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SOCIAL INTERACTIONS OF *Bacillus subtilis*

DOCTORAL DISSERTATION

SOCIALNE INTERAKCIJE BAKTERIJE *Bacillus subtilis*

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AB Bacteria communicate using freely diffusible signals which are constitutively secreted during growth. This process is known as quorum sensing (QS) and its traditional model assumes that after reaching threshold concentration, QS signals activate their cognate receptors and induce coordinated response across many cells. In this dissertation the link between QS signaling and QS response in the ComQXPA QS system of *Bacillus subtilis* was studied. This system consists of the QS signal processing enzyme ComQ, the QS signal ComX, the QS receptor ComP and the transcription factor ComA, which activates the expression of surfactin and competence genes. We discovered that QSS- mutants (Δ*comQ* or Δ*comX*) are overly responsive to ComX produced by QS+ population and this manifested in overproduction of surfactin and increased genetic competence for transformation. The increased response of the mutant was associated with high fitness costs and its failure in competition with QS+ population in coculture. However, under the selection pressure for DNA exchange in mixed planktonic culture ComX serves as public good shared by the wild type and the signal deficient population. In these conditions overly responsive mutant acts as hypercheater revealing higher competence as compared to the wild type and surviving better thanks to the uptake of new genes. Next, the QS response of the QS+ and QSS- was compared in planktonic and colony cocultures and results suggest that in the colony ComX produced by QS+ may not be fully shared with the mutant. Since in general the influence of environmental factors on QS is poorly understood, the influence of temperature and salinity on QS was accessed here. We show, for example that high salinity of medium differently affects QS response in planktonic culture and in biofilm. Finally, the diversity of *B. subtilis* QS systems, biocontrol properties and plant growth promoting properties of tomato rhizoplane isolates was addressed. These reveal remarcable diversity of pherotypes, surfactin production and plant growth promoting traits within the population of strains isolated from a rhizoplane of a single plant.

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AB Bakterije komunicirajo s pomočjo majhnih signalnih molekul, ki jih izločajo tekom rasti. Ta proces poimenujemo zaznavanje kvoruma (ang. Quorum sensing - QS) Klasičen model predpostavlja, da se QS signali pri kritični koncentraciji vežejo na specifične receptorie kar sproži koordiniran odziv na nivoju populacije. V tej disertacij, smo preučevali povezave med sintezo signala in odzivom na ta signal QS sistema ComQXPA *Bacillus subtilis*. Ta je sestavljen iz encima ComQ, ki procesira, QS signala ComX, receptorja ComP ter transkripcijskega faktorja ComA, ki aktivira izražanje surfaktina in kompetenčnih genov. Ugotovili smo, da se mutante QSS- (Δ*comQ* ali Δ*comX*) prekomerno odzovejo na ComX, ki ga producira populacija QS+, kar se odraža v povečani produkciji surfaktina in povečani genetski kompetenci za transformacijo. Povečan odziv mutant je povezan z visokim stroškom odziva, kar zniža fitnes mutante in s tem sposobnost sobivanja v kokulturi s populacijo QS+. Pod selekcijskim pritiskom, ki je vezan na sposobnost izmenjave DNA med partnerjema v kokulturi pa ComX postane skupno dobro, ki ga divji tip deli s populacijo »goljufivih« mutant. V teh pogojih je QSS- mutanta hipergoljuf, saj zaradi višje genetske kompetence prevzame več selekcijskih genov divjega tipa kar zviša njeno frekvenco preživetja. Vrednotili smo tudi odziv QS+ in QSS- populacij v koloniji in ugotovili, da zaradi prostorske ločenosti obeh sevov ComX divjega tipa ni v popolnosti dosegljiv mutante. Ugotovili smo, da je pri visoki slanosti QS odziv v planktonski kulturi drugačen od odziva v biofilmu. Pokazali smo tudi, da sevi *B. subtilis* izolirani iz rizosfere paradižnika, kažejo podobno diverzifikacijo ComQXPA sistema, kot tisti izolirani iz tal in da imajo nekateri med njimi značilnosti, ki so pomembne za vlogo te bakterije kot biokontrolnega agenta za zaščito in pospeševanje rasti rastlin.

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ABBREVATIONS AND SYMBOLS

AHL	acyl homoserine lactone
ATP	adenosine triphosphate
Cfp	cyan fluorescent protein
Cm	chloramphenicol
DNA	deoxyribonucleic acid
eDNA	extracellular deoxyribonucleic acid
ESS	evolutionary stable strategy
Gfp	green fluorescent protein
HPLC	high performance liquid chromatography
IAA	indole-3- acetic acid
LacZ	β-galactosidase
Mls	macrolide antibiotics (in this work lincomycin and erythromycin)
MS	Murashige and Skoog medium
OD	optical density
PGP	plant growth promoting
PGPR	plant growth promoting rhizobacteria
SE	standard error
QS	quorum sensing
QS+	wild type; produces QS signal and QS response
QS+A-	surfactin-null mutant (Δ*srfA*); produces QS signal and QS response; cannot produce surfactin
QSR-	receptor-null mutant (Δ*comP*); can produce QS signal, cannot respond to the signal
QSS-	signal-null mutant (Δ*comQ* or Δ*comX*); cannot produce QS signal, can respond to the signal
QSS-A-	signal-null surfactin-null mutant (Δ*comQ*Δ*srfA*); cannot produce QS signal; can respond to the signal; cannot produce surfactin
QSS-K-	signal-null competence-null mutant (Δ*comQ*Δ*comK*); cannot produce QS signal; can respond to the signal; cannot develop competence for transformation
Spec	spectinomycin
T1	time 1 – one hour after transition of bacterial culture into stationary phase
T2	time 2 – two hours after transition of bacterial culture into stationary phase
Yfp	yellow fluorescent protein

1 INTRODUCTION

1.1 PROBLEM DESCRIPTION

In the second half of the 20th century we have become aware that bacteria are not only self-contained, constitutively dividing entities, but rather social beings which live in communities, communicate with each other and act collectively (Tomasz, 1965; Nealson et al., 1970). Communication of microbes, named quorum sensing (QS), is possible due to secretion of small, diffusible signals (Waters & Bassler, 2005). These signals can accumulate at high local density of the population (Platt & Fuqua, 2010). Signals then reach critical concentration and activate specific receptors triggering expression of target genes in a coordinated manner (Waters & Bassler, 2005). It was shown that QS can be important for survival of bacteria (Diggle et al., 2007a) and that both QS signals and QS regulated behaviors can serve as public goods, shared within the community and benefiting even those who do not contribute to their production (Diggle et al., 2007a; Xavier et al., 2011; Yang et al., 2010). Such dishonest mutants can be isolated from natural populations of *Pseudomonas aeruginosa* (Rumbaugh et al., 1999; Denervaud et al., 2004). It was shown that in laboratory conditions QS mutants may invade the wild populations and by being released from metabolic costs of QS functions, they divide faster (Diggle et al., 2007a; Yang et al., 2010). One of the major questions related to evolution of social interactions is how can QS remain stable and what keeps such cheats from invasion in natural populations? For example peptide QS signals of gram positive bacteria were predicted to be costly to synthesize (Keller & Surette, 2006) and ComX produced by *Bacillus subtilis* could also be considered public good in conditions when it can increase fitness of others in the group (West et al., 2006). ComX is a component of ComQXPA QS system (Manguson et al., 1994; Schneider et al., 2002). This system consists of the QS signal processing enzyme ComQ, the QS signal ComX (Manguson et al., 1994; Schneider et al., 2002), the QS receptor ComP and the transcription factor ComA (Weinrauch et al., 1990), which activates the expression of surfactin (Nakano et al., 1991), competence genes (Dubnau, 1991) and many other genes (Comella et al., 2005). It was shown that surfactin helps *B. subtilis* to compete with other species like *Pseudomonas syringae* (Bais et al., 2004) and that competence development may promote survival under specific conditions (Johnsen et al., 2009). But is the ComQXPA QS system essential for survival of *B. subtilis* and if so, how do the wild type populations resists invasion by QS mutants? These questions remain to be answered and important for understanding evolutionary stability of bacterial communication. For example we know that bacteria mostly live and communicate on solid surfaces (Costerton et al., 1995), but QS has mostly been investigated in planktonic cultures. Very little is known about how QS operates in colonies or biofilms and whether in densely packed communities, peptide QS signals can freely diffuse away from the producing cells and be shared within the

whole community. We also do not know how is QS influenced by environmental conditions. For example acyl-homoserine lactone (AHL) signals of gram negative bacteria are sensitive to high temperature, which decreases their production (Latour et al., 2007; Tait et al., 2010). On the other hand peptide signals of gram positive bacteria show higher temperature-stability (Manguson et al., 1994), but their production at different temperatures was never accessed. In addition *B. subtilis* likely encounters high osmolarity in its natural habitat such as plant rhizosphere (Miller & Wood, 1996) or desert soils, but QS in high osmolarity condition was never examined. Finally, thriving on roots, *B. subtilis* can promote the growth of plants (Earl et al., 2007; Barea et al., 2005, van Elsas & Mandic-Mulec, 2013) however mechanisms behind its biocontrol mechanisms and plant growth promoting properties are not well-understood. It was also suggested that expression of biocontrol traits may be regulated by QS (Joshi & McSpadden Gardener, 2005). In fact surfactin, which is induced by ComQXPA, is an important anti-pathogenic agent (Bais et al., 2004), and a signaling molecule that induces colonization of roots (Zeriouh et al., 2014). Moreover, the ComQXPA QS system manifests diversification into distinct "communication'' groups (pherotypes). Strains within a pherotype communicate efficiently but communication between these groups is impaired (Ansladi et al., 2002). It was shown that pherotypes can coexist in soil even at a millimeter scale (Stefanic & Mandic-Mulec, 2009). Still, complementary studies exploring *B. subtilis* pherotype diversity in other habitats, like rhizoplane, where these bacteria may exist in vegetative state, would add to understanding of the pherotype puzzle and the ecological meaning of pherotype diversification in bacterial species. This dissertation will focus on regulation of QS, its evolutionary stability, performance of QS in different growth modes and environmental conditions and its diversity of the ComQXPA QS system within rhizoplane. The results will benefit our knowledge on sociomicrobiology and bring us closer to understanding the communication of microbes, their behaviors and possibly provide new tools to exploit these interesting bacteria to our favor.

1.2 HYPOTHESIS

Working hypotheses that were provided in this dissertation are as follows:

- QS response dynamics of signal-null mutants (e.g. Δ*comQ*) is different as compared to the wild type when both strains are facing the same concentrations of QS signal
- the presence of signal-null mutants in *B. subtilis* population will influence the QS response in this population
- ComX can serve as public good which helps in survival of *B. subtilis* and which is freely shared by the producers with the non-producers
- QS is influenced by growth mode and by environmental factors:
    - QS in planktonic cultures differs from that in colonies
    - high temperature (51°C) increases the accumulation of the ComX pheromone in the condition medium
    - high salinity (8%) influences QS signaling and QS response in *B. subtilis*
- diversity of QS systems can be found within rhizoplane of a single plant

1.3 GOALS

The goal of this dissertation is to show that:

- QS signaling and QS response are not independent events and the lack of signaling function influences the response function
- QS signals of *B. subtilis* can serve as public goods and help in survival of those who do not produce them
- QS is different in planktonic and in colony growth mode
- temperature influences QS in *B. subtilis*
- salinity influences QS in *B. subtilis*
- genetic polymorphisms of *comQXPA* loci, previously identified in soil isolates of *B. subtilis* is also associated with strains of this species isolated from the rhizoplane of one plant

2 LITERATURE REVIEW

2.1 QUORUM SENSING – DO BACTERIA SENSE QUORUM?

Quorum sensing (QS) is the most wide spread mechanism of communication in bacteria (Witzany, 2008). During this process microbes regulate expression of certain genes in response to signaling molecules that are constitutively secreted from cells during growth (Miller & Bassler, 2001; Platt & Fuqua, 2010). Extracellular-signal-regulated behavior of a bacterium was for the first time named QS when referring to process of bioluminescence induction in marine bacterium *Vibrio fisheri* (Fuqua et al., 1994). For next several years it had been accepted that bacteria use QS to monitor population density and that QS signals allow them to estimate when population reaches the size of minimal behavioral unit called ,,quorum" (Miller & Bassler, 2001; Witzany, 2008).

A. B.

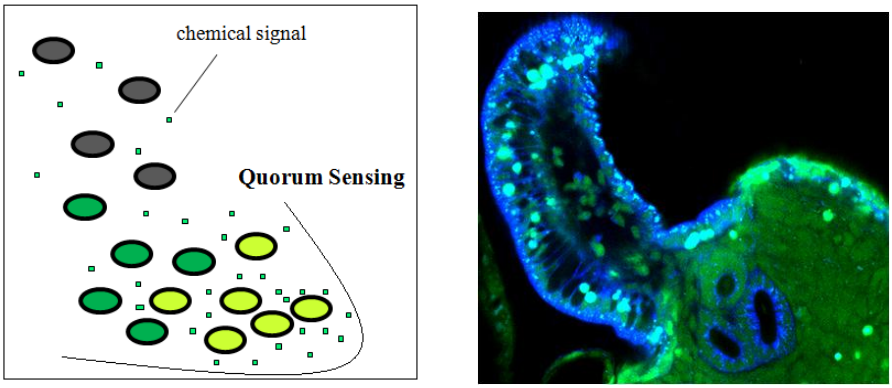


Figure1. **Illustration of quorum sensing**. A. Simple model of the quorum sensing phenomena. That shows that the response (yellow>green> grey) increases with the concentration of the signal and cell density. B. The squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* within the light organ, which is a typical example of quorum sensing (MEDMINUS9, 2010).

Slika 1. **Pojav zaznavanja kvoruma**. A. Preprost model pojava zaznavanja kvoruma, ki izpostavi, da se z večanjem gostote celic in posledično z večjo koncentracijo signala poveča tudi odziv na signal (rumeno > zeleno >sivo) B. Lignji *Euprymna scolopes* in luminescentna bakterija *Vibrio fischeri*, ki je tipičen primer zaznavanja kvoruma (MEDMINUS9, 2010).

V. fischeri is a symbiont of the Hawaiian bobtail squid *Euprymna scolopes* where it exists at high cell density in the squid light organ. Light production by the bacteria enables the squid to eliminate its shadow on the shallow ocean floor and thus light is used by the squid to evade predators (Figure 1B). Inside the organ *V. fischeri* secretes autoinducers, and in response to these molecules, it produces luciferase enzyme which enables the light emission. By contrast,

autoinducers do not accumulate to any significant level in the open ocean, so under this condition, *V. fischeri* does not luminescence. Luciferase production by single dispersed cells would be unnecessary metabolic cost as the light can be detected only when emitted in a group (Dunlap, 1999).

Bioluminescence is a classic example of how bacteria make use of QS molecules to monitor cell-density, however since this discovery, many other QS-regulated behaviors have been described. For example, QS regulates the synthesis of degrading enzymes (Kunst et al., 1994), surfactants (Nakano et al., 1991), exopolymers (Stanley & Lazazzera, 2005, Lopez et al., 2009b; Dogsa et al., 2014), bacteriocins (Marahiel et al., 1993) or development of genetic competence for transformation (Nakano et al., 1991; D'Souza et al., 1994; Comella & Grossman, 2005). Variability of QS-regulated behaviors opened fierce discussion on an evolutionary role of this process, questioning its cooperative nature of sensing density or population size (Redfield, 2002; Hense et al., 2007).

In fact cells can encounter high density of QS signals even when cell numbers are low (Conell et al., 2010). In extreme cases, QS can even operate at level of a single cell, when it is entrapped in a very small space (Carnes et al., 2010). Logically in terms of low diffusion rate it would be directly beneficial for the cell to induce QS-response, because enzymes or other secreted products will not diffuse away (Redfield, 2002). It has now been accepted that QS can be useful from both points of view, the social one, where it is beneficial for the group and the selfish one, where it directly benefits the cell. The character of QS benefits may depend on species and environmental conditions which it encounters (Hense et al., 2007, Platt & Fuqua, 2010). Whatever the main benefits are, it is at least clear that the benefits of QS-regulated gene expression exists what was experimentally demonstrated for several times. For example Darch and colleagues (2012) demonstrated that regulation of extracellular proteases release by QS benefits *P. aeruginosa.* Similar was shown by the model and experiments of Pai and colleagues (2012) that tested fitness gains of QS-dependent production of costly detoxifying agent by *Escherichia coli*. It was also shown by modeling, that bacteria which employ QS to regulate (turn on or turn off) the production of exopolymeric substances, can outcompete strains which do not employ such local density-dependent regulation, indicating again that signal-dependent release of costly products pays off (Nadell et al., 2008).

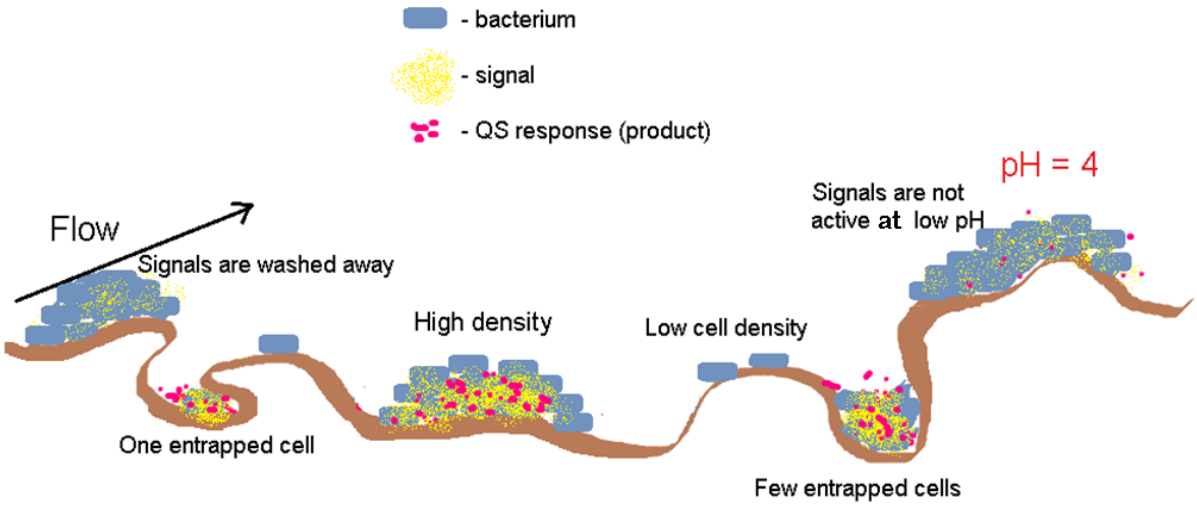


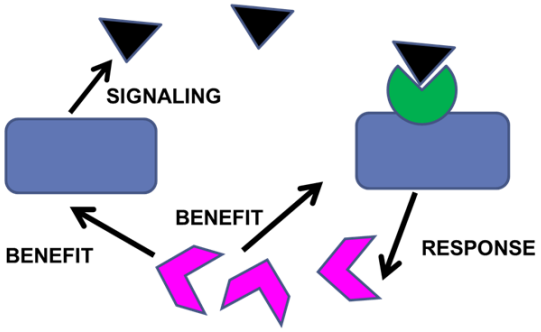
Figure 2. **Different scenarios of QS response induction in relation to signal accumulation**. The accumulation may take place under many conditions: high cell density or low number of cells entrapped in a small space. Despite high population density signals will not accumulate when they are washed away by flow, or when they are not stable in certain environment (like at low pH).

