structure and organization of SinR (beyond the scope of this thesis) interacting with SinI and with DNA specific sites in the eps and yqxM operons are recently described in Colledge *et al.* 2011[31].

SlrR

A new gene was found to be involved in biofilm formation by Chu *et al.* in 2008. By extending the regulatory aspects from the abrB gene, described earlier by Hamon and Lazazzera 2001 and Hamon *et al.* 2004[25, 26], the authors found an positive activator of the yqxM-operon that is known to be repressed by AbrB. An experiment with a promoterless lacZ gene fused into the yqxM-operon with mutated AbrB binding sites, the expected lacZ expression would be higher as the control with AbrB-binding sites. However this was not the case, the expression of lacZ lowered compared to the control. Thus, the authors hypothesized that important regulatory sites would be near or in the AbrB-binding sites that are necessary for an, yet to be described, activator of the yqxM-operon. Two extra mutations in the construct that resulted in no transcription selected a possible DNA-site for the activator. A direct DNA repeat was found that possibly would be the binding site of the activator. Both 5- and 3' mutations in those direct repeats resulted in a lower expression of lacZ. This suggests that the yqxM-operon is also under control of a positive regulator that binds to those direct repeats. The authors described a gene called slrR that is similar to sinR and when mutated disrupts biofilm formation. With a promoterless lacZ gene fused into the yqxM-operon, expression of LacZ was measured in absence of slrR and indeed the expression levels dropped suggesting that SlrR promotes the yqxM operon. To test whether the yqxM-operon is the major gene regulated by SlrR, a IPTG inducible promoter was constructed in front of the yqxM-operon. In absence of the slrR gene no biofilm was formed, but when IPTG was added the yqxM-opereon restored the biofilm formation suggesting that yqxM is the major or only operon under regulation of SlrR. To test whether SlrR binds to the yqxM operon, parts of SlrR protein (full length protein was insoluble) were used in EMSA and showed to bind to the yqxM-operon[32]. Interesting is that the earlier identified SinR binding sites described in Kearns *et al.* 2005 were also found upstream of slrR. This suggests that SinR would also repress slrR. With a promoterless lacZ fused into the slrR gene, expression of LacZ increased significantly in absence of SinR. This observation suggests that slrR is under the negative control of SinR[32].

In the same year Kobayashi described two new genes related to slrR, slrA and ywcC. Kobayashi suggested that slrR and slrA form a complex that antagonizes the SinR/SinI system. In Bacillus subtilis ATCC 6051 a frameshift mutation in the ywcC was found (unlike 168 and NCIB 3610) this YwcC mutation decreased the SlrA expression which enhanced biofilm formation. This suggests that the ywcC frameshift is the genotype of the wild type *B. subtilis*[33]. More thorough and

evident results were found in Chai et al 2009. The authors found that SlrA (a paralogue of sinI) binds and represses SinR and that SrlR binds a represses SrlA. This regulation is described as a negative feedback loop wherein SlrA indirectly promotes the synthesis of SlrA and SlrA inhibits the activity of SlrA. In a ywcC mutant the colony grew as a rigid colony. In a slrA mutant a biofilm-like colony was formed lacking the typical architecture of a biofilm. In a double mutant of both genes, ywcC and slrA, the phenotype was indistinguishable from the single slrA mutant suggesting that ywcC is repressing srlA transcription. To test what the expression effect on the biofilm responsible operons was, eps, yqxM, and slrR lacZ fusions were made. In a srlA mutant the expressions of those genes lowered a little, suggesting that slrA plays a minor role in biofilm formation. In contrast when a ywcC mutant was followed, the expression increased majorly. Under normal conditions SrlA concentrations are low (explaining the minor effect of biofilm formation) and in absence of YwcC the SrlA concentration becomes rather high what will results in major repression of SinR and sequentially a major expression of the eps, yqxM, and slrR operons. At final, a pull-down assay combined with EMSA showed that SlrA binds SinR and prevents it from binding to the eps and yqxM operons[34].

Galactose pathway involved in biofilm formation.