Slika 2. **Različni scenariji za indukcijo QS odziva v odvisnosti od dinamike kopičenja signala**. Do kopičenja lahko pride pod različnimi pogoji: pri visoki celični gostoti ali takrat ko je majhno število celic ujeto v majhnem prostoru. Kljub visoki gostoti populacije lahko signale spere tok tekočine, kar prepreči njihovo kopičenje. Signali lahko v določenem okolju (kot je okolje z nizkim pH) izgubijo stabilnost.

Conell and colleagues showed that single cell of *Staphylococcus aureus* can benefit from QS autoinduction and survive better (Conell et al., 2010), showing that QS can be beneficial regardless of high population size (Redfield, 2001). In fact the name ,,quorum sensing'' carries an imprint of oversimplification, suggesting to us that bacteria aim to monitor, or even count the cell numbers (Platt & Fuqua, 2010). It is more accurate to think of QS as sensing critical concentration of active QS signals. Whether these signals accumulate and remain stable or not, depends on many environmental conditions bacteria are facing at a given time (Figure 2) (Platt & Fuqua, 2010).

2.2 WHAT IS A SIGNAL?

Signal is a molecule secreted by an individual which alters the behavior of other individual and that has evolved for this purpose and which is effective because the response to it has also evolved (Figure 3) (Diggle et al., 2007b). Definitely not all molecules secreted by microbes and influencing other microbes are signals. For example colicin released by *E. coli* during competition with other species influences other individuals by causing depolarization of their cytoplasmic membrane or inhibition of murein synthesis (Feldgarden & Riley, 1999). It is very likely that colicin evolved exactly for this purpose however its efficiency does not depend on specific feedback of the bacteria being under attack. In this case rather than a signal, colicin acts as a coercion, negatively affecting recipient and benefiting the producer by increasing its chances for survival in so called interference competition (Case & Giplin, 1974). Another type of secreted molecules that can influence others, are metabolic by-products like glutamate secreted by *Corynobacterium glutamicum.* Glutamate may positively influence other species providing food resource, however it does not mean that *C. glutamicum* secretes this amino acid in order to help others. Glutamate therefore acts as a cue, providing benefits to the recipient but being neutral for the secreting cell. Finally, *B. subtilis* secretes polypeptide ComX which binds to receptor ComP leading to activation of genetic competence for transformation (Comella & Grossman, 2005). ComX is highly specific to ComP (Manguson et al., 1994, Ansaldi et al., 2002), ComX peptide and ComP receptor coevolved (Ansaldi et al., 2002; Stefanic & Mandic-Mulec, 2009) and both, sender and receiver that also carries ComP, can benefit from the response by competence development (Johnsen et al., 2007). All this allows us to consider ComX as a typical signaling molecule (Diggle et al., 2007b).



MOLECULE	BENEFITS SENDER	BENEFITS RECIPIENT
SIGNAL	+	+
CUE	-	+
COERCION	+	-

Figure 3. **Evolutionary meaning of the signal.** Scheme on the left represents signaling, which evolved in order to induce response and this response was selected for due to positive effects on the sender and on the recipient. Table on the right assumes different types of secreted molecules depending on their effect on the sender and recipient. A signal is beneficial for both, a cue only benefits the recipient and coercion only benefits the sender. „-'' in the table indicates both, neutral or even negative effects (especially in case of coercion).

Slika 3. **Evolucijski pomen signala.** Shema na levi prikazuje signaliziranje, ki se je razvilo z namenom indukcije odziva. Ta se je evolucijsko ohranil zaradi pozitivne selekcije, ki je delovala na pošiljatelja in prejemnika signala. Preglednica na desni povzame različne tipe izlitih molekul na osnovi njihovega učinka na pošiljatelja in prejemnika signala. Signal koristi obema. Navedba (cue) koristi samo prejemniku, medtem ko izsiljevanje (coercion) koristi le pošiljatelju. „-'' v tabeli lahko pomeni nevtralne ali celo negativne učinke (predvsem v primeru izsiljevanja).

How can a signal-response interaction evolve? It is believed that signals originated from non-communication molecules in so-called ritualization process, where communication events were emphasized by gradually increasing the response of the receiver (Tinbergen, 1952). Constitutive release of non-communication molecules, like waste-products, by cell A incidentally informs cell B on the presence of A providing a starting point for the evolution of communication. Reaction of cell B to this by-product may arise as a result of mutation and if this reaction is somehow beneficial to cell A, evolution towards signaling would be expected (Steiger et al., 2011). Efficiency of such sender/recipient interactions can be further increased by increasing quantity of emitted by-products or by increasing their reliability/specificity by adding modifications (Steiger et al., 2011) which typically increases costs of signal synthesis (Keller & Surette, 2006). For example, the ComX peptide is modified by isoprenylation and the type of isoprenyl added affects the specificity of ComX (Ansaldi et al., 2002).

2.3 PROBLEM OF EVOLUTIONARY STABILITY OF QS

Why would then a cell pay the cost of secreting and sharing any costly molecule that benefits other cells? Benefiting other individuals contradicts original Darwinian model of survival of

the fittest (Darwin, 1859), however Darwin did not know that behaviors are determined by genes that can be inherited the altruistic sharing was explained by Hamilton more than hundred years later (Hamilton, 1964). He hypothesized that an individual can increase its own fitness by promoting spread of its own genes that it also shares with its relatives. Therefore gene A encoding for altruism can remain in the population, but only if costs are lower than benefits multiplied by relatedness (c<br). Therefore behavior indirectly benefits an individual, by benefiting its relatives.

Another explanation how sharing and cooperation remains stable is direct benefits. For example secretion of costly enzyme by one of two closely related cells that are sticking together would benefit the producer and non-producer and the first has both selfish and indirect benefit of secretions.

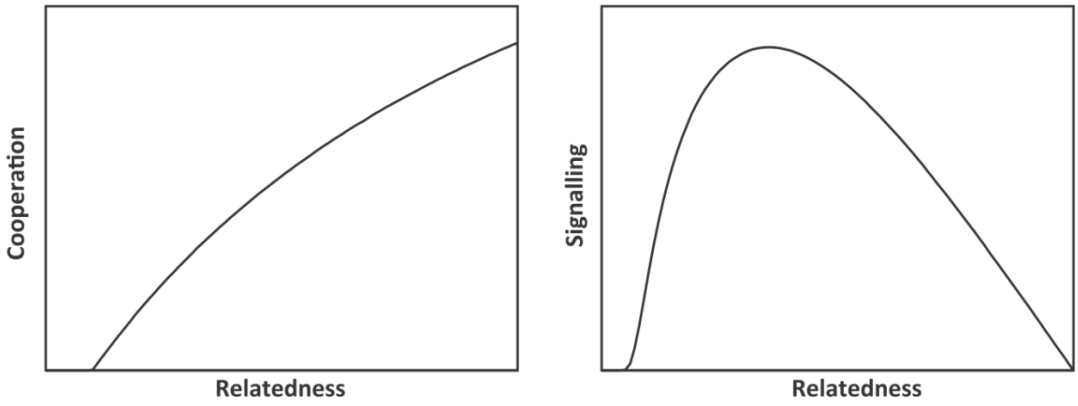


Figure 4. **Brown and Johnstone's model of signaling development (Brown & Johnstone, 2001).** A. According to kin selection theory cooperation increases with increasing relatedness, because the inclusive fitness benefits of cooperation are maximal at high relatedness. B. In terms of intermediate relatedness costly, advanced signaling should develop, because cooperation is still worthwhile, yet there is also a scope for conflict. At low relatedness there is little inclusive fitness benefit of signaling for cooperation so no signaling to mediate it will develop and at high relatedness there is little conflict so that a cheap signal is favored.

Slika 4. **Model Browna in Johnstona, ki povzame evolucijo signaliziranja (Brown & Johnstone, 2001).** A. V skladu z teorijo sorodstvene selekcije, sodelovanje narašča z naraščajočo sorodnostjo, zato ker je dobiček oziroma fitnes za vključevanje sorodnikov maksimalnen pri visoki sorodnosti. B. V pogojih srednje sorodnosti, naj bi se razvilo sofisticirano in s tem tudi energetsko dražje signaliziranje. V tem območju sorodnosti se sodelovanje še vedno splača, vendar je potencialno možen tudi konflikt. Pri nizki sorodnosti je dobiček za vključen fitnes nizek, zato se signaliziranje, ki posreduje sodelovanje, ne bo razvilo. Pri visoki sorodnosti je konflikt majhen, kar spodbuja evolucijo energetsko nezahtevnega signaliziranja.

Brown and Johnstone (2001) using a model of QS evolution proposed how natural selection would influence both the production and the response to signaling molecules. They predicted

conditions under which QS can be an evolutionarily stable strategy (ESS) and cannot be beaten by any other strategy. They found that at high relatedness, there is a shared interest in the cooperative production of public goods (Figure 4A) and cheap signaling to coordinate this (Figure 4B). At low relatedness, there is no selection for cooperation (Figure 4A), so also no selection for signaling to coordinate this (Figure 4B). With intermediate relatedness, there can be selection for secretion of public goods (Figure 4A), but it is in the individual's interest to produce lower levels of them than the other local cells because the relatedness is not very high. This favors strong signaling to manipulate the competitors and make them produce more public goods (Figure 4B). This in turn leads to the signals being increasingly ignored what is called competitive devaluation of signal strength.

Evolutionary stability of QS has been challenged experimentally. For example Diggle and colleagues (2007a) have shown that in *P. aeruginosa* QS signaling and response are costly and that in mixed planktonic cultures of low relatedness between cells wild type can be exploited by QS mutants that avoid the costs of signal production or QS response. However, fitness of the mutants decreases with increasing relatedness in the population suggesting that QS is stabilized here by the kin selection mechanisms (Diggle et al., 2007a). On the other hand, it was shown that signal-null mutants of *V. fisheri* are for some reason outcompeted by the wild type which is able to synthesize AHL suggesting that in this case QS can be stabilized by direct benefits of signaling (Chong et al., 2013). These can be related to pleiotropic effects of signaling or response loss-of function mutations, which have been recently detected in several signaling-dependent systems such as *Dictyostelium discoideum* (Foster et al., 2004) or *P. aeruginosa* (Wilder et al., 2011). Finally QS itself can stabilize cooperative public good production because it ensures their prudent release minimizing the costs and the vulnerability to cheaters invasion (Xavier et al., 2011). The vast majority of experimental studies addressing stability of QS were performed on gram negative systems, probably because QS regulation of gram positive bacteria remains less understood. The stability of QS and QS-mediated social behaviors of gram positive bacteria therefore remains a black box waiting to be opened.

2.4 ComQXPA QS SYSTEM OF *B. subtilis* – A MODEL TO OPEN BLACK BOX OF GRAM-POSITVE'S SOCIAL BEHAVIORS

The discovery of microbial cell-cell communication and coordination of group behaviors has brought new light on microbes that similarly to higher animals, occurred to be social (Bassler & Losick, 2006). Microbes are easy to manipulate, relatively cheap to grow and multiply fast, therefore they are an excellent model to study communication and social behaviors. Although

both gram negative and gram positive bacteria use QS to regulate gene expression, the systems differ in several key issues. Gram negative bacteria use AHLs signals that are small, metabolically cheaper to produce and less specific than peptides used by gram positive bacteria (Keller & Surette, 2006). Another important difference is that AHLs are synthesized as typical secondary metabolites – non-ribosomally, while signals of gram positive bacteria are directly encoded and in most cases posttransionally modified by a processing enzyme. Finally in gram negative bacteria a signal can not only act on the extracellular receptor but it can also freely enter the cell and autoinduce its own expression – which is not the case in most gram positive bacteria (reviewed in Sifri, 2008). These differences indicate that there is an emerging need for addressing QS-related problems on both gram negative and gram positive models.

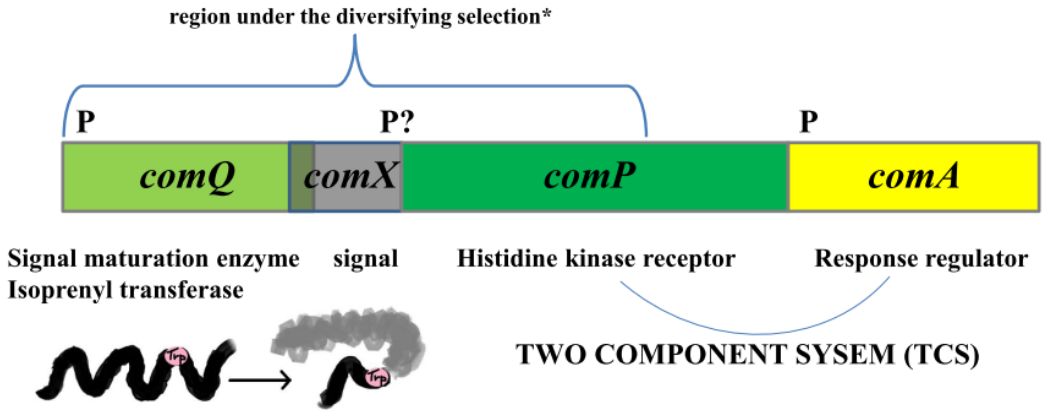


Figure 5. **Genetic architecture of the *comQXPA* QS genetic locus of *B. subtilis***. Overlapping region of the *comQ* and *comX* genes are marked as overlapping as indicated by positioning of the light green and grey boxes. Promoters and putative promoters are marked with P or P?, respectively. Also the region under the diversifying selection responsible for pherotype specificity is indicated (chapter 2.5.5).

Slika 5. **Organizacija lokusa *comQXPA*, ki kodira sistem za zaznavanje kvoruma pri *B. subtilis.*** Prekrivanje genov *comQ* in *comX* je označeno s prekrivanjem barvnih odsekov. Promotorji so označeni s P, domnevni promotorji pa s P?. Označeno je tudi območje ki je pod selekcijo diverzifikacije in kaže povečan polimorfizem znotraj vrste in je osnova za evolucijo ferotipov (poglavje 2.2.2).

The ComQXPA QS system of *B. subtilis* is a typical QS system of gram positive bacteria and it directly or indirectly controls the expression of nearly 200 genes (Ogura et al., 2001; Comella & Grossman, 2005). This system involves four proteins: the ComQ isoprenyl transferase, the ComX signal peptide, the ComP histidine kinase, and the ComA response regulator, all encoded by a tandem of genes in the *comQXPA* operon (Figure 5).

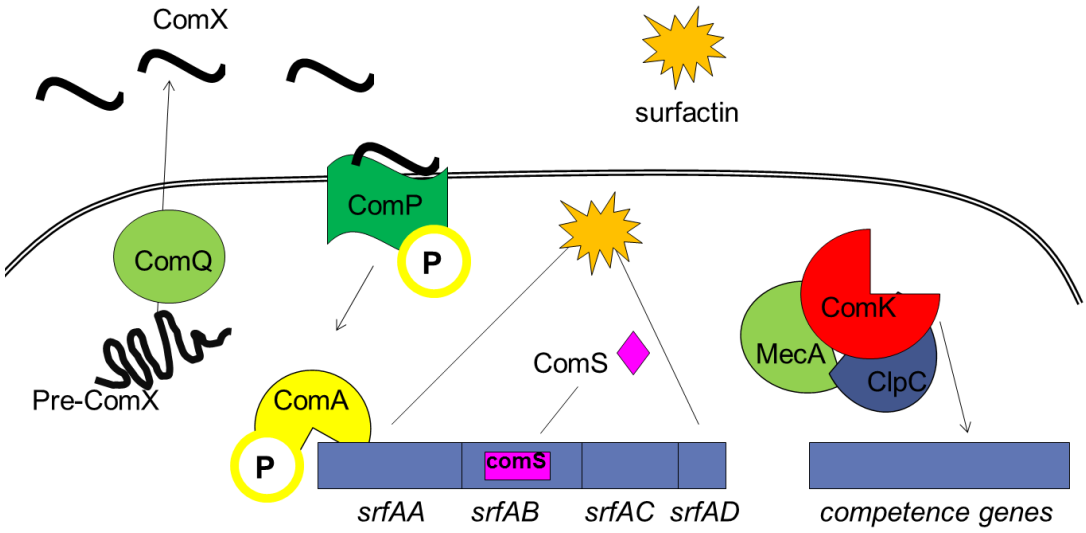


Figure 6. **A model of the ComQXPA QS system functioning.** ComQ modifies Pre-ComX and probably assists in secretion of ComX from the cell. ComX by binding to the membrane receptor ComP induces its autophosphorylation. ComP then phosphorylates ComA. ComA activates transcription of the *srfAA* operon which encodes enzymes responsible for the synthesis of the lipopeptide antibiotic surfactin. On the same operon the *comS* gene encodes the ComS peptide, which is crucial for release of ComK from the proteolytic complex (MecA-ComK-ClpC). This release stabilizes ComK, which then induces development of competence for transformation in subpopulation of cells.

Slika 6. **Model delovanja ComQXPA sistema za zaznavanje kvoruma.** ComQ modificira pre-ComX in verjetno tudi pomaga pri izlivu ComX iz celice. ComX se veže na membranski receptor ComP, kar sproži njegovo avtofosforilacijo. ComP nato fosforilira ComA. ComA aktivira transkripcijo operona *srfAA*, ki kodira encime za sintezo lipopeptidnega antibiotika surfaktina. Na istim operonu se nahaja tudi gen *comS*, ki kodira peptid ComS. Ta je ključen za sprostitev ComK iz proteolitičnega kompleksa (MecA-ComK-ClpC), kar stabilizira ComK in pripelje do razvoja genetske kompetence za transformacijo v subpopulaciji celic.

The signaling peptide ComX is initially synthesized as a 55-residue propeptide and then processed and modified by the isoprenyl transferase ComQ (Manguson et al., 1994; Schneider et al., 2002). The isoprenylated ComX is then secreted and upon reaching the critical concentration, it activates autophosphorylation of the membrane-bound ComP, which then phosphorylates the transcriptional activator ComA (Weinrauch et al., 1990). Phosphorylated ComA directly modulates the expression of various genes including the operon *srfA* (Nakano et al., 1991). This operon encodes enzymes for nonribosomal synthesis of the lipopeptide antibiotic surfactin (Comella & Grossman, 2005; Roggiani & Dubnau, 1993). The *srfA* operon also encodes a small peptide ComS that is essential for competence development (Figure 6) (Comella & Grossman, 2005; D'Souza et al., 1994; Hamoen et al., 1995). The evolutionary role of the coupling between *comS* and *srfA* expression remains a mystery. A failure to

synthesize the surfactin does not prevent development of competence for transformation (Nakano et al., 1991) yet ComS peptide induces competence through release of the major competence regulator ComK from the inhibitory proteolytic complex. In turn, ComK activates its own transcription (van Sinderen & Venema, 1994) and transcription of late competence genes (Hahn et al., 1996).

2.5 UNANSWERED „WHY" AND „HOW" QUESTIONS ABOUT QS

2.5.1 Are QS signal production and QS response functionally independent?

It is known that QS systems are wide spread among bacteria (Bassler & Losick, 2006). Putative ComQXPA QS systems can be predicted from genome sequences of phylogenetically distinct species indicating that this operon and the mode of genetic regulation are evolutionary ancient (Dogsa et al., 2014). Coevolution of signals and their cognate receptors has then a long history, sometimes resulting in very specific and polymorphic signals like those of *B. subtilis* (Tortosa et al., 2001). It has been shown several times that mutants with signal deletion can respond to the signal provided and also that the mutants with receptor deletion can produce QS signals (Diggle et al., 2007a; Schneider et al., 2002; Piazza et al., 1999). However it has never been tested to which extend these two functions are intracellularily independent. In other words, it has never been examined whether cell with signal deletion sense extracellular signal equally well as signal-proficient strains.

2.5.2 How does QS work in colonies or biofilms?

Over the past decades QS has been examined in laboratory conditions using planktonic cultures where cells grow in a shaken liquid suspension with cell density and concentration of QS signals gradually increasing yet remain homogeneous all the time. However life of bacteria in nature is far from that, because microbes usually live in a form of biofilms, attached to solid surfaces or floating on the liquid-surface interfaces where conditions for communication differ dramatically from those in the laboratory flasks (Decho et al., 2010).

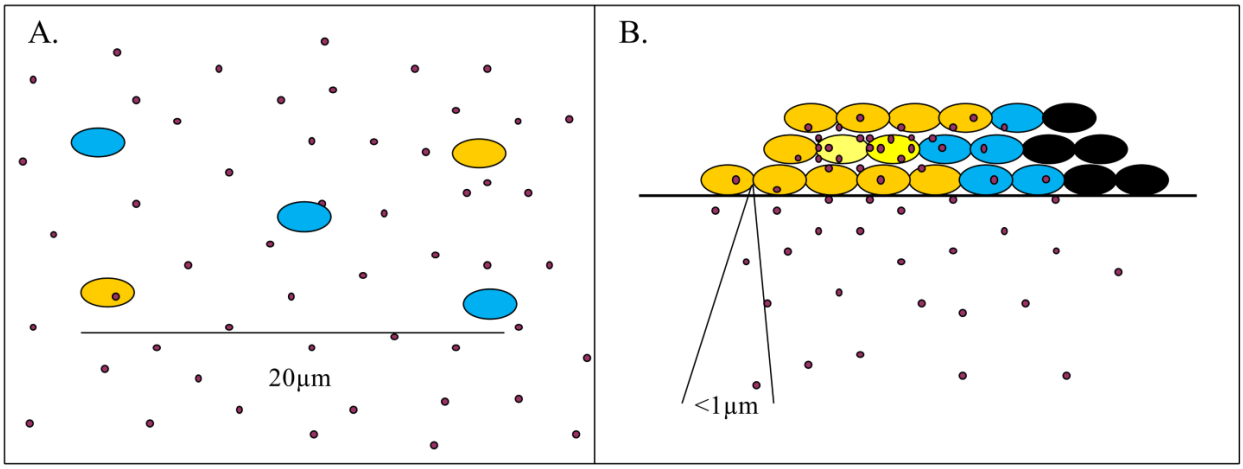


Figure 7. **Schematic representation of the QS conditions in a mixed culture of the QS+ wild type and QSS-signal-null mutant in planktonic and attached growth mode**. A. In planktonic coculture at maximal cell density (~10^8 cells·ml^{-1}) bacteria are dispersed and an individual cell occupies approximately 8 x 10^3 μm^3 volume. The average intercellular distance of 20 µm in one dimensional scale significantly reduces the probability of direct cell-cell contact. B. The distance between cells in a colony is smaller than the length of the bacterial cell. QS+ wild type cells are coloured yellow, QSS- signal deficient cells blue, QSS- cells responding to signal are shown in blue and those not sensing the signal are shown in black, the signal is shown as violet dots.

Slika 7. **Shematski prikaz pogojev za QS v mešanih kulturah sestavljenih iz divjega tipa QS+ in mutante, ki ne proizvaja signal QSS-.** A. V stresani planktonski kokulturi so pri maksimalni celični gostoti (~10^8 cells·ml^{-1}) bakterie razprašene in posamezna celica zasede približno 8 x 10^3 μm^3 volumna. Proprečna razdalja med celicami v enodimenzionalni lestvici znaša 20 µm, kar značilno zmanjšuje verjetnost neposrednega stika celic. B. Razdalja med celicami v koloniji je manjša kot dolžina posamezne celice. Celice divjega tipa QS+ so obarvane z rumeno, celice mutante, ki ne producira signala (QSS-) are se nanj odzivajo so obarvane z modro, QSS- celice ki se ne odzovejo na signal so obarvane s črno, signal je prikazan kot vijolične pike.

In biofilms or colonies distances between cells are dramatically smaller as those in planktonic cultures (Figure 7), cells encounter gradients of nutrients, pH, oxygen or ions (Decho et al., 2010). It is also possible that cells encounter gradients of QS signals since it is not known whether signal production rate within the population is homogenous at single cell level and whether peptide QS signals diffuse freely between cells that stick together.

2.5.3 How does QS work in different environmental conditions?

In general the influence of environmental factors on QS is poorly understood. It is not known whether the crucial environmental variables like temperature or salinity influence the synthesis or the activity of peptide signaling molecules, because usually the experiments are performed at optimal conditions. It is known that the ComX pheromone is heat stable (80°C, 12 hours) (Manguson, 1994). It is also known that QS response of *B. subtilis* is negatively affected by

low pH (Cosby et al., 1998). The bioinformatics analysis revealed that *B. subtilis* grown in low temperature induces so called cold-shock proteins (Csp) which work as chaperons and bind to mRNA reducing formation of secondary structures (Buddle et al., 2006). In addition, in order to deal with decreased membrane fluidity at low temperature *B. subtilis* induces synthesis of non-saturated fatty-acids (Aguilar, 1998). Finally there is a global repression of genes involved in glycolysis, oxidative phosphorylation, ATP synthesis, purine and pyrimidine biosynthesis, and haem and fatty acid biosynthesis. Taken together, these observations reflect the reduced catabolic and anabolic needs of slow-growing, low-temperature-cultivated *B. subtilis* cells (Buddle et al., 2005). Is then the rate of ComX synthesis also reduced? Analogically, when grown at high temperatures (48-50°C) *B. subtilis* induces heat-shock proteins, mostly chaperons or ATP-dependent proteases, which either assist in proper folding or in degradation of temperature-affected proteins (Arnosti et al., 1986). It is known that ComX-regulated surfactin production is significantly influenced by temperature. Highest concentrations of surfactin are produced at around 30°C and they decrease with increasing temperature to almost no surfactin being produced at 50°C (Abushady et al., 2005). This decrease could be due to induction of Spx protein during heat-shock. Spx is a repressor of srfA transcription (Zhang et al. 2006) and during heat shock Spx is released from degradation by ClpXP complex (Nakano et al., 2002)which is needed for degradation of damaged proteins during heat shock (Helmann et al., 2001) and this presumably leads to repression of srfA transcription. It could also be that temperature-dependent changes in *srfA* expression are caused by changes in ComX concentration, but this remains to be tested. Expression of *srfA* is also under positive regulation by PerR during oxidative stress. However, this regulation is independent of the ComQXPA QS system (Hayashi et al., 2005).

B. subtilis is able to grow in very high salt concentration and it was shown that in shaken planktonic culture, after adaptation period to salt stress (6% NaCl) the synthesis of majority of vegetative proteins is repressed (Hoper et al., 2006). Interestingly, TCA cycle is repressed, except for the enzymes converting glucose to 2-oxoglutarate which is probably needed to supply glutamate, a proline precursor (Hoper et al., 2006). Proline is known osmoprotectant, which is produced or taken up by *B. subtilis* facing salt stress (Brill et al., 2011). In addition transcriptomic data indicated changes in the membrane fatty acids metabolism (up-regulation of enzymes responsible for fatty acid chain elongation, or degradation of amino acids needed for branched fatty-acid synthesis) (Hoper et al., 2006). Besides, synthesis of general stress proteins of σ^B regulon is up-regulated (similarly as during heat, cold or acidic stress or glucose, phosphate or oxygen limitation). Also PerR regulon is upregulated, which links salt stress response with regulation of oxidative stress response (Hoper et al., 2006). All these analyses were performed in liquid cultures and it is not understood how *B. subtilis* reacts to salt stress in biofilm. In biofilms bacteria probably face high osmotic pressure due to the

presence of high concentrations of exopolymers and other substances entrapped in the matrix even if they are not exposed to high salt (Rubinstein et al., 2012), so one would expect different adaptation to salt stress in biofilms as compared to planktonic growth mode. However salt response in biofilm has not been addressed yet and QS functions at high salt were not examined either in planktonic nor in biofilm growth modes.

2.5.4 Why does *B. subtilis* perform QS?

Although it is known that ComQXPA QS system of *B. subtilis* regulates at least 20 genes directly and additional 150 indirectly (Comella & Grossman, 2009) it was never shown that this system helps in survival of *B. subtilis*. It was shown that QS-regulated traits like surfactin can help *B. subtilis* to outcompete *P. syringae* from cocultures (Bais et al., 2004). It was also shown that competence can help survive penicillin treatment (Johnsen et al., 2009). Both surfactin production and competence are QS-dependent therefore lack of QS should also handicap the population in terms of competition or penicillin treatment. Can we then show that in such conditions ComX serves as public good? Will QS remain stable or will it be vulnerable to cheating by QS-deficient mutants?

2.5.5 Why are some QS systems diverse?

A striking feature of ComQXPA QS is the existence of distinct „language'' groups (pherotypes) where strains can communicate efficiently within but not between groups (Ansladi et al., 2002). Similar phenomena is found in other Gram positive bacteria, including *Staphylococcus aureus, Streptococcus pneumoniae* (Pozzi et al., 1996; Ji et al., 1997; Whatmore et al., 1999), and *B. cereus* (Slamti et al., 2005). The sequences of *comQ, comX* and *comP* genes were aligned using either Neighbour Joining or Minimum Evolution distance based methods and these revealed that these sequences cluster into 4 distinct groups. These groups were then analyzed for cross activation by specific sensor strains. These indicated that sequence clusters correlate with 4 communication specificity groups or pherotypes and that strains within these clusters can exchange signals while strain across clusters do not communicate efficiently (Ansaldi et al., 2002; Stefanic & Mandic-Mulec, 2009; Tran et al., 2000). It was shown that all pherotypes can coexist in soil at a millimeter scale (Stefanic & Mandic-Mulec, 2009). It was also shown that strains that reside very close to each other (mm distances) can be classified to different phylogenetically and ecologically related clusters (ecotypes) with a different pherotype dominating each ecotype (Stefanic et al., 2012). This supports the hypothesis that pherotype diversity is an adaptation to ecological diversity within *B. subtilis* (Stefanic et al., 2012). Still, complementary studies exploring *B. subtilis* pherotype diversity in another microhabitat, like rhizosphere would help to better understand the pherotype puzzle and ecological meaning of multilingual bacterial species.

2.5.6 Is there a link between QS and plant-growth-promotion in *B. subtilis*?

Bacillus subtilis is one of the most diverse and environmentally widespread bacterium (Earl et al., 2008; Mandic-Mulec & Prosser, 2011). It can persist in soil mostly in a form of dormant spores and its vegetative form is believed to be associated with plant rhizosphere (Norris & Wolf, 1961). *B. subtilis* can colonize roots, and promote the growth of plants (Bais et al., 2004; Zeriouc et al., 2013; Barea et al., 2005, van Elsas & Mandic Mulec, 2013), however its biocontrol mechanism is not well-understood. Based on gene sequence analysis of known PGPRs and competition experiments, it was suggested that their potential to protect plants from pathogens relies on antibiotics production (Bais et al., 2004, Joshi & Gardener, 2005), disruption of the pathogen cell-cell signaling (Dong et al., 2000) or an activation of the plant defense system (Ongena et al., 2005). It was also suggested that expression of biocontrol traits may be regulated by QS (Joshi & Gardener, 2005). This is in line with QS-dependent expression of lipopeptide antibiotic surfactin, which acts against *P. syringae* (Bais et al., 2004). In addition surfactin serves as a signaling molecule that triggers biofilm formation (Lopez et al., 2009b) which is essential for colonization of roots (Beuregard et al., 2012; Zeriouh et al., 2014) and protection against pathogens (Zeriouh et al., 2014).

ComQXP QS system (Ansladi et al., 2002; Stefanic & Mandic-Mulec 2009) through modulation of surfactin production and biofilm formation may then be important for biocontrol potential of *B. subtilis*. Therefore knowledge on its diversity within rhizosphere and how this diversity manifests in QS-regulated traits could help in optimal design of biocontrol formulations. It is widely accepted that these are an ecofriendly alternative to chemical pesticides and they have been in commercial use since several years (Nakkeeren et al., 2005). However formulations are most often based on monocultures while suppression of plant disease does not depend on single species, but rather on whole community of microbes (Mendes et al., 2011).

Here we ask first, whether different QS pherotypes of *B. subtilis* can coexist within a rhizoplane of a single plant. We further ask whether being a member of a certain pherotype in the rhizoplane translates into similar expression of known ComQXPA-regulated biocontrol properties like lipopeptide antibiotic production or biofilm formation. In addition, we compare direct plant growth promotion and potential PGP traits between isolates derived from a single plant or within a pherotype.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents

- 1,3,5 - triphenyl tetrazolium formazan *(*TTF*)* M_w = 303.37 (Fluka, Switzerland)
- 2-({2-[Bis(carboxymethyl)amino]ethyl}(carboxymethyl)amino) acetic acid (EDTA) $C_{10}H_{16}N_2O_8$ M_w = 292.24 (Sigma, USA)
- 2,3,5 - Triphenyl tetrazolium chloride (TTC) $C_{19}H_{15}ClN_4$ M_w = 334.81 (Merck, Germany)
- 2-mercaptoethanol C_2H_6OS M_w = 78.13 (Sigma, USA)
- 96% (V/V) ethanol C_2H_2OH M_w = 46.07 (Merck, Germany)
- acetonitrile C_2H_3N M_w = 41.05 (Sigma, USA)
- acid hydrolyzed casein (BD, USA)
- agarose (Sigma, USA)
- ammonium chloride NH_4Cl M_w = 53.49 (Merck, Germany)
- ammonium sulfate $(NH_4)_2SO_4$ (Merck, Germany)
- calcium chloride dehydrate $CaCl_2$ x $2H_2O$ M_w = 147.01 (Sigma, USA)
- calcium phosphate monobasic $Ca(H_2PO_4)_2$ M_w = 234.05 (Sigma, USA)
- carboxymethylcellulose (CMC) sodium salt (Sigma, USA)
- chloroform $CHCl_3$ M_w = 119.38 (Sigma, USA)
- chrome azurol S (CAS) $C_{23}H_{13}Cl_2Na_3O_9S$ M_w = 605.29 (Sigma, USA)
- D-(+)-glucose $C_6H_{12}O_6$ M_w = 180.16 (Sigma, USA)
- destiled water
- dichloromethane CH_2Cl_2 M_w = 84.93 (Sigma, USA)
- dipotassum phosphate K_2HPO_4 (Sigma, USA)
- disodium hydrogen phosphate dihydrate $Na_2HPO_4 \cdot 2H_2O$ M_w = 141.96 (Merck, Germany)
- fluorescein sodium $C_{20}H_{10}Na_2O_5$ M_w = 376.27 (Sigma, USA)
- glycerol $C_3H_8O_3$ M_w = 92.09
- hexadecyltrimetyl ammonium bromide (CTAB) $C_{19}H_{42}BrN$ M_w = 364.45 (Sigma, USA)
- hydrogen chloride HCl M_w = 36.5 (Merck, Germany)
- iron chloride $FeCl_3$ M_w = 162.2 (Fluka, Switzerland)
- iron sulphate heptahydrate $FeSO_4$ x $7H_2O$ M_w =278.01 (Merck, Germany)
- isoamyl alcohol $C_5H_{12}O$ M_w = 88.15 (Fluka, Switzerland)
- LB broth (Sigma, USA)
- LB-agar (Sigma, USA)
- L-histidine M_w = 155.15 (Fluka, Switzerland)
- L-leucin M_w = 131.17 (Merck, Germany)
- L-methionine M_w = 149.21 (Merck, Germany)
- magnesium sulphate heptahydrate $MgSO_4 \cdot 7H_2O$ M_w = 246.48 (Merck, Germany)
- manganese sulphate MnSO4 M_w = 151 (Merck, Germany)
- mangenesium chloride hexahidrate $MgCl_2$ x $6H_2O$ M_w = 203.3 (Sigma, USA)
- methanol CH_3OH M_w = 32.04 (Merck, Germany)

- MiliQ water (deionized water)
- N-acetyl-β-D- glucosaminide (MUF) $C_{14}H_{18}N_2O_8$ M_w = 342.30 (Sigma, USA)
- ortho-nitrophenyl-β-galactoside $C_{12}H_{15}NO_8$ M_w = 301.25 (Sigma, USA)
- orthophosphoric acid H_3PO_4 M_w = 98 (Merck, Germany)
- pectin from citrus peel (Sigma, USA)
- peptocomplex (Biolife, Italy)
- perchloric acid $HClO_4$ M_w = 100.46 (Merck, Germany)
- piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) $C_8H_{18}N_2O_6S_2$ M_w = 302.37 (Merck, Germany)
- Poly-L-lysin (Sigma, USA)
- potassium chloride M_w = 74.55 (Sigma, USA)
- potassium phosphate monobasic KH_2PO_4 M_w = 136.09 (Sigma, USA)
- proteose peptone No.3 (BD, USA)
- SlowFade (Invitrogene, USA)
- sodium bicarbonate $NaHCO_3$ M_w = 87 (Merck, Germany)
- sodium chloride NaCl M_w = 58.5 (Merck, Germany)
- sodium dodecyl sulphate (SDS) $NaC_{12}H_{25}SO_4$ M_w = 288.37 (Fluka, Switzerland)
- sodium hydroxide NaOH M_w = 40 (Merck, Germany)
- sodium nitrate $NaNO_3$ M_w = 84.99 (Sigma, USA)
- sucrose $C_{12}H_{22}O_{11}$ M_w = 342.29 (Merck, Germany)
- thiamine $C_{12}H_{17}ClN_4OS$ M_w = 136.3 (Fluka, Switzerland)
- toluene C_7H_8 M_w = 92.14 (Sigma, USA)
- trifluoroacetic acid $C_2HF_3O_2$ M_w =114.02 (Sigma, USA)
- Tris-base $NH_2C(CH_2OH)_3$ M_w = 121.4 (Sigma, USA)
- Tris-hydrochloride $C_4H_{11}NO_3 \cdot HCl$ M_w = 157.6 (Sigma, USA)
- tri-sodium citrate dihidrate $C_6H_5Na_3O_7 \cdot 2H_2O$ M_w = 294.1 (Merck, Germany)
- yeast extract (Biolife, Italy)
- zinkum chloride $ZnCl_2$ M_w = 136.3 (Sigma, USA)

3.1.2 Enzymes and active compounds

- indole-3-acetic acid (IAA) (Sigma, USA)
- lysozyme (Sigma, USA)
- nystatin (Fluka, Switzerland)
- proteinase K (Sigma, USA)
- RNase (Chromosomal DNA isolation kit; Invitrogen, USA)
- surfactin (Sigma, USA)

3.1.3 Kits

- Live/Dead BacLight Kit (Invitrogene, USA)
- Chromosomal DNA isolation kit (Invitrogen, USA)
- Qiagen plasmid extraction kit (Qiagen, Netherlands)

3.1.4 Antibiotics

- Ampicilin (Sigma, USA)
- Chloramphenicol (Sigma, USA)
- Erythromycin (Sigma, USA)
- Kanamycin (Sigma, USA)
- Lincomycin (Sigma, USA)
- Spectinomycin (Sigma, USA)
- Tetracycline (Sigma, USA)

3.1.5 Media

CM medium (Competence medium):

- 100 ml of SS buffer (0.6 g KH_2PO_4, 1.4 g K_2HPO_4, 0.2 g $(NH_4)_2SO_4$, 0.1 g Na-citrate · $2H_2O$, 0.02 g $MgSO_4 \cdot 7H_2O$)
- 1 ml 50% (v/w) glucose
- 1 ml 2% (v/w) acid hydrolyzed casein
- 1 ml 10% (v/w) yeasts extract
- 0.5 ml methionine [10 mg/ml]
- 0.5 ml leucine [10 mg/ml]
- 0.5 ml histidine [10 mg/ml]

For solid media preparation CM was supplemented with 1.5 g of agar per 100 ml of the medium.