Recently, Chai *et al.* 2012 described the involvement of a galactose pathway (figure 2) in biofilm formation. The authors found that in a *B. subtilis* galE mutant galactose causes toxicity that leads to cell lysis. The toxic effect from the galactose in this mutant was due to one of the intermediates Galactose-P or UDP-Galactose. To rule out one of the intermediates D-oxy-galactose was used as galactose metabolite. (D-oxy-galactose can be converted to the phosphorylated D-oxy-galactose-P by GalK but cannot be futher processed by GalT to UDP-galactose). D-oxy-galactose-P is in *E. coli* identified as toxic. When the galE mutant and wild type were grown in LB with D-oxy-galactose no significant growth defect was observed, suggesting that galactose-P is not toxic and UDP-galactose would be the toxic candidate. The two genes that are involved in conversion of galactose to galactose-P, galK, and from galactose-P to UDP-galctose, galT were mutated in the galE mutant. The triple galEKT mutant, when grown in LB/galactose, showed no growth defect indicating that indeed one of both genes are involved in the toxic conversion of galactose. In a mutant with only galK as intact gene, the mutant was still insensitive to galactose. This indicates that, as the results before already suggested, that UDP-galactose is the toxic compound in *B. subtilis*. When the galE mutant was grown on LB with galactose the expectation was that the cells would lyse, however some colonies grew on the agar plate. The authors found in these resistant colonies suppressor genes, galK, galT and interestingly sinR showed frameshifts and point mutations. Found was that a sinR mutation restored the growth of a galE mutant growing on LB/galactose. This made the authors hypothesize that the toxic UDP-galactose is shunt into the EPS by the eps-operon. To test this hypothesis EPS was isolated from the galE mutant and from the wildtype, galactose was identified in the galE mutant's EPS but not in the wildtype's EPS. The authors conclude that an EPS producing *B. subtilis* strain can shunt toxic metabolites such as galactose into its EPS-matrix.

To find out which genes regulate this galactose pathway, galEKT mutants were grown under biofilm promoting circumstances. A galE mutant formed without galactose no pellicles, when galactose was present it did form a pellicle. This is because galK and galT are still present. When a galK and galT mutant was grown under biofilm promoting circumstances it formed a pellicle with or without galactose present. This is because galE can still convert UDP-glucose to UDP-galactose. A triple, galEKT, mutant did not form a pellicile in both cases because galE is also not present. These results are concluding that UDP-galactose is necessary for biofilm formation.

A promoterlless lacZ gene was fused to galE and expression was monitored. When spo0A was disrupted the expression of lacZ lowered, indicating that spo0A is activating galE transcription. The galK and galT genes are clustered in an operon with ywcC (a known repressor described above) and gtcA. The authors found that the YwcC protein can autoregulate its own operon. In an experiment with lacZ as reporter the expression of lacZ was two times higher in a ywcC mutant than in a wild type with an intact ywcC gene. This indicates that the whole operon (including galK and galT) is negatively regulated by YwcC[35].

Discussion and Conclusion.

More than a decade ago Branda *et al.* and Hamon & Lazazzera found simultaneously in 2001 that spo0A and spo0H have major roles in biofilm formation. Thereby the genetic research began in development of biofilms.

In this thesis the emerging gene regulatory systems, which were found in the past decade, have been studied to create a genetic map of biofilm formation in *B. subtilis*. The authors of Branda *et al.* 2001 described besides spo0A and spo0H two other genes necessary for EPS formation called yveQ and yveR, later renamed as epsG and epsH respectively. Simultaneously Hamon & Lazazerra found besides spo0A and spo0H a gene, repressed by Spo0A, called abrB that negatively regulates the yqxM-operon, necessary for TasA production. TasA is a major protein in the extracellular matrix and is also required for the architecture of the matrix[18]. Various genes, stimulating and depressing the biofilm formation, that were negatively regulated by the AbrB protein have been found but not further discussed[26]. No studies about those genes involved in biofilm formation have been done yet. A new biofilm master regulator, the SinR/SinI complex, was found in 2005. The authors showed that SinR can bind to, and negatively regulate the eps-operon. Also two genes, ylbF and ymcA, were found to, negatively regulate sinR, positively regulate sinI or positively regulate directly on the eps-operon[24]. Soon after this discovery it was shown that sinR also negatively regulates the yqxM-operon [29] and the slrR-operon[32]. All the described regulatory systems were mapped in figure 1.

The first conclusion to be mentioned is the fact that biofilm formation involves an intriguing and complex genetic intertwined regulation between genes for various lifestyles in *B. subtilis*. Probably much more new identified genes will follow and differences between *B. subtilis* strains will be better mapped. An interesting feature described in Chai *et al.* 2008[30] is that even in one strain that formed a biofilm subpopulations exists that have different expression patterns then other subpopulations in the same biofilm. The authors found for example that (only) 2% of a biofilm subpopulation expressed the sinI gene suggesting that only a part of the biofilm community produces the EPS matrix for the whole biofilm.

Recently a galactose pathway was described to be a part in biofilm formation. The toxic UDP-galactose intermediate produced from galactose by GalT and GalK or produced by GalE was found to be incorporated in the EPS-matrix from *B. subtilis*. This self-protecting mechanism will succeed only when *B. subtilis* grows in its multicellular form. Many
B. subtilis laboratory strains lost this ability. I think that wild type *B. subtilis* strain with the ability to form biofilms should be used more often when we want to reveal the functions and regulations of the large quantity of genes simply because of its intertwined genetic systems that already are involved in biofilm formation.

In future the differences between various *B. subtilis* strains and (laboratory or wild type strains) and differences between subpopulations inside the biofilm should be further revealed to have more insight into *B. subtilis* its great adaptability and bistability.

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