M9 medim:

- 200 ml 5xM9 salt (64 g $Na_2HPO_4 \cdot 2H_2O$, 15 g KH_2PO_4, 5 g NH_4Cl, 150 g NaCl, 1000 ml H_2O)
- 2 ml 1 M $MgSO_4 \cdot 7H_2O$
- 0.1 ml 1 M $CaCl_2 \cdot 2H_2O$
- 20 ml 50% (v/w) glucose
- 775 ml dH_2O

PVK medium (Pikovskaya medium):

- 0.5 g yeast extract
- 10 g glucose
- 5 g $Ca(H_2PO_4)_2$
- 5 g $(NH_4)_2SO_4$
- 0.2 g KCl

- 0.2 g $MgSO_4 \cdot 7H_2O$
- 0.0001 g MnSO4
- 0.00018 g $FeSO_4 \cdot 7H_2O$
- 15 g agar
- Per 1 liter of destilled water

MSN medium (Minimal salts nitrogen):

- 5 mM potassium phosphate buffer pH 7
- 0.1 M Mops pH 7
- 2 mM $MgCl_2$
- 0.05 mM $MnCl_2$
- 1 μM $ZnCl_2$
- 2 μM thiamine
- 700 μM $CaCl_2$
- 0.2% NH_4Cl

CMC agar:

- 2 g $NaNO_3$
- 1 g K_2HPO_4
- 1 g $MgSO_4 \cdot 7H_2O$
- 0.5 g KCl
- 2 g carboxymethylcellulose (CMC) sodium salt,
- 0.2 g peptocomplex
- 17 g agar
- Per 1 liter of destilled water

CPG medium:

- 1 g Casamino Acids
- 10 g Bacto Peptone
- 5 g glucose
- 15 g agar
- Per 1 liter of destilled water
- pH 7.2

King's B medium:

- 10 g proteose peptone
- 1.5 g K_2HPO_4
- 15 g glycerol
- 5 mL $MgSO_4$ (1 M; sterile)

- Per 1 liter of destilled water

Yeast peptone glucose agar (YPGA):

- 5 g yeast extract
- 5 g proteose peptone
- 10 g glucose
- 12 g agar
- Per 1 liter of destilled water
- pH 7.2-7.4

MS medium:

- 2.2 g/l Murashige and Skoog basal salts mixture (Sigma, USA)
- 1% sucrose

3.1.6 Bacterial strains and plasmids

Engineered *B. subtilis* and *E. coli* strains:

- **BD2833** *his leu met srfA-lacZ* (*tet*) (Tortosa et al., 2001)
- **BD2876** *his leu met srfA-lacZ* (*tet*) *comQ::kan* (Tortosa et al., 2001)
- **BD2913** *his met srfA-lacZ* (*tet*) *amyE***::***xylR Pxyl-comK* (*ery*) (*comQ comX comP* replaced by genes from *B. mojavensis* RO-H-1) (Tortosa et al., 2001)
- **BD2915** *his met srfA-lacZ* (*tet*) *amyE***::***xylR Pxyl-comK* (*ery*) (*comQ comX comP* replaced by genes from *B. subtilis* natto NAF4) (Tortosa et al., 2001)
- **BD2936** *his met srfA-lacZ* (*tet*) *amyE***::***xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. mojavensis* RO-B-2) (Tortosa et al., 2001)
- **BD2940** *his leu met srfA-lacZ* (*tet*) *amyE***::***xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. subtilis* RO-E-2) (Ansladi et al., 2002)
- **BD2949** *his leu met srfA-lacZ* (*tet*) *amyE***::***xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. subtilis* RS-D-2) (Ansladi et al., 2002)
- **BD2877** *his leu met srfA-lacZ (tet)* (*comQ::phl comX comP* replaced by genes from *B. subtilis natto* NAF4) (Tortosa et al., 2001)

- **BD2962** *his met srfA-lacZ (tet) amyE::xylR Pxyl-comK* (*ery*) (*comQ::pED345 comX comP* replaced by genes from *B. mojavensis* RO-H-1) (Tortosa et al., 2001)
- **BD2983** *his leu met srfA-lacZ (tet) amyE::xylR Pxyl-comK* (*cat*) (*comQ::pED345 comX comP* replaced by genes from *B. mojavensis* RO-B-2) (Ansladi et al., 2002)
- **BD3019** *his leu met srfA-lacZ* (*tet*) *amyE::xylR Pxyl-comK* (*cat*) (*comQ::pED375 comX comP* replaced by genes from *B. subtilis* RS-D-2) (Ansladi et al., 2002)
- **BD3020** *his leu met srfA-lacZ* (*tet*) *amyE::xylR Pxyl-comK* (*cat*) (*comQ::pED375 comX comP* replaced by genes from B. subtilis RO-E-2) (Ansladi et al., 2002)
- **BD4720 (QS+)** *his leu met srfA-yfp* (*spec*)
- **BD4729 (QSS-)** *his leu met srfA-lacZ* (*tet*) *comQ::kan srfA-cfp* (*cat*)
- **BD4726 (QS+)** *his leu met srfA-cfp* (*spec*)
- **BD4720_Q (QSS-)** *his leu met srfA-yfp* (*spec*) *comQ::kan*
- **PS-216 (QS+)**Undomesticated wild-type isolate (Stefanic & Mandic-Mulec, 2009)
- **PS216 (QS+)** *srfA-cfp* (*cat*)
- **PS216ΔcomQ (QSS-)** *comQ::kan srfA-cfp* (*cat*)
- **PS216ΔcomP (QSR-)** *comP::cat*
- **PS216ΔsrfA (QS+A-)** *srfA::Tn917* (*mls*) *srfA-cfp* (*cat*)
- **PS-216ΔsrfA (QS+A-)** *srfA::Tn917* (*mls*)
- **PS-216ΔcomQXP** (**QSS-R-)** *comQXP::kan*
- **PS-216ΔcomQΔcomK (QSS-K-)** *comQ::kan comK::spec*
- **PS-216ΔcotA (QS+)** *cotA::cm*
- **PS216ΔskfA (QS+)** *skfA::spec*
- **PS216ΔcomQΔcotA (QSS-)** *comQ::kan cotA::cm*

- **PS216ΔcomQΔskfA (QSS-)** *comQ::kan skfA::spec*
- **PS216ΔsrfAΔcotA (QS+A-)** *srfA::Tn917* (*mls*) *cotA::cm*
- **PS216ΔsrfAΔskfA (QS+A-)** *srfA::Tn917* (*mls*) *skfA::spec*
- **PS216ΔcomQΔsrfA (QSS-A-)** *comQ::kan srfA::Tn917* (*mls*) *srfA-cfp* (*cat*)
- **PS-216ΔcomQΔsrfA (QSS-A-)** *comQ::kan srfA::Tn917* (*mls*)
- **PS216ΔcomX (QSS-)** *ΔcomX pKB68* (*comXW53A cat mls*)
- **KTB308** *ΔcomX amyE::*(*srfA-lacZΩ 682 neo*) *pKB68* (*comXW53A cat mls*) (Schneider et al., 2002)
- **OKB120** *pheA1 sfp srfA::Tn917* (Nakano et al., 1988)
- **BD4679** *comK::spec* (gift from D. Dubnau)
- **BD2711** *comK-gfp* (*cat*)
- **BD2899** *comGA-gfp* (*cat*)
- **BD1658** *his leu met comP::cat* (Tortosa et al., 2001)
- **PS-216ComK-GFP (QS+)** *comK-gfp* (*cat*)
- **PS-216ΔcomQComK-GFP (QSS-)** *comQ::kan comK-gfp* (*cat*)
- **PS-216ComGA-GFP (QS+)** *comGA-gfp* (*cat*)
- **PS-216ΔcomQComGA-GFP (QSS-)** *comQ::kan comGA-gfp* (*cat*)
- **PS-216ΔsrfAComK-GFP (QS+A-)** *srfA::Tn917* (*mls*) *comK-gfp* (*cat*)
- **PS-216ΔcomQΔsrfA (QSS-A-)** *comQ::kan srfA::Tn917* (*mls*) *comK-gfp* (*cat*)
- **ED367** BL21 + pED367 (= pET22 + *comQ* and *comX*) (Ansaldi et al., 2002)

Rizosphere isolates and control strains (*Bacillus* spp.*)*:

- **T12-1** *B. subtilis*
- **T14-1** *B. subtilis*

- **T14-3** *B. subtilis*
- **T14-4** *B. subtilis*
- **T14-5** *B. subtilis*
- **T15-1** *B. licheniformis*
- **T16-2** *B. subtilis*
- **T16-3** *B. subtilis*
- **T16-4** *B. subtilis*
- **T16-5** *B. subtilis*
- **T16-6** *B. licheniformis*
- **T16-7** *B. amyloliquefaciens*
- **T16-8** *B. subtilis*
- **T16-10** *B. subtilis*
- **T17-1** *B. subtilis*
- **T19-1** *B.megaterium*
- **T21-2** *B. subtilis*
- **T24-5** *B. pumilus*
- **T26-2** *B. licheniformis*
- **T31-1** *B. licheniformis*
- **GB03** *B. subtilis* (BGSC 3A37)
- **FZB42***B. amyloliquefaciens* (BGSC 10A6)
- **6051** *B. subtilis* (ATCC DSM 10)

3.2 METHODS

3.2.1 Growth conditions

Growth experiments were performed in CM medium (Albano et al., 1987). Overnight cultures were grown at 37°C in 50 ml tubes (Duran) containing 5 ml LB broth with vigorous shaking (200 rpm) and were supplemented with chloramphenicol (5 $\mu g \cdot ml^{-1}$), kanamycin (5 $\mu g \cdot ml^{-1}$), spectinomycin (100 $\mu g \cdot ml^{-1}$), erythromycin (0.5 $\mu g \cdot ml^{-1}$), lyncomycin (12$\mu g \cdot ml^{-1}$) or tetracycline (20 $\mu g \cdot ml^{-1}$) as appropriate. For experiments in cocultures the cell numbers were estimated by OD 650 and the strains were mixed in Eppendorf tubes in a 1:1 ratio in fresh medium. Cultures were prepared in 10 ml fresh CM medium with 2% (vol/vol) inoculums and were incubated at 37°C with shaking at 200 rpm. For colony growth mode colonies were grown on CM medium solidified with 1.5% (w/v) agar. Colony cocultures were prepared by spotting 2 µl of the prepared coculture on solid medium and they were also incubated at 37°C. To monitor colony growth by fluorescent microscopy, thin CM-agar medium was prepared on glass microscope slides, inoculated with 1 µl of 1:1 coculture and grown in a humidified chamber at 37°C.

For testing the effects of different temperature or salinity on QS, overnight cultures were prepared as described above. In case of experiments addressing influence of temperature on QS, an overnight culture was transferred to three separate Erlenmeyer flasks that were then incubated with shaking at 200 rpm at 24, 37 and 51°C respectively. For experiments addressing the influence of salinity on QS, an overnight culture was transferred to three separate flasks (or Petri dishes in case of biofilm studies) each containing different NaCl concentration (non supplemented control, 4% and 8% of added NaCl) and incubated at 37°C (with shaking at 200 rpm in case of planktonic cultures).

3.2.2 Slides preparation and fluorescence microscopy

Cells were collected, washed twice by centrifugation at 7,000 × g for 5 min with 0.5 ml 0.9% NaCl and immobilized on 10-well poly-L-lysine covered diagnostic slides (Thermo Scientific). In case of colony cocultures cell suspensions were prepared by gently scratching colony cells from the solid agar medium and dispersing cells in saline solution [0.9% (w/v) NaCl]. Fluorescence bleaching was reduced by applying the antifading agent SlowFade (Invitrogen).

Fluorescence images were taken using a Zeiss Axio Observer Z1 with 100×/1.40 oil Plan apochromat objective and equipped with an AxioCam MRm Rev.3 camera. The fluorescent light source was an HBO 100 Illuminator using 47HE and 48HE filters for excitation and emission, respectively. Flat-field correction and calibration was performed using sodium

fluorescein (0.75 $g \cdot ml^{-1}$) as a standard (Model & Burkhardt, 2001). The captured images were analyzed with ImageJ (Version 1.43u) software (Schneider et al., 2012) and artifact objects were removed manually before calculating fluorescence. The weighted average of the mean normalized intensities of objects was calculated for individual images with the fluorescence of each sample determined in five technical replicates obtained by imaging five different fields of 50- to 700-cell samples. Histograms were obtained using the OriginPro (OriginLab Corporation) program. The *B. subtilis* BD2833 (QS+) and B. subtilis BD2876 (QSS-) strains, which do not carry respective fluorescence markers, were used to determine autofluorescence

3.2.3 Spectro-fluorimetry

The cells were centrifuged and resuspended in equal volumes of 0.9% NaCl. Fluorescence was measured using a Safire II microplate reader (Tecan). The excitation/emission wavelengths for CFP and YFP were 455/500 nm and 513/527 nm respectively. The data are expressed as relative fluorescence units and then normalized to OD 650.

3.2.4 β-Galactosidase assay

β-Galactosidase was assayed using a Multiscan Spectrum Microplate Reader (Thermo Scientific). The absorbance at 420 nm was measured at 30°C immediately after the addition of ortho-nitrophenyl-β-galactoside substrate. Responsiveness of the wild type and signal deficient strain to exogenous ComX was determined by growing the wild-type BD2833 (*srfA-lacZ*) and the QS signal-deficient BD2876 (*srfA-lacZ*) strains with shaking for 16 h at 28°C in 100µl fresh CM medium and 100 µl of ComX (purified from *E. coli*) 0.005% to 0.05% (vol/vol) suspensions diluted in sterile MiliQ. The ComX activity in conditioned media of different cocultures or in conditioned media produced in different physical conditions was tested by incubating the BD2876 tester strain in 100µl of fresh CM medium and 100 µl of conditioned media sample. After the incubation cells were centrifuged (4°C; 1,800 × g) and resuspended in 200 µl Z-buffer with 5.6% (vol/vol) β -mercaptoethanol before adding 10 µl toluene and incubating the cultures on ice for 30 min. The plate was then warmed to 30°C, 50 µl ortho-nitro-phenyl-β-galactoside substrate was added and the absorbance (420 nm) was immediately determined at 30°C.

3.2.5 Purification of QS signal ComX and surfactin

Expression and purification of ComX from the *E. coli* ED367 producer strain was carried out according to the procedure of Ansaldi et al. (2001), except that primary purification was performed using HPLC with gradient elution on a chromatographic column (NUCLEODUR C 18 Gravity, Macherey-Nagel) in two mobile phases (0.1% TFA and acetonitrile with 0.1% TFA). The approximate concentration of ComX was calculated from the area under the

chromatogram (two ComX corresponding peaks) using an estimated extinction coefficient. This coefficient was calculated as a sum of absorbance (λ = 214 nm) of amino acids of ComX pheromone 168, peptide bonds, and squalene (similar to an isoprenoid tail) dissolved in acetonitrile and 0.1% TFA. Surfactin was isolated from the spent media according to the protocol described by Cooper et al. (1981) and was dissolved in pure methanol. The HPLC system was operated on the NUCLEODUR C 18 Gravity column at a flow rate of 1.0 ml/min with 80% (vol/vol) acetonitrile and 0.1% TFA in water. Eluting fractions were detected by their absorbance at 214 nm. The concentrations of surfactin in the spent media were estimated from the integrated area under representative peaks.

3.2.6 Hemolytic assay

Bovine red blood cells (RBCs) were washed two times with isotonic buffer [140 mM NaCl and 20 mM Tris (pH 7.4)] and once with 0.9% NaCl. The RBCs were then resuspended in 0.9% NaCl to OD 650 0.7 a.u. One hundred microliters of spent media, 30 µl 96% (vol/vol) ethanol, and 100 µl RBCs were mixed on a microtitre plate. OD 650 was measured immediately after the addition of RBCs and after 15 min of incubation at room temperature. The percentage of hemolysis was calculated as the decrease of OD 650 divided by OD 650 obtained for the bacterial cultures before spent media sampling (Moran et al., 2002).

3.2.7 Dehydrogenase activity

To measure dehydrogenase activity, 1200 µl cells were harvested, centrifuged at 7,000 × g for 5 min, and washed in 0.1 M Tris · HCl buffer (pH 7.7). Cell pellets were resuspended in 150 µl of the same buffer mixed with 1 ml 1% (vol/vol) 2,3,5-triphenyltetrazolium chloride (TTC), 7.5 µl 1 M glucose, and 7.5 µl 0.5 M monopotassium phosphate. The control samples were prepared in the same manner only without the addition of TTC to the Tris · HCl buffer. All samples were incubated for 1 h shaking at 100 rpm at 37°C in the dark. After incubation, cells were harvested by centrifugation at 7,000 × g for 5 min at room temperature. The supernatant was discarded and 2,3,5-triphenyltetrazolium formazan (TTF) was extracted from the cell pellet with 300 µl methanol with continuous vortex mixing for 5 min. Methanol extracts were centrifuged at 7,000 × g for 5 min at room temperature to remove cell debris, and supernatant absorbance at 485 nm was measured spectro-photometrically. The concentration of TTF produced was calculated based on a calibration curve of freshly prepared TTF in methanol. The cell dehydrogenase activity was expressed in micrograms of TTF produced per viable cell.

3.2.8 DNA extraction

Chromosomal DNA for transformation of *B. subtilis* strains and for competence assay was extracted using phenol-chloroform protocol. Briefly cells were grown in LB supplemented with 1% glucose for 6 hours, and 3 ml of culture was harvested, centrifuged (7,000 g for 5 min) and the supernatant were discarded. Cells were then resuspended in 300 µl of TES buffer, 6µl of lysozyme (from 50 $mg \cdot ml^{-1}$ stock solution) and 1µl of RNaseA (from 20 $mg \cdot ml^{-1}$ of stock solution) were added and samples were incubated for 30 min at 37°C. Next 2µl (from 20 $mg \cdot ml^{-1}$ stock) of proteinase K and SDS to final 1% concentration were added and samples were incubated overnight at 50°C. Next day the DNA was extracted with 200 µl of phenol and after centrifugation (16,000 g, 10 min) the supernatant was carefully collected and again extracted with 24:1 solution of chloroform: isoamyl alcohol. After centrifugation (16,000 g, 10 min) the supernatant was collected and the DNA was precipitated with 2.5 volumes of cold ethanol. The supernatant was discarded after few-second centrifugation and 500 µl of cold ethanol was added. After 15 min of incubation on ice the supernatant was discarded after few-second centrifugation, samples were air-dried and resuspended in sterile MiliQ water. Plasmid DNA was extracted using Qiagen plasmid extraction kit. DNA concentration was determined by spectrophotometry (A_{260}/A_{280}).

3.2.9 Transformation of *B. subtilis* and genetic competence assay

For standard transformation of *B. subtilis,* strains were grown in CM medium for 6 hours and 0.5 ml of culture was transferred to 2 ml eppendorf tube. DNA was added to final concentration of 1 $ng \cdot ml^{-1}$ and culture was incubated for 30 minutes at 37°C with vigorous shaking. Next 0.5 ml of LB medium was added and sample was incubated for additional 60 minutes at 37°C with vigorous shaking. Next 250, 100 and 50 µl of the culture was transferred to LB-agar medium supplemented with appropriate selection marker. In addition control experiment with no DNA added was always performed.

For genetic competence assay cultures were sampled after 4, 6 and 8 hours and transformation was performed as described above, except that each volume of transformed cultures was tested in triplicates and CFU assay for the cultures before transformation was performed.

3.2.10 Extracellular DNA measurement

B. subtilis strains were grown in CM medium at 37°C with shaking at 200 rpm and conditioned media were sampled after 4, 6 or 8 hours of growth. Cells were centrifuged (7,000 g, 5 min) and 3 ml of the filter-sterilized supernatant was used to precipitate the e-DNA with sodium acetate and cold ethanol as described by Sambrock & Russel (2001). Precipitate was resuspended in 80 µl of MiliQ water and the concentration was determined by

spectrophotometry (A_{260}/A_{280}) using Nanodrop Spectrophotometer ND-1000 (Thermo Scientific).

3.2.11 Plant material and growth conditions

Arabidopsis ecotype Col-0 seeds were surface-sterilized using 2% sodium hipochlorite solution. Briefly, seeds were incubated in 2% sodium hipochlorite with mixing on a orbital mixer for 20 minutes and then washed 5 times with sterile distilled water.

Seeds were germinated and grown on agar plates containing MS medium (Murashige and Skoog basal salts mixture; Sigma) (2.2 $g \cdot l^{-1}$) supplemented with 1% sucrose. After 3 days of incubation in 4°C plates were transferred to plant-growth chamber (photoperiod of 16 h of light, 8 h of darkness, light intensity 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperature of 24°C) and placed vertically at an angle of 65 degrees. After 10 days homogenous 1cm long seedlings were selected for growth promoting experiments.

3.2.12 Plant growth promotion assay

Bacterial strains were grown in LB medium until late exponential phase, cells were washed twice with 0.9% NaCl and 50µl suspensions were inoculated on MS agar plates (see chapter 3.2.11) in a line, approximately 2 cm from the bottom of the plate. Plates were incubated overnight in 37°C. Next, 16, 1cm-long Col-0 seedlings (see chapter 3.2.11) were transplanted to the MS plates, approximately 5 cm away from the bacterial line and arranged similarly as described by Lopez-Bucio and colleagues (2006). After 10 days of incubation in plant-growth chamber, the seedlings were removed from the agar, the roots were washed with distilled water, separated from the leaves and measured with a ruler. Roots and leafs from each MS plate were collected and weighted before and after drying. Roots and leafs mass obtained from bacteria-inoculated plates was compared with control, where seedlings were grown on sterile plates.

3.2.13 Indole-3-acetic acid production

Strains were growth in LB medium supplemented with tryptophan (1 $mg \cdot ml^{-1}$) for 48 h at 28°C with 200 rpm shaking. Supernatants were collected and indole-3-acetic acid (IAA) production was determined with the use of iron and perchloric acid according to modified method of Solon and coworkers (1950). Briefly $FeCl_3$-$HCl0_4$ reagent (1.0 ml of 0.5 M $FeCl_3$, 50 ml 35% $HC10_4$) was mixed with the culture supernatant in 1:2 ratio and incubated for 15 minutes. 5 µl of orthophosphoric acid were added for reaction enhancement and absorbance at 510 nm was determined. IAA concentration was calculated from a standard curve prepared using commercial IAA (Sigma).

3.2.14 Siderophore production

Strains were grown overnight on solid media prepared as follows: 100 ml of Minimal Media 9 (M9) stock solution (15 g KH_2PO_4, 25 g NaCl, 50 g NH_4Cl dissolved in 500 ml of ddH_2O) was mixed with 750 ml of MiliQ and supplemented with 15 g of agar. After autoclaving and cooling to 50°C the medium was supplemented with 30 ml of 10% sterile casamino acid solution and 10 ml of sterile 20% glucose solution (Schwyn & Neilands, 1987).

Siderophore detection was carried out with an overlay of the following medium: 60.5 mg Chrome azurol S (CAS), 72.9 mg hexadecyltrimetyl ammonium bromide (CTAB), 30.24 g Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and 1 mM $FeCl_3 \cdot 6H2O$ in 10 mM HCl with 0.9% agar. 10 ml overlays of this medium were applied over M9 agar plates containing cultivated strains. After a maximum period of 15 min, a change in color from blue to purple around the colonies indicated the siderophore producers (Perez-Miranda et al., 2007). Qualitative estimation of siderophore production was performed as follows: – negative (no orange halo), from +/- to ++, positive.

3.2.15 Phosphate solubilization

The ability of the isolates to solubilize inaccessible phosphate was determined using Pikovskaya agar (Pikovskaya, 1948). Cells were grown overnight in LB medium, next they were washed twice with 0.9% NaCl and resuspended in 0.9% NaCl to produce equal cell densities. Solutions were inoculated on the agar plates and incubated in 37°C for 7 days. The size of halo (zone of solubilization) around the bacterial colony indicated phosphate solubilizing abilities of each strain. Qualitative estimation of phosphate solubilization was performed as follows: – negative (no halo), positive from +/- to ++.

3.2.16 Biofilm formation assay

Assay was performed using MSN medium (5 mM potassium phosphate buffer pH 7, 0.1 M Mops pH 7, 2 mM $MgCl_2$, 0.05 mM $MnCl_2$, 1 μM $ZnCl_2$, 2 μM thiamine, 700 μM $CaCl_2$, 0.2% NH_4Cl) supplemented with 0.5% pectin as performed by Beauregard et al. (2012). Strains were grown overnight in LB, and suspensions were inoculated (2%) into MSN pectin media distributed in 10ml Petri dishes and grown for 48 hours at 28°C. Pellicles were harvested, dried and weighted. Conditioned media were sampled for hemolytic assay.

3.2.17 Cell-wall degrading enzymes

Protease assay was performed according to the method of Saran and colleagues (2007). The detection medium was prepared with 20 g of skim milk and 20 g of agar–agar each dissolved in 200 ml distilled water, and 600 ml of 0.2 M Phosphate buffer at pH 7. All the three medium components were autoclaved separately and later mixed under sterile conditions. Twenty milliliter of culture medium were then poured per plate and allowed to harden. The plates were subsequently inoculated and incubated in 37°C for 48 h. To observe the zone of hydrolysis, 10% tannic acid was flooded on the milk agar plate. Qualitative estimation of was performed as follows: – negative (no halo around the colony), positive from +/- to ++.

Chitinase assay was performed using MUF-N-acetyl-β-D-glucosaminide substrate. Cells obtained from 1 ml of overnight cultures (LB) were washed twice with 0.9% NaCl and resuspended in 0.9% NaCl to produce equal cell densities. Next 20 µl of MUF substrate was added to 0.5 ml of prepared suspensions. After 10 min incubation in 37°C, 2 drops of saturated $NaHCO_3$ were added and samples were transferred into 96-well microtiter plate. Fluorescence was determined using a Safire II microplate reader (Tecan) with 360 nm excitation and 450 nm emission and relative chitinase activities were determined from the obtained fluorescence values.

Cellulase assay was performed as described previously (Kasana et al., 2008). Cells were grown overnight in LB, washed twice with 0.9%NaCl and resuspended in 0.9%NaCl to produce equal cell densities before inoculated on CMC agar (0.2% $NaNO_3$, 0.1% K_2HPO_4 , 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose (CMC) sodium salt, 0.02% peptone, and 1.7% agar). Plates were incubated at 28°C for 48 hours and flooded with 1% hexadecyltrimethyl ammonium bromide (HAB) for 30 to 40 minutes. Qualitative estimation of was performed as follows: –negative (no yellow dye), positive from +/- to ++.

3.2.18 Antagonistic activity against *Ralstonia solanacearum*

Antagonistic activity of the *Bacillus* isolates against *R. solanacearum* was tested on two types of solid media: Non-selective casamino acid-peptone-glucose medium (CPG) where glucose is a main carbon source and selective King B medium where glycerol is a main carbon source.

Suspension of *R. solonacearum* (10^8) was spread on the agar plate and left to absorb into the medium. Next 3x5 µl of *Bacillus* suspensions (10^8) were applied on 3 filter papers arranged in a triangle on the plate. The plates were incubated at 28°C for 2 days before the diameter of the clear halo surrounding the filter was measured. As negative control, sterile medium was used in place of the suspension.

Except the experiments on solid media, the spent media activity of selected strains against *R. solanacearum* was tested in liquid CPG medium. For this purpose *Bacillus* strains were grown in liquid CPG medium for 2 days in 28°C and its conditioned media were sterilized by filtering it through the 0.2 μm filter (Milipore). *R. solonacearum* growth was followed on 96-well microtiter plate in presence of various concentrations of the conditioned media. After 24 hours of growth cells were inoculated on solid CPG medium to determine if they were able to grow after conditioned media treatment.

4 RESULTS

4.1 INTRACELLULAR LINK BETWEEN QS SIGNALING AND QS RESPONSE IN *B. subtilis*

4.1.1 Differences in dynamics of QS response between the QS wild type and QS signal-deficient mutant

The ComX-dependent QS response (measured as *srfA* expression) is induced at the beginning of the stationary phase (Nakano et al., 1991; Hahn & Dubnau, 1991). We investigated the dynamics of *srfA* expression in the QS proficient (QS+) *B. subtilis* BD4720 (*srfA-yfp)* and QS signal-deficient (QSS-) *B. subtilis* BD4729 (*srfA-cfp*) strains. The latter strain carries a mutation in *comQ*, which is responsible for signal processing and modification.

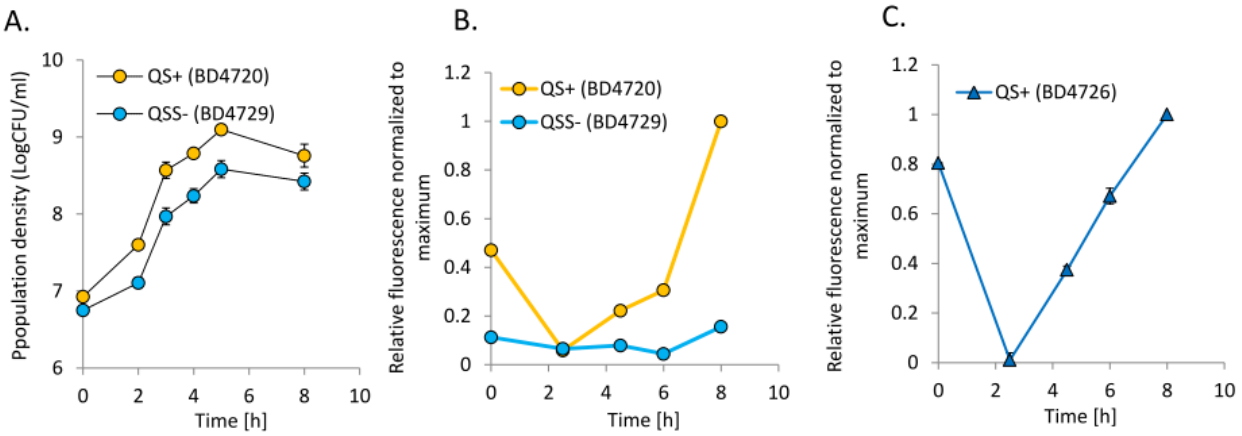


Figure 8**. Growth and fluorescence of QS+ and QSS- strains in monocultures**. A. Bacterial growth was monitored by viable cell counts (CFU) of QS proficient (QS+) *B. subtilis* BD4720 (*srfA-yfp*) and QS signal deficient (QSS−) *B. subtilis* BD4729 (*srfA-cfp*) in monocultures. B. The relative QS response was monitored by single-cell fluorescent microscopy during growth of the same QS+ and QSS- strains in monocultures. C. Changes in relative QS response during growth of control QS proficient (QS+) *B. subtilis* BD4726 (*srfA-cfp*). The fluorescence of the *srfA-yfp* and *srfA-cfp* reporter fusions was normalized to maximal fluorescence for each fluorophore, and presented as relative fluorescence (Chapter 3.2.2). Data are presented as mean values of at least three biological replicates and the SE is indicated for every time point.

Slika 8. **Rast in fluorescenca sevov QS+ in QSS- v monokulturah**. A. Rast bakterij smo spremljali preko štetja živih celic (CFU) pri sevu z neokvarjenim QS sistemom (QS+) *B. subtilis* BD4720 (*srfA-yfp*) in pri sevu, ki ne proizvaja QS signala (QSS−) *B. subtilis* BD4729 (*srfA-cfp*) v monokulturah. B. Relativen QS odziv smo spremljali preko fluorescenčne mikroskopije na nivoju posamezne celice tekom rasti omenjenih sevov QS+ in QSS−. C. Spremembne v relativnem QS odzivu tekom rasti kontrolnega seva z neokvarjenim QS sistemom (QS+) *B. subtilis* BD4726 (*srfA-cfp*). Fluorescenca *srfA-yfp* in *srfA-cfp* reporterskih fuzij je bila normalizirana na maksimalno fluorescenco posameznega fluoroforja in prikazana kot relativna fluorescenca (Poglavje 3.2.2) Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

First QS+ and QSS- were monitored in monocultures. Both strains showed very similar growth kinetics (Figure 8A) but differed significantly in their QS response (Figure 8B). The QS response of the signal-producing population was already induced during the overnight growth. After inoculation into fresh medium expression decreased transiently and was induced again at 4 hours, reaching a maximum after incubation for 8 hours (during the late stationary phase). The comparable result was observed for the control isogenic strain carrying different fluorescent fusion, *B. subtilis* BD4726 (*srfA*-*cfp*) (Figure 8C). The QS response of the QS signal-deficient mutant, however, remained very low throughout the same incubation period confirming the ComX-dependent expression of *srfA* (Figure 8B).

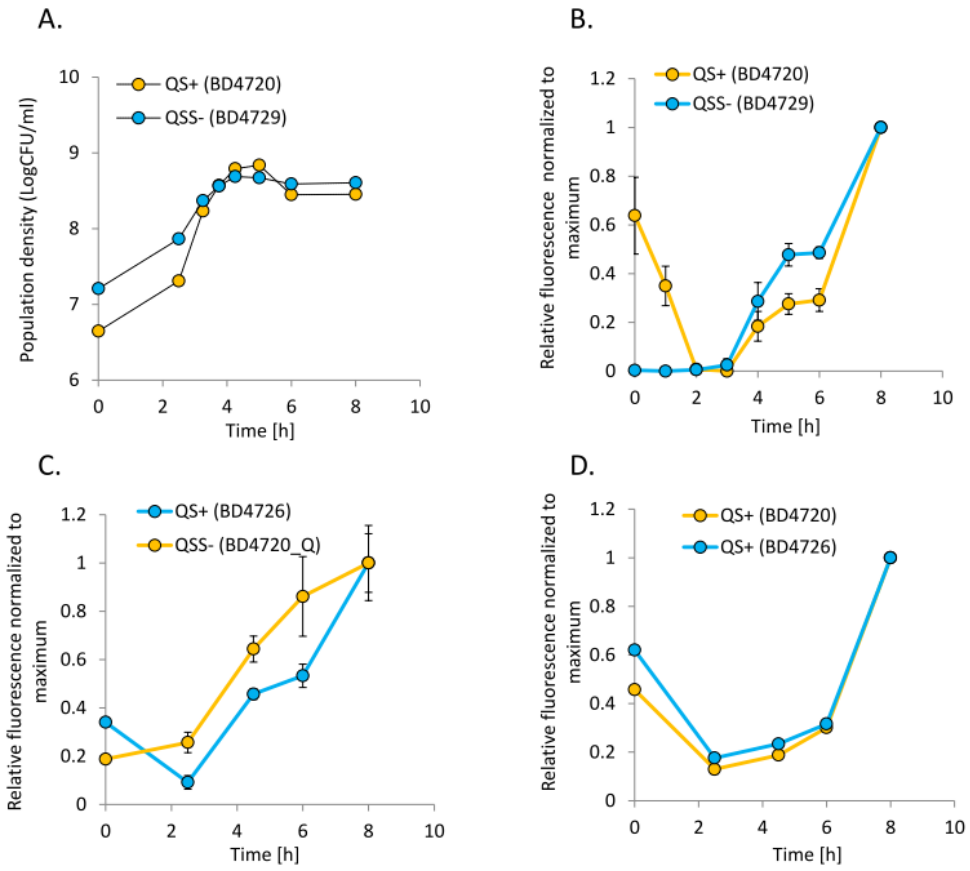


Figure 9**. Growth and fluorescence of QS+, QSS- and controls in cocultures**. A. Bacterial growth was monitored by viable cell counts (CFU) of QS proficient (QS+) *B. subtilis* BD4720 (*srfA-yfp*) and QS signal deficient (QSS−) *B. subtilis* BD4729 (*srfA-cfp*) in 1:1 cocultures. B. The relative QS response was monitored by single-cell fluorescent microscopy during growth of the same QS+ and QSS− strains in 1:1 coculture. C. Changes in relative QS response during growth of control pair of strains: domesticated QS+ *B. subtilis* BD4726 (*srfA-cfp*) and QS signal deficient (QSS−) *B. subtilis* BD4720BD4720_Q (*ΔcomQ; srfA-yfp*) with switched fluorophores, in 1:1 coculture. D. Changes in relative QS response during growth of two domesticated QS+ strains of *B. subtilis* BD4720 (*srfA-yfp*) and *B. subtilis* BD4726 (*srfA-cfp*) in coculture. The fluorescence of the *srfA-yfp* and *srfA- cfp* reporter fusions was normalized to maximal fluorescence for each fluorophore and presented as relative fluorescence (Chapter 3.2.2). Data are presented as the mean of biological triplicates with SE indicated for each time point.

Slika 9. **Rast in fluorescenca QS+, QSS- in kontroli v kokulturah.** A. Rast bakterij smo spremljali preko štetja živih celic (CFU) seva z neokvarjenim QS sistemom (QS+) *B. subtilis* BD4720 (*srfA-yfp*) in seva, ki ne proizvaja signala (QSS−) *B. subtilis* BD4729 (*srfA-cfp*) v kokulturi 1:1. B. Relativen QS odziv smo spremljali s fluorescenčno mikroskopijo na nivoju posamezne celice tekom rasti istih sevov QS+ in QSS- v kokulturi 1:1. C. Spremembe v relativnim QS odzivu tekom rasti kontrolnega para sevov: QS+ *B. subtilis* BD4726 (*srfA-cfp*) in QSS− *B. subtilis* BD4720BD4720_Q (*ΔcomQ; srfA-yfp*) z zamenjanimi fluorescenčnimi makrerji, v kokulturi 1:1. D. Spremembe v relativnim QS odzivu dveh sevov QS+ *B. subtilis* BD4720 (*srfA-yfp*) in *B. subtilis* BD4726 (*srfA-cfp*) v kokulturi. Fluorescenca *srfA-yfp* in *srfA-cfp* reporterskih fuziji je bila normalizirana na maksimalno fluorescenco vsakega fluoroforja in predstavljena kot relativna fluorescenca (Chapter 3.2.2) Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko.

Next, we monitored growth and the QS response of QS+ and QSS- in 1:1 coculture. Growth kinetics of the two strains in coculture was similar (Figure 9A). Signal producers showed very similar relative QS response dynamics in monoculture and in coculture. On the other hand the QSS- population showed a 2-fold higher relative QS response than the QS+ after 5 hours of incubation ($P<0.0001$). This difference in the relative QS response was lost after incubation for 8 hours (Figure 9B). The comparable results were obtained in cocultures containing two strains with switched fluorescent markers (Figure 9C). Additionally, when two QS-proficient strains were labeled with different fluorescent fusions [*B. subtilis* BD4720 (*srfA-yfp*) and BD4726 (*srfA-cfp*)] and cocultured in a 1:1 ratio, the QS response dynamics of both strains were highly comparable (Figure 9D), suggesting that the observed responses are not due to the intrinsic properties of the fluorescent proteins.

4.1.2 Signal production and QS response in QS+ and QSS- cocultures of different ratios

To further test the dynamics of QS response of the wild type and signal deficient mutant in cocultures we inoculated QS+ and QSS- in different rations ranging from 1:10 (QS+ to QSS-) to 10:1 (QS+ to QSS-). In this way we wanted to create conditions with different concentrations of QS signal available in the medium (Figure 10A).

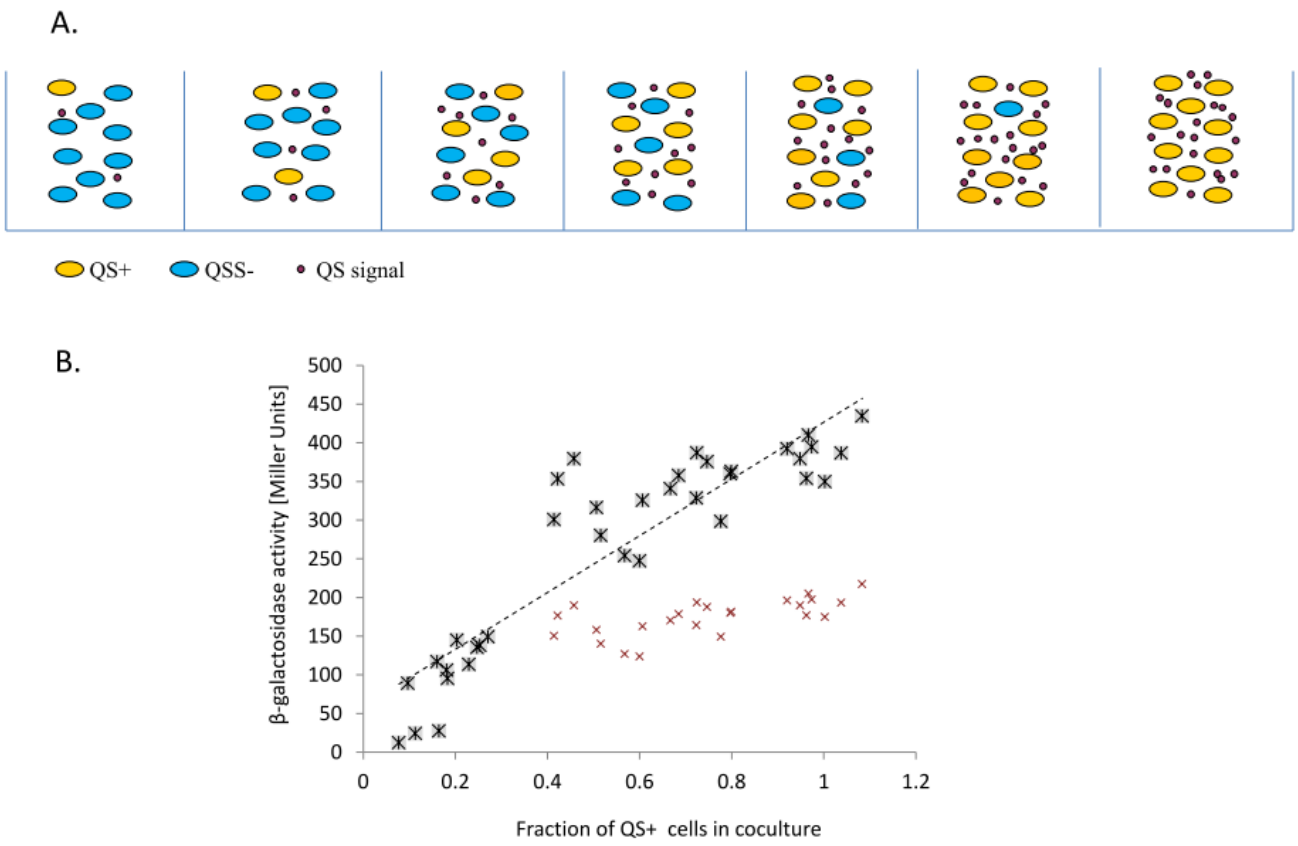


Figure 10. **ComX activity in conditioned media of QS+ and QSS- cocultures inoculated at different ratios.** A. The relative frequencies of the two strains and the relative concentration of the signal are schematically represented. B. the QS response of the tester strain (*B. subtilis* BD2876, *srfA-lacZ*) was monitored by β-galactosidase activity. Conditioned media were taken in late stationary phase from planktonic cocultures with different ratios of the wild type (QS+) and the signal deficient populations (QSS-) and indicated on the x axis. Tester strain was then incubated in this medium until late stationary phase and the response was determined as β-galactosidase activity. Red markers represent results for non-diluted and grey markers for 2-fold diluted spent media. Data collected for all biological replicates are presented on the graph.

Slika 10. **Aktivnost ComX v izrabljenih gojiščih kokultur QS+ in QSS-, ki smo jih inokulirali v različnih razmerjih**. QS odziv testerskega seva (*B. subtilis* BD2876, *srfA-lacZ*) smo spremljali preko testa aktivnosti β-galaktozidaze. Izrabljena gojišča so bila pridobljena v pozni stacjonarni fazi iz stersanih planktonskih kokultur z različnimi razmerjami divjega tipa (QS+) in seva, ki ne proizvaja QS signala (QSS-) (kot prikazano zgoraj). Testerski sev smo inkubirali v izrabljenih gojiščih do pozne stacionarne faze in potem spremljali njegov odziv preko testa aktivnosti β-galaktozidaze. Rdeče oznake prikazujejo rezultat za neredčen vzorec, sive oznake prikazujejo rezultat za 2-krat redčeno izrabljeno goojišče Graf vsebuje podatke pridobljene za vsako biološko ponovitev.

We tested this assumption by evaluating the active concentrations of the QS signal in spent media of prepared cocultures after 16 hours of incubation. This was performed by incubating the ComX specific biosensor (*B. subtilis* BD2876 tagged with *srfA-lacZ*) with spent media of

the cocultures. With undiluted spent media the maximum β-galactosidase activity (150 Miller units) was detected when it originated from cocultures containing 20% of the wild type (Figure 10B). Further linear increase of QS response was obtained only if the spent media from cocultures with higher proportion of the wild type cells were diluted two fold. This suggested that there was an excess of QS signal in cocultures which contained more that 20% of the wild type cells.

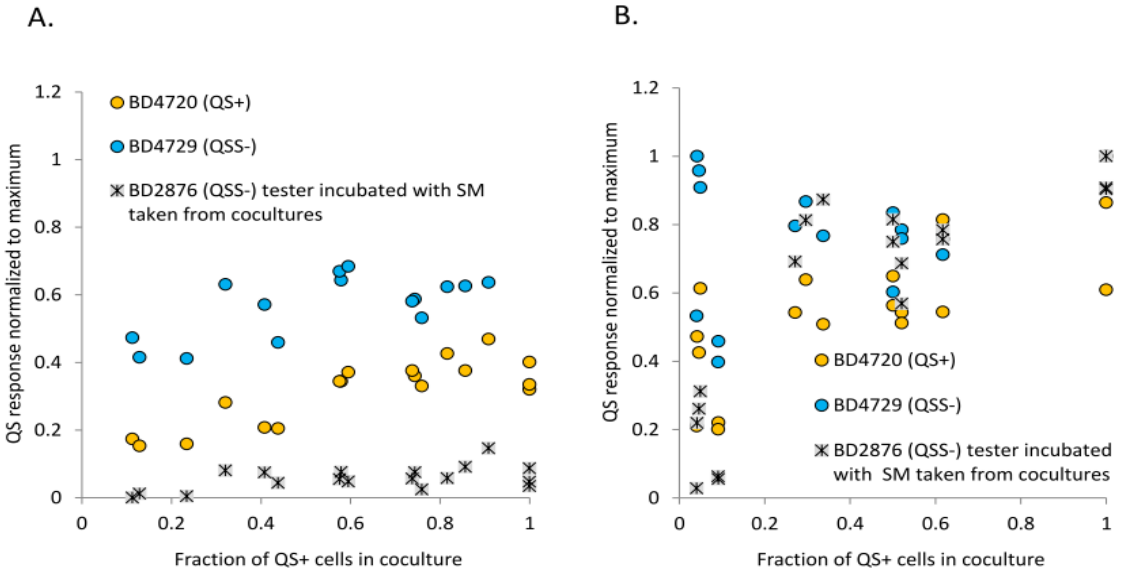


Figure 11. **The relative QS response of QS+ and QSS- in cocultures of different ratios**. QS response was monitored by single-cell fluorescent microscopy after (A) 5 and (B) 11 hours of growth of domesticated QS proficient (QS+) *B. subtilis* BD4720 (*srfA-yfp*) and QS signal deficient (QSS−) *B. subtilis* BD4729 (*srfA-cfp*) in planktonic cocultures with different ratios of QS+ to QSS-. Additionally, grey markers represent the relative QS response of the tester strain BD2876 (*srfA-lacZ*) to conditioned media (SM) prepared from indicated cocultures with different percent of QS+ cells.The fluorescence of each reporter was normalized to its maximal value, and presented as relative QS response. Data are presented as mean values of at least three biological replicates and the SE is indicated for every time point.

Slika 11. **Relativen QS odziv v stresanih kokulturah QS+ in QSS- sevov nacepljenih v različnih razmerjih.** QS odziv smo spremljali s pomočjo fluorescenčne mikroskopije na nivoju posamezne celice po (A) 5 in (B) 11 urah rasti seva z neokvarjenim QS sistemom (QS+) *B. subtilis* BD4720 (*srfA-yfp*) (rumeni krogec) in seva, ki ne proizvaja QS signala (QSS−) *B. subtilis* BD4729 (*srfA-cfp*) (modri krogci) v stresanih planktonskih kokulturah. Seva QS+ in QSS- smo nacepili v različnih razmerjih, ki so prikazana na x osi. Dodatno, sive oznake prikazujejo relativen QS odziv testerskega seva BD2876 (*srfA-lacZ*), ki smo ga gojili v izrabljenih gojiščih pridobljenih iz zgoraj omenjenih kokultur. Fluorescenca vsakega reporterja je bila normalizirana na njihovo maksimalno vrednost in je predstvljena kot relativen QS odziv. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko.

We next monitored the relative QS response of QS+ and QSS- populations grown in cocultures with different ratio of the two strains after 5 hours and after 16 hours of incubation.

After 5 hours the relative fluorescence of the signal deficient mutant (indicating QS response) was approximately 2-fold higher as compared to the fluorescence of the wild type (Figure 11A). After 16 hours we observed that the QSS- population reached its maximum response in coculture with the 20% of the wild type cells while the wild type reached only 70% ($P<0.005$) of its maximum response at this point (Figure 11B). Additionally we compared the relative QS response that was induced in the tester strain (BD2876 tagged with *srfA-lacZ*) by the spent media of the cocultures. Results are presented as relative QS response. It is worth to notice that after 5 hours of the incubation the response of the tester strain was very low (did not reach 10% of the maximal response) as compared to the response of the QS+ and QSS- strains monitored directly in coculture that reached over 40% or over 60% of the maximal response, respectively.

4.1.3 Increased sensitivity of the signal-deficient mutant to exogenous ComX purified from *E. coli*

The results described above indicated a difference in the QS response dynamics of the QS signal proficient (QS+) and signal deficient (QSS-) populations. We speculated that QS+ and QSS- strains differ in their sensitivity to ComX. Therefore, we aimed to test the responsiveness of the ComX signal proficient (BD2833) and signal deficient (BD2876) strains tagged with the *srfA-lacZ* reporter fusion to different concentrations of the ComX purified form *E. coli* strain ED687 (Ansaldi et al., 2001) that expressed ComX under IPTG control.

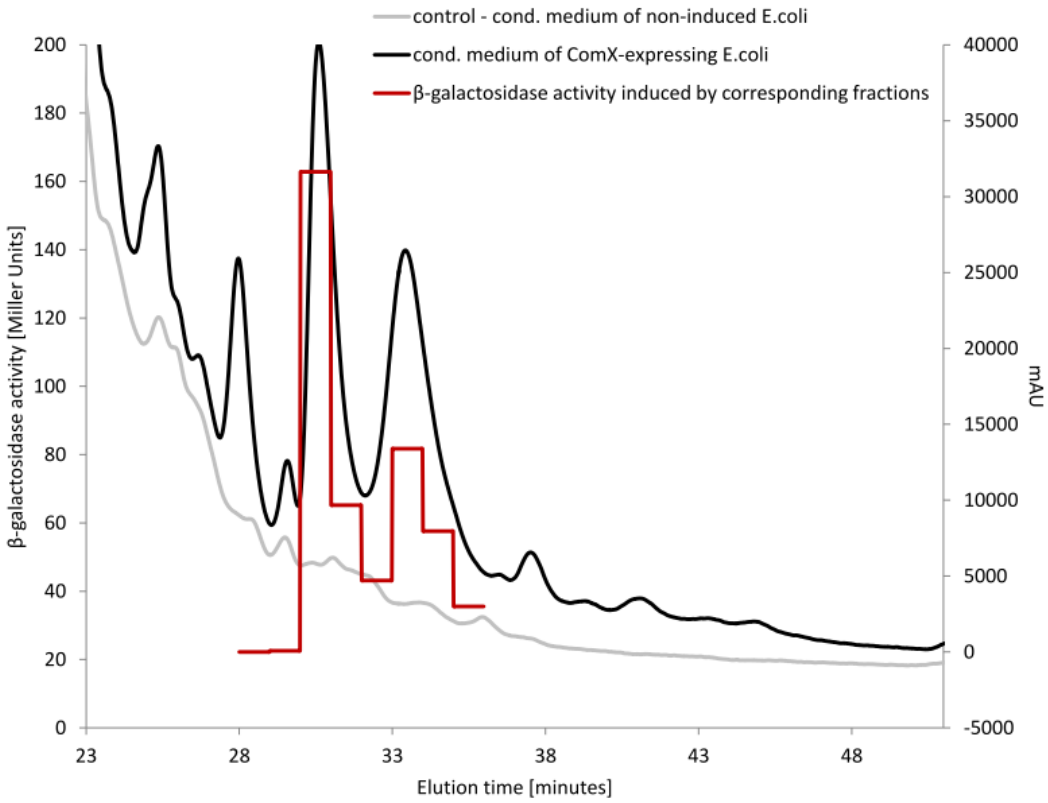


Figure 12. **RT-HPLC elution profile of ComX purified from *E. coli* conditioned media.** 214 nm elution profile after the second reverse-phase chromatography step for the media sample obtained from *E. coli* ED 687 induced with 0.5 mM IPTG (black line) after reaching OD650 value 0.7 and for sample obtained for the same strain with no addition of IPTG (grey line). The elution profile was correlated with the QS response monitored in the tester strain (BD2876 *srfA-lacZ*) in the presence of the corresponding elution fractions.

Slika 12**. Elucijski profil RT-HPLC za ComX očiščen iz izrabljenega gojišča *E. coli.*** Elucijski profil pri 214 nm je bil pridobljen v drugem koraku kromatografije reverznih faz za vzorec izrabljenega gojišča seva *E .coli* ED 687 induciranega z 0.5 mM IPTG (rdeča črta), pri OD_{650} 0,7 ter za vzorec izrabljenega gojišča istega seva brez dodatka IPTG (siva črta). Elucijski profil smo korelirali s QS odzivom testerskega seva (BD2876 *srfA-lacZ*), ki je bil izpostavljen ustrezni elucijskih frakciji.

We collected ComX-containing fractions using HPLC and confirmed their activity with the use of the tester strain (BD2876 *srfA-lacZ*) (Figure 12). We next incubated QS+ (BD2833) and QSS- (BD2876) with estimated ~10 nM ComX, which corresponds to the saturating levels of ComX produced by the signal-proficient strains (Manguson et al., 1994).

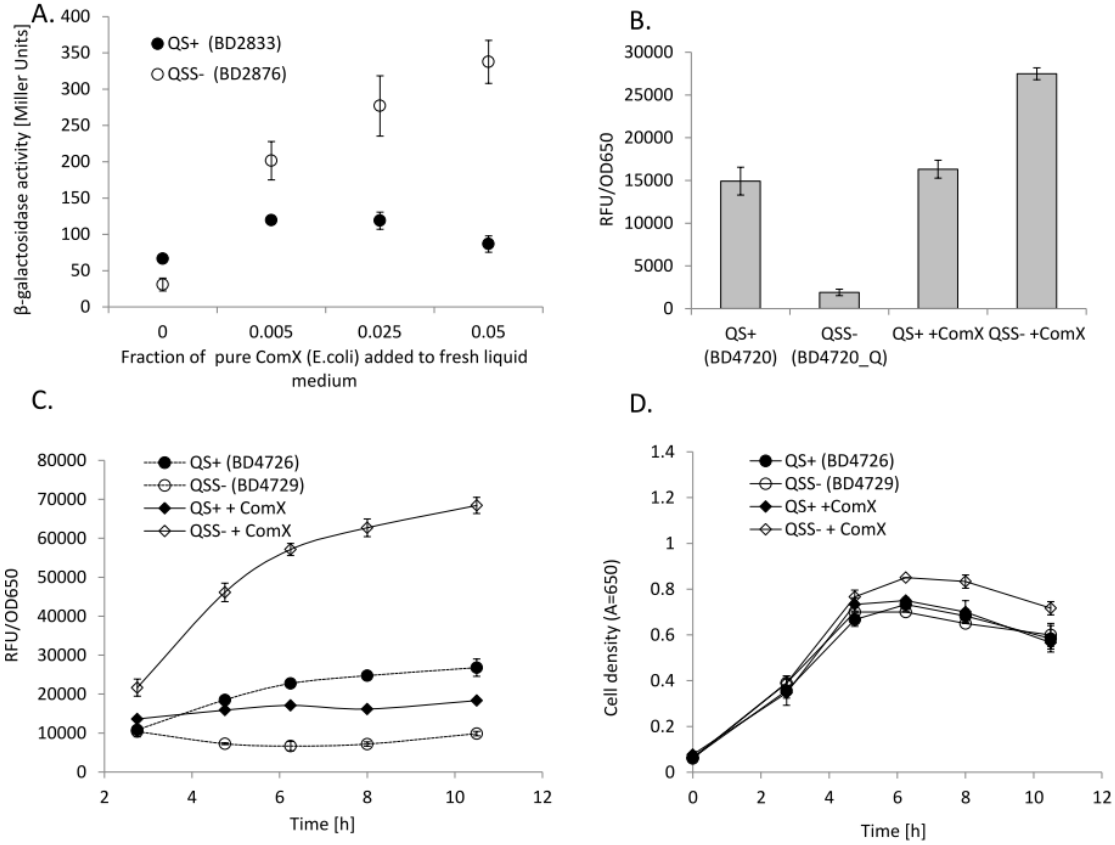


Figure 13. **QS response of QS+ and QSS- strains monoculturs to saturating concentration of ComX.** A. The QS response of the QS+ (*srfA-lacZ*) QSS− *B. subtilis* BD2876 (*srfA-lacZ*) was monitored by β-galactosidase activity. Cultures were incubated until the stationary phase with a range of concentrations of the ComX pheromone purified from recombinant *E. coli* ED367. B. Cultures of QS+ *B. subtilis* BD4720 (*srfA-yfp*) and QSS− *B. subtilis* BD4720BD4720_Q (*srfA-yfp*) were incubated without or with 0.05 fraction (~10nM) of the ComX pheromone purified from *E. coli* and the QS response was measured by fluorimeter after 8 h. C. Cultures of QS+ *B. subtilis* BD4726 (*srfA-cfp*) and QSS− *B. subtilis* BD4729 (*srfA-cfp*) were incubated without or with 0.05 fraction (~10nM) of ComX pheromone purified from *E. coli* ED386 and the QS response was monitored spectrofluorimetrically over time. D. Growth curves of indicated strains obtained during the experiment presented in 13C. Data are presented as the mean of biological triplicates and SE is indicated.

Slika 13. **QS odziv monokultur sevov QS+ in QSS- izpostavljenih nasičeni koncentraciji ComX.** A. QS odziv seva QS+ *B. subtilis* BD2833 (*srfA-lacZ*) in QSS− *B. subtilis* BD2876 (*srfA-lacZ*) smo spremljali preko testa aktivnosti β-galaktozidaze. Kulture smo inkubirali do stacionarne faze v prisotnosti različnih koncetracij feromona ComX, ki je bil očiščen iz rekombinantnega seva *E. coli* ED367. B. Kulturi QS+ *B. subtilis* BD4720 (*srfA-yfp*) in QSS− *B. subtilis* BD4720BD4720_Q (*srfA-yfp*) smo inkubirali brez ali z 0.05 frakcijo feromona ComX očiščenega iz *E. coli* in QS izmerili po 8. urah s spektrofluorimetrom. C. Kulture Cultures QS+ *B. subtilis* BD4726 (*srfA-cfp*) in QSS− *B. subtilis* BD4729 (*srfA-cfp*) smo inkubirali brez ali z 0.05 frakcijo feromona ComX očiščenega iz *E. coli* ED386 in QS odziv izmerili ob različnih časih tekom rasti s pomočjo spektrofluorimetra. D. Rastne krivulje secoc omenjenih pod točko C. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko.

Indeed, the signal-deficient strain was more responsive to exogenous ComX than the QS signal-producing strain at all fractions tested (0.005-0.05) and was 4-fold ($P<0.000001$) more responsive with the 0.05 fraction (~10nM) of ComX (Figure 13A). In a complementary approach, the 0.05 fraction (~10nM) of exogenous ComX was added to the monocultures of the fluorescently labeled signal-producing strain BD4726 (*srfA-cfp*) and signal-deficient strain BD4729 (*srfA-cfp*) and the QS response was measured during growth. At each time point the fluorescence of the QS signal-deficient mutant grown with exogenous ComX was higher than that of the signal producer, which even showed a small decrease in fluorescence in the presence of exogenous ComX (Figure 13C). Exchange of fluorescent reporters did not influence the results (Figure 13B) and confirmed the greater sensitivity of the signal-deficient mutant to ComX. Despite the pronounced difference in the ComX-induced *srfA* response, the specific growth rates of the signal-producing and -deficient strains were not significantly different (Figure 13D).

4.2 METABOLIC CONSEQUENCES OF PRIVATE LINK BEWTEEN QS SIGNALING AND RESPONSE IN *B. subtilis*

4.2.1 Expression of *srfA* gene in undomesticated *B. subtilis* QS+ and QSS-

We next examined whether the same effects that we observed for domesticated *B. subtilis* can be also observed for undomesticated strains PS-216 (Stefanic & Mandic-Mulec, 2009) that, unlike the domesticated strain, synthesizes and secretes surfactin in response to ComX. It was previously reported that surfactin may regulate its own expression acting like a paracrine signal (Lopez et al., 2009a), therefore different pattern could be expected for undomesticated QS+ and QSS- in the presence of ComX as compared to domesticated strains.

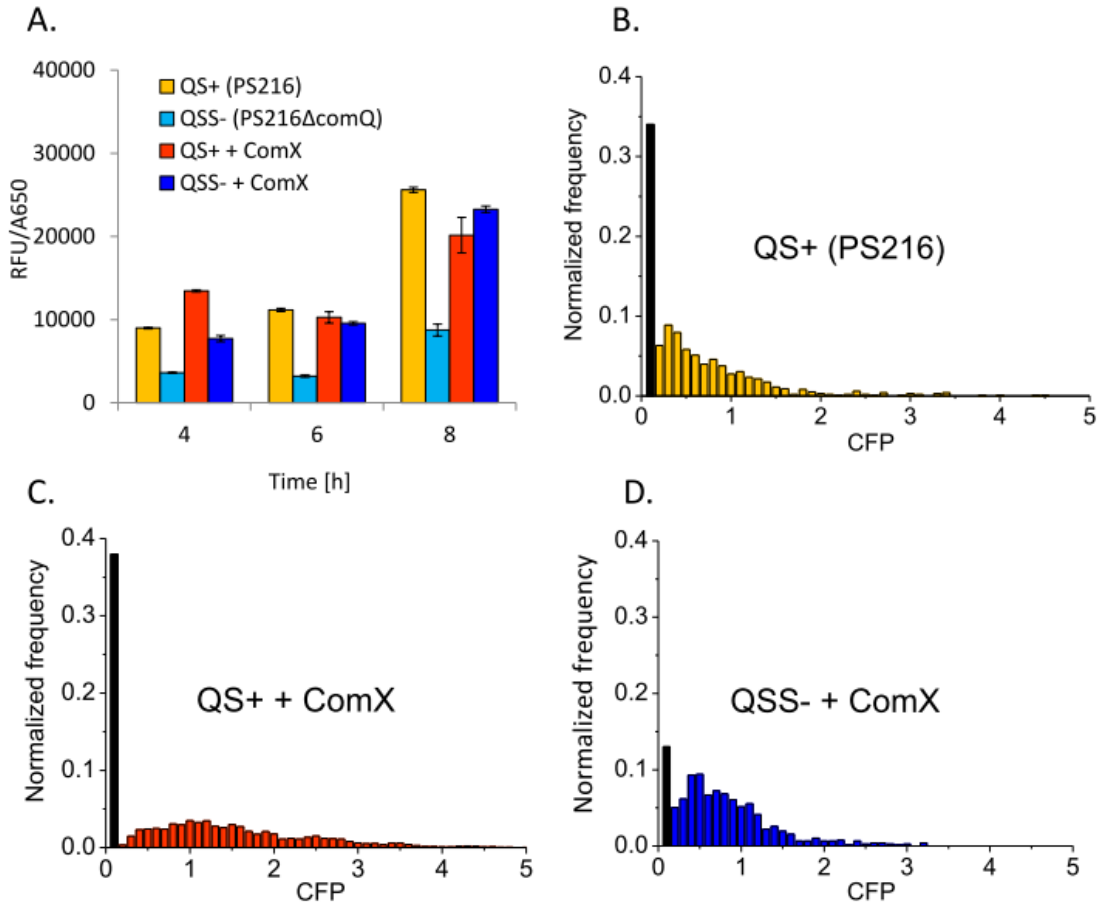


Figure 14. **ComX-induced expression of *srfA-cfp* in undomesticated QS+ and QSS- *B. subtilis***. A. Expression of *srfA* was measured spectrofluorimetrically at three different time points in *srfA-cfp*–labeled strains: QS+ *B. subtilis* PS216 and QSS−PS216ΔcomQ incubated with or without purified ComX. Data are presented as the mean of biological triplicates with SE indicated. B. Histograms represent the distribution of *srfA-cfp* expression in the QS+ *B. subtilis* PS216 (yellow), (C) in the same strain incubated with ComX (red) and (D) in the QSS- *B. subtilis* PS216ΔcomQ (blue) incubated with ComX. Non-induced population (on the left) is marked with black.

Slika 14. **Izražanje *srfA-cfp* inducirana z ComX v neudomačenih sevih *B. subtilis* QS+ in QSS-**A. Izražanje *srfA-cfp* smo zmerili spektrofluorimetrično v treh časovnih točkah pri sevih *B. subtilis* PS216 (QS+) in PS216ΔcomQ (QSS−) ki smo ju inkubirali z ali brez očiščenega ComX. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko. B. Histogrami prikazujejo distribucijo izražanja *srfA-cfp* pri QS+ *B. subtilis* PS216 (rumena), (C) pri istim sevu inkubiranim z dodanim ComX (rdeča) in (D) pri QSS- *B. subtilis* PS216ΔcomQ (modra) inkubiranim z dodanim ComX. Neinducirana populacija (na levi) je označena s črno.

Surprisingly, total fluorescence of *srfA-cfp* in the PS216ΔcomQ background with exogenous ComX was comparable to that of the QS-proficient PS216, which is different to that of

domesticated strains (Figure 14A). Interestingly, single-cell microscopy analysis revealed that, despite similar cumulative fluorescence of PS216 and the signal-deficient derivative PS216ΔcomQ in the presence of ComX, the distributions of *srfA* expression differed between the two strains: in PS216 30% ±7% of cells did not express *srfA-cfp* regardless of ComX exposure, but only 14% ±1% of the mutant population remained uninduced in the presence of ComX (Figure 14B-D).

4.2.2 Secretion of lipopeptide antibiotic - surfactin

We next measured the secretion of surfactin in the undomesticated QS+ and QSS- strains. To compare surfactin secretion in the PS216 (*srfA-cfp*) and signal deficient PS216ΔcomQ (*srfA-cfp*) mutants, we took the advantage of the hemolytic property of this lipopeptide antibiotic (Kracht et al., 1999) and measured its hemolytic activity in spent medium of the four experimental variants described above: PS216 signal-producer alone, signal-producer with added ComX, signal-deficient mutant *B. subtilis* PS216ΔcomQ and signal-deficient mutant with ComX and for the same cultures grown with exogenously provided ComX signal.

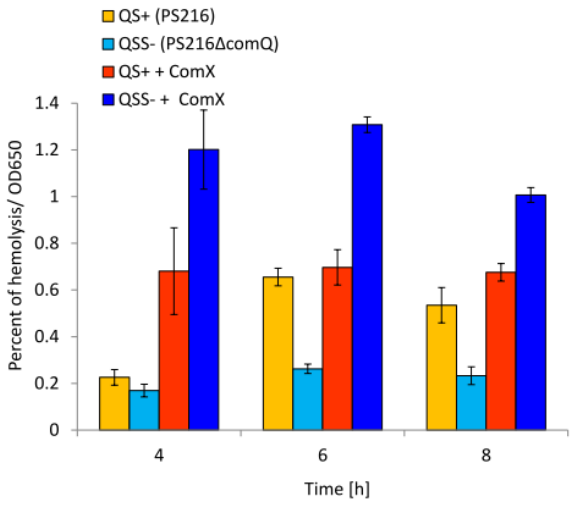


Figure 15. **ComX-induced hemolytic activity in QS+ and QSS- conditioned media**. Hemolytic activity of biosurfactants in conditioned media of undomesticated QS+ *B. subtilis* PS216 and QSS− *B. subtilis* PS216ΔcomQ was measured after 4, 6, and 8h. Both strains were grown with or without 0.05 fraction (~10nM) of ComX. Data are presented as mean of biological triplicates with SE indicated.

Slika 15. **Hemolitična aktivnost izrabljenih gojišč QS+ in QSS- inducirana z ComX**. Hemolitično aktivnost izrabljenega gojišča sevov QS+ *B. subtilis* PS216 in QSS− *B. subtilis* PS216ΔcomQ smo izmerili po 4, 6, in 8 urah inkubacije. Oba seva sta bila gojena z ali brez ComX (~10nM). Podatki predstavljajo povprečne vrednosti treh bioloških ponovitev s standardno napako prikazano za vsako točko.

Indeed, the spent medium taken from the signal-deficient mutant grown in the presence of ComX produced at least a 2-fold (P<0.0006) stronger hemolytic activity at several time points during the growth than the signal-producing *B. subtilis* PS216 grown in the presence or absence of ComX (Figure 15).

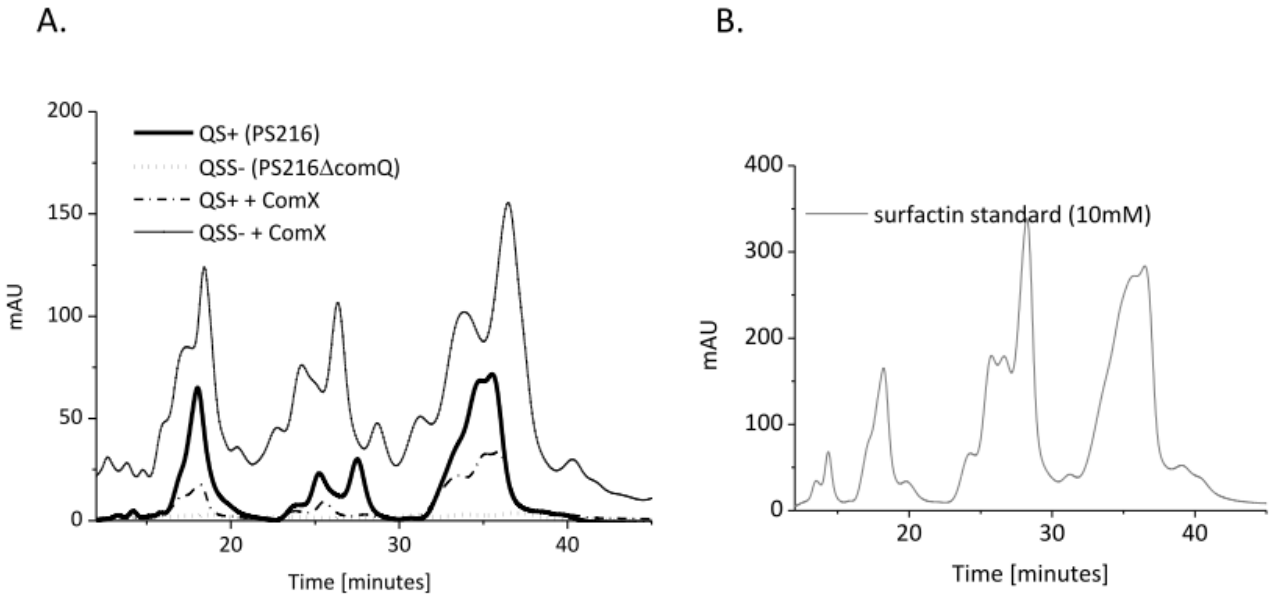


Figure 16. **Comparison of elution profiles of surfactin extracted from QS+ and QSS- conditioned media.** A. Chromatograms were obtained after HPLC analysis of surfactin isolated from the conditioned media of the *B. subtilis* PS216 and PS216ΔcomQ control cultures (no ComX) and cultures supplemented with 0.05 fraction (~10nM) purified ComX. B. The HPLC chromatogram of the commercially available surfactin standard is presented.

Slika 16. **Primerjava elucijskih profilov surfaktina ekstrahiranega iz izrabljenih gojišč QS+ in QSS- sevov**. A. Kromatogrami pridobljeni z HPLC analizo surfaktina izoliranega iz izrabljenih gojišč sevov *B. subtilis* PS216 in PS216ΔcomQ (brez in z ComX). B. HPLC kromatogram za komercialni standard surfaktina.

To link further the hemolytic activity to surfactin itself, the concentration of this lipopeptide antibiotic was determined by reverse-phase HPLC in the supernatant of all four experimental variants. Chromatographic peaks corresponding to peaks of commercially available surfactin standard were evident in all experimental variants except the *B. subtilis* PS216ΔcomQ culture without added ComX. Again, concentration of surfactin was highest in the supernatant of the PS216ΔComQ strain with added ComX, reaching almost 80 μM after 8 hours (Figure 16). In contrast, the signal-producer PS216 with and without ComX produced only up to 10 μM of surfactin at the same time (P<0.02) (Figure 16).

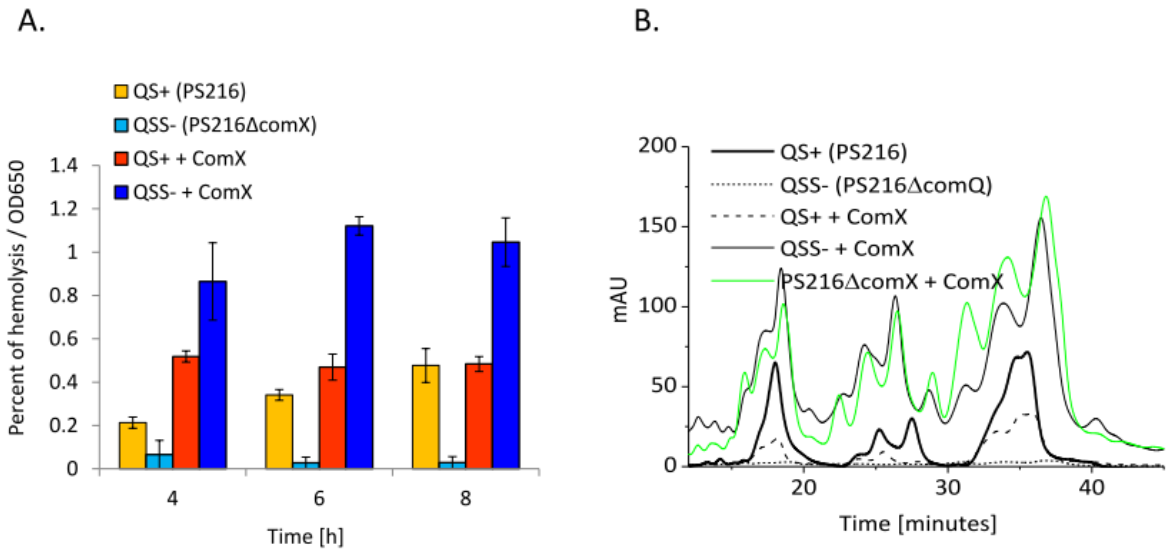


Figure 17. **ComX-induced hemolytic activity in QS+ and QSS- (Δ*comX*) conditioned media** A. Hemolytic activity of biosurfactants was measured after 4, 6, and 8 h in conditioned media of undomesticated QS+ *B. subtilis* PS216 and QSS− PS 216ΔcomX strains which were incubated without (control) or with 0.05 fraction (~10nM) of purified ComX. Data are presented as mean of biological triplicates with SE indicated. B. HPLC chromatogram (green) represents surfactin which was obtained from the conditioned media of undomesticated QSS- *B. subtilis* PS216ΔcomX grown in the presence of 0.05 fraction (~10nM) of ComX.

Slika 17. **Hemolitična aktivnost izrabljenih gojišč QS+ in QSS- (Δ*comX*) inducirana z ComX**. Hemolitično aktivnost izrabljenih gojišč, v katerih smo gojili neudomačene seve *B. subtilis* PS216 (QS+) in *B. subtilis* PS216ΔcomX (QSS−) smo izmerili po 4, 6, in 8 urah. Oba seva smo gojili z ali brez ComX. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko. B. Kromatogram HPLC (zelena) prikazuje surfaktin pridobljen iz izrabljenega gojišča neudomačenega seva *B. subtilis* PS216ΔcomX (QSS-), ki smo ga gojili v prisotnosti ComX [0,05 frakcija (~10nM)].

Similarly, addition of ComX to the signal-deficient PS216comX mutant resulted in increased surfactin production (Figure 17). This mutant has the crucial tryptophan residue changed to alanine (Schneider et al., 2002) and is unable to produce an active ComX, but has an intact ComQ protein. This result indicates that the synthesis or/and secretion of active ComX is important for modulation of the QS response.

4.2.3 Expression of *comK* and *comGA*in undomesticated *B. subtilis* QS+ and QSS-

Since we showed that in response to ComX QSS- mutant secretes more surfactin as compared to the QS+, we further hypothesized that this could also lead to higher expressiothe *comS* gene, which lies within the *srfA* operon , more precisely within the *srfAB* gene (D'Souza et al., 1994) ComS modulates stability of ComK (Turgay et al., 1998) and indirectly also expression

of the competence gene *comK* (Hameon et al., 1995). We used fluorescent fusions *comK-gfp* and *comGA-gfp* to monitore the expression of competence genes at the level of single cell in QS+ and QSS- population and in these populations supplemented with exogenous ComX.

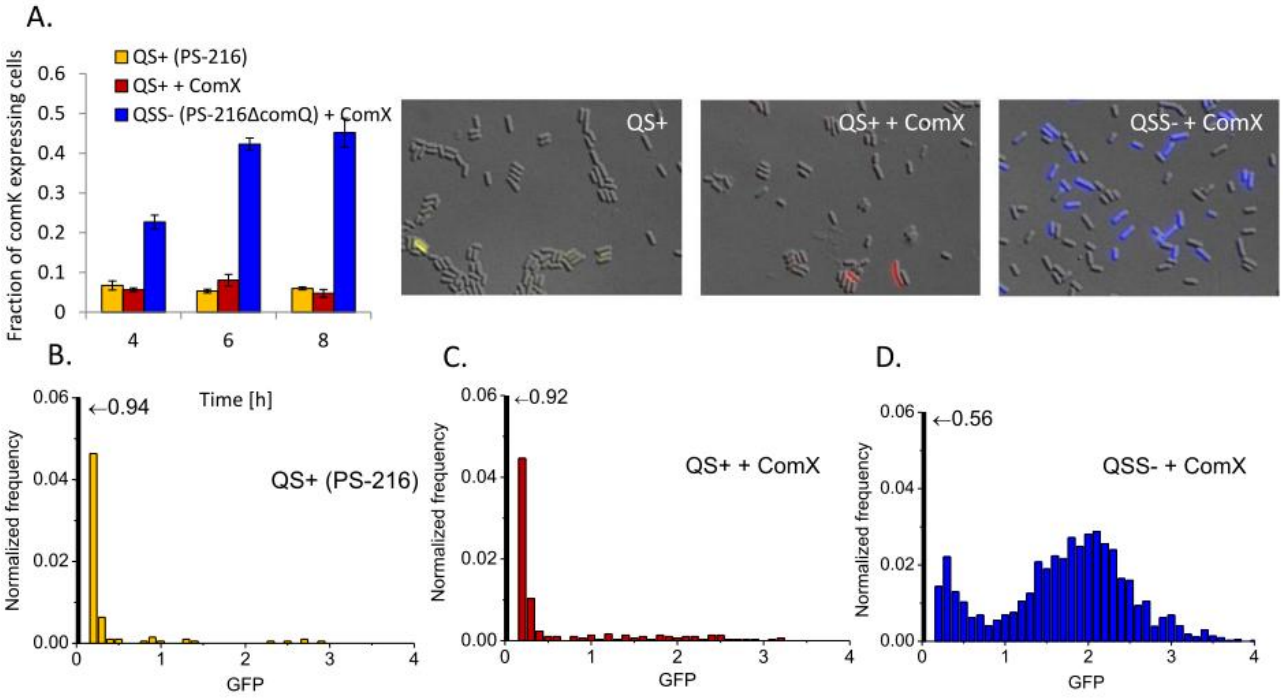


Figure 18. **Influence of ComX on expression of *comK-gfp* in undomesticated QS+ and QSS- *B. subtilis*** A. Fractions of *comK-gfp* expressing cells were determined by single-cell fluorescent microscopy at three timepoints. The QS+ (*B. subtilis* PS216) strain was incubated with no (yellow) or with ComX (red), while QSS- *B. subtilis* PS216ΔcomQ was incubated with ComX (blue). Data are presented as the mean with SE of biological triplicates. B-D.Histograms and corresponding pictures represent the distribution of *comK-gfp* expression in the (B) QS+ *B. subtilis* PS216 (yellow), (C) in the same strain incubated with ComX (red) and (D) in the QSS- *B. subtilis* PS216ΔcomQ (blue) incubated with ComX. Non-induced population (on the left) is marked with black.

Slika 18. **Vpiv ComX na izražanje *comK-gfp* pri neudomačenih sevih *B. subtilis* QS+ in QSS.** A. Izražanje *comK-gfp* smo spremljali s pomočjo fluorescenčne mikroskopije na nivoju posamezne celice v treh časovnih točkah. Seva *B. subtilis* PS216 (rdeča) in za QSS- *B. subtilis* PS216ΔcomQ (modra) smo inkubirali z ComX. Vrednosti označene z rumeno bravo smo izmerili za sev PS216 brez dodanega ComX. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko. B-D. Histogrami in ustrezne slike prikazujejo distribucijo izražanja *comK-gfp* pri (B) QS+ *B. subtilis* PS216 (rumena), (C) pri istem sevo inkubiranim z ComX (rdeča) in (D) pri QSS- *B. subtilis* PS216ΔcomQ (modra) inkubiranim z ComX. Neinducirana populacija (na levi) je označena s črno.

We discovered that in QS+ the subpopulation of *comK-gfp* expressing cells was smaller than 10% in all three timepoints measured regardless of exogenous ComX presence in the medium (Figure 18A). No *comK-gfp* expressing cells were found in QSS- , but when ComX was added

the subpopulation expressing fluorescence was more than 7-fold larger than these of the QS+ ($P<0.008$) and exceeded 40% of all cells after 6 and 8 hours of growth (Figure 18A). It is also interesting to notice that in each case two major subpopulations of cells were found: subpopulation of non-fluorescing cells and subpopulation showing relatively weak (less than 1 fluorescent unit) *comK-gfp* expression (Figure 18B-C). However the distribution of *comK-gfp* in QSS- grown with ComX differed because another major subpopulation of hyper *comK-gfp* expressing cells was found with app. 2-fold higher mode of *comK-gfp* fluorescence (Figure 18D).

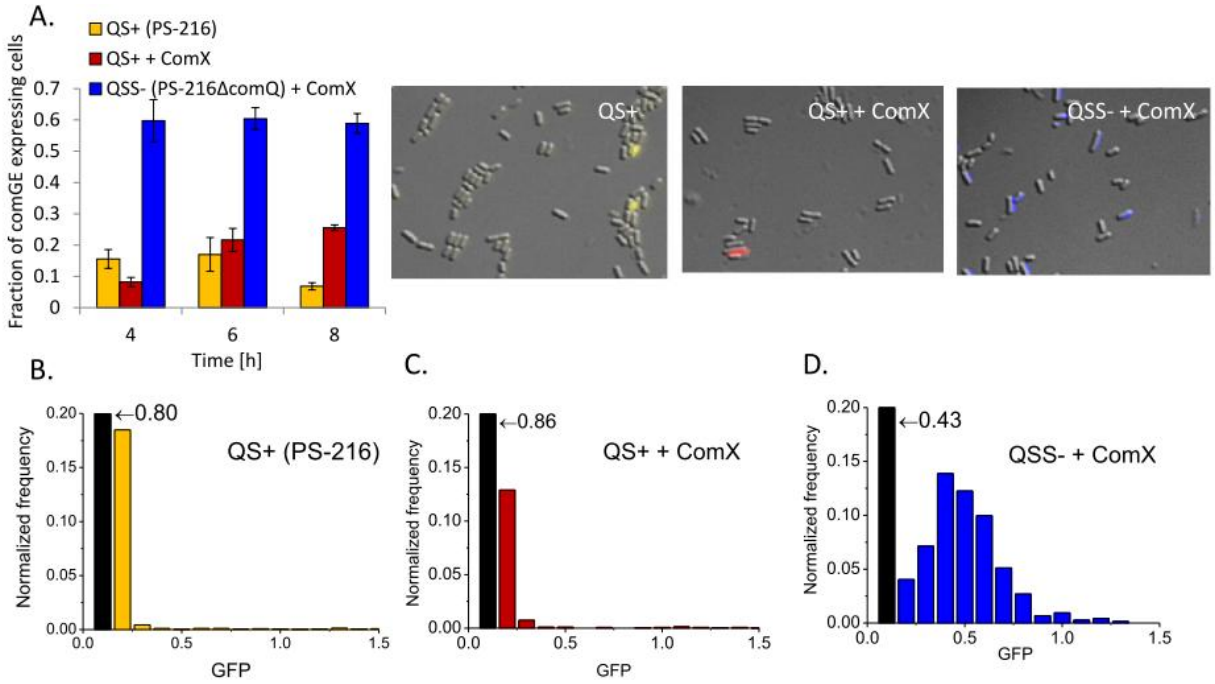


Figure 19. **Influence of ComX on expression of *comGA-gfp* in undomesticated QS+ and QSS- *B. subtilis*** A. Fractions of *comGA-gfp* expressing cells were determined by single-cell fluorescent microscopy at three timepoints for QS+ *B. subtilis* PS216 (yellow), in the same strain incubated with ComX (red) and in the QSS- *B. subtilis* PS216ΔcomQ (blue) incubated with ComX. Data are presented as the mean with SE of biological triplicates. B-D. Histograms and corresponding pictures represent the distribution of *comK-gfp* expression in the (B) QS+ *B. subtilis* PS216 (yellow), (C) in the same strain incubated with ComX (red) and (D) in the QSS- *B. subtilis* PS216ΔcomQ (blue) incubated with ComX. Non-induced population (on the left) is marked with black.

Slika 19. **Vpliv ComX na izražanje *comGA-gfp* pri neudomačenih QS+ in QSS- sevih *B. subtilis*.** A. Izražanje *comGA-gfp* smo spremljali s fluorescenčno mikroskopijo na nivoju posamezne celice v treh časovnih točkah pri QS+ sevu *B. subtilis* PS216 (rumeno), pri istem sevu inkubiranem z ComX (rdeča) in pri QSS- sevu *B. subtilis* PS216ΔcomQ (modra) inkubiranim z ComX. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko B-D. Histogrami in ustrezne slike, ki prikazujejo distribucijo izražanja *comGA-gfp* pri (B) QS+ *B. subtilis* PS216 (rumena), (C) pri istem sevu inkubiranem z ComX (rdeča) in (D) pri QSS- *B. subtilis* PS216ΔcomQ (modra) inkubiranem z ComX. Neinducirana populacija (na levi) je označena s črno.

Similar effects were observed when *comGA-gfp* expression was monitored (Figure 19). Here fluorescnece values were lower as compared to *comK-gfp*, but still QS+ subpopulation of *comGA-gfp* expressing cells did not exceed 20% while QSS- supplemented with ComX made up for over 50% of all cells (Figure 19A). Also the fluorescence of *comGA-gfp* in this culture was higher as compared to the QS+ and QS+ grown with ComX (Figure 19B-D).

4.2.4 Genetic competence for transformation of QS+ and QSS-

We assumed that since in the presence of exogeniously added QS signal, QSS- (PS-216ΔcomQ) population generates remarkably high numbers of *comK* and *comGA* expressing cells, the transformation frequency of this population should be higher as compared to the wild type when equal amounts of DNA are provided. We tested this by transforming the QS+ cells, QSS- cells and the same cultures supplemented with ComX, with chromosomal DNA from OKB120 strain (Nakano et al., 1988) with Tn19 transposone carrying lincomycin and erythromycin resistance.

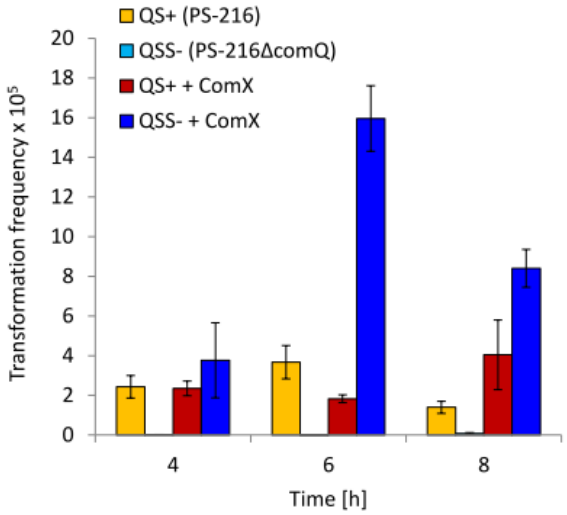


Figure 20. **ComX-dependent transformation frequency of undomesticated QS+ and QSS- *B. subtilis*.** The frequency of transformation was determined at three timepoints, by transforming cultures of QS+ *B. subtilis* PS-216 and QSS− PS-216ΔcomQ grown without (control) or with 0.05 fraction (~10nM) of exogenous ComX, with chromosomal DNA (added to final concentration 1ng·µl^{-1}) carrying *mls* resistance. Transformants were selected on *mls* (12.5 µg·ml^{-1} lincomycin and 0.5 µg·ml^{-1} erythromycin) LB-agar medium and their number was devided by total cell number of the population (determined by CFU palted on LB agar without antibiotics). Data are presented as the mean with SE of biological triplicates.

Slika 20. **Od ComX-odvisna frekvenca transformacije neudomačenih QS+ in QSS- sevov *B. subtilis*.** Frekvenco transformacije smo določili v treh časovnih točkah za seva *B. subtilis* PS-216 (QS+) in PS-216ΔcomQ (QSS−), ki smo ju gojili z [0.05 frakcija (~10nM) ComX] ali brez ComX in transformirali s kromosomalno DNA (dodana do končne koncentracije 1ng·µl^{-1}), ki je nosila lokus *mls*. Transformante smo selekcionirali na LB- agarskem gojišču z *mls* (12.5 µg·ml^{-1} linkomicina in 0,5 µg·ml^{-1} eritromicina) in število zraslih kolonij (CFU) na selekcijskem gojišču delili s številom celic celotne populacije (določili kot CFU, ki so zrasle na LB agarju brez antibiotikov). Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

As expected the transformation frequency of the mutant grown with ComX was the highest in all 3 timepoints and it reached 0.016% (± 0.0017%) after 6 hours (T2) which was more that 4-fold higher as compared to both wild populations accounting for 0.0037% (± 0.00083%) and 0.0018% (±0.0002%) (with or without ComX respectively) (Figure 20).

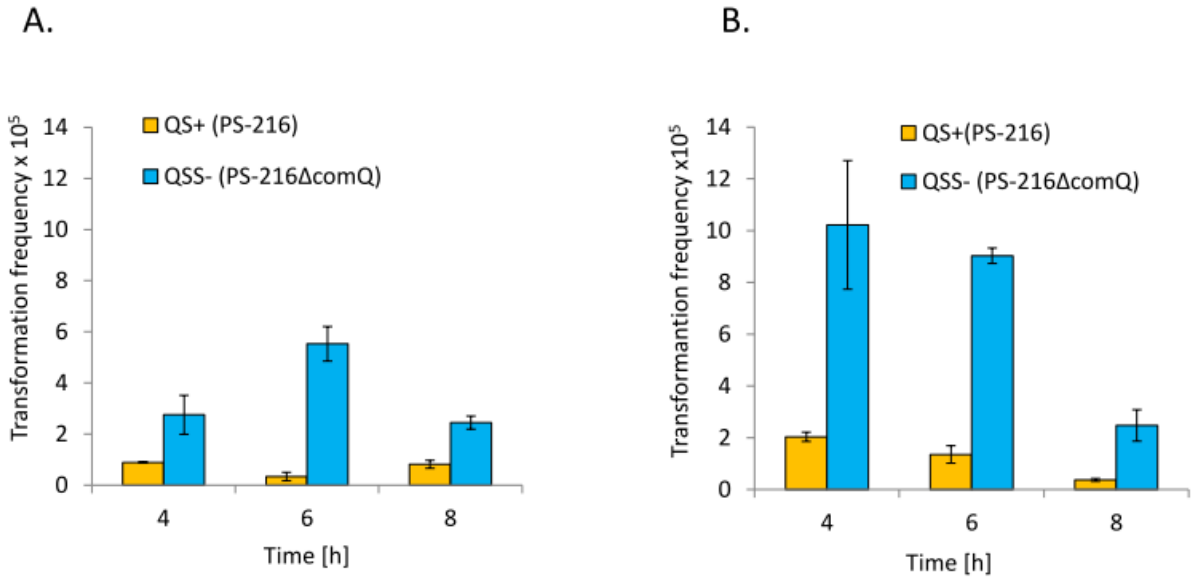


Figure 21. **Frequency of trasnformation for in 1:1 cocultures of *B. subtilis* QS+ and QSS−**. Strains. The frequency of transformation was determined at three timepoints, by transforming cocultures of QS+ PS-216 and signal-deficient (QSS−) PS-216ΔcomQ with (A) chromosomal or (B) plasmid DNA (added to final concentration 1ng·µl^{-1}) carrying *mls* resistance. Transformants were selected on *mls* (12.5 µg·ml^{-1} lincomycin and 0.5 µg·ml^{-1} erythromycin) LB-agar medium and their number was devided by total cell number of the population (exporessed as CFU on LB agar without antibiotics). Data are presented as the mean with SE of biological triplicates.

Slika 21. **Frekvenca transformacije v kokulturah dveh sevov *B. subtilis* (QS+ in QSS-) , ki smo ju inokulirali v razmerju 1:1.** Frekvenco transformacije smo določili v treh časovnih točkah, kjer smo kokulturo QS+ *B. subtilis* PS-216 in QSS− PS-216ΔcomQ izpostavili (A) kromosomalni DNA ali (B) plazmidni DNA (dodani do končne koncentracije 1ng·µl^{-1}), ki sta nosili odpornost *mls*. Transformante smo selekcionirali na *mls* (12.5 µg·ml^{-1} linkomicina in 0.5 µg·ml^{-1} eritromicina) LB-agarskih gojiščih in njihovo število delili s številom celotne populacije (določeno kot CFU na LB agarju brez antibiotikov). Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako izračunano za vsako točko.

We next examiend whether the same is true in coculture of the QS+ and QSS- , by transforming the mixed culture with the same chromosomal DNA and additionally with multicopy plasmid pHP13 (Harwood et al., 1990) carrying the same transposon. Again dramaticly higher transformation frequency was observed for QSS- population accounting for 0.0055% (± 0.00068%) while only 0.00034% (± 0.000016%) of transformants were found in the QS+ population in case of experiment with chromosomal DNA (Figure 21A). If multicopy plasmid pHP13 was used the transformation frequencies were higher for both strains, but still the mutant showed incresed (0.009% (±0.0003%) fraquency while the wild type only 0.0013% (±0.00033%) frequency of transformation (Figure 21B). We concluded the QSS- in the presence of ComX represented a hyper-competent phenotype.

4.2.5 Primary/secondary metabolic imbalance of the QSS- mutant

Surfactin production and competence development will be further referred to as secondary metabolic activity. Surfactin is a typical secondary metabolite – nonrybosomally produced organic compound that is not directly involved in normal growth and reproduction of the bacterium and its loss does not result in immediate death of *B. subtilis*. By analogy development of genetic competence for transformation can be considered secondary metabolic activity; because it is not absolutely required for survival of *B. subtilis*, it is rather specialized (not basic) function of the spicies and adaptive for survival during stationary phase. Since we observed that signal-deficient strain have strongly increased secondary metabolic activity (increased production of surfactin, increased competence for transformation) we evaluated the differences in primary metabolic activity between QS+, QSS- and the same strains grown in presence of ComX. To do this we used dehydrogenase activity assay, which is routinely used for this purpose (Maness et al., 1999).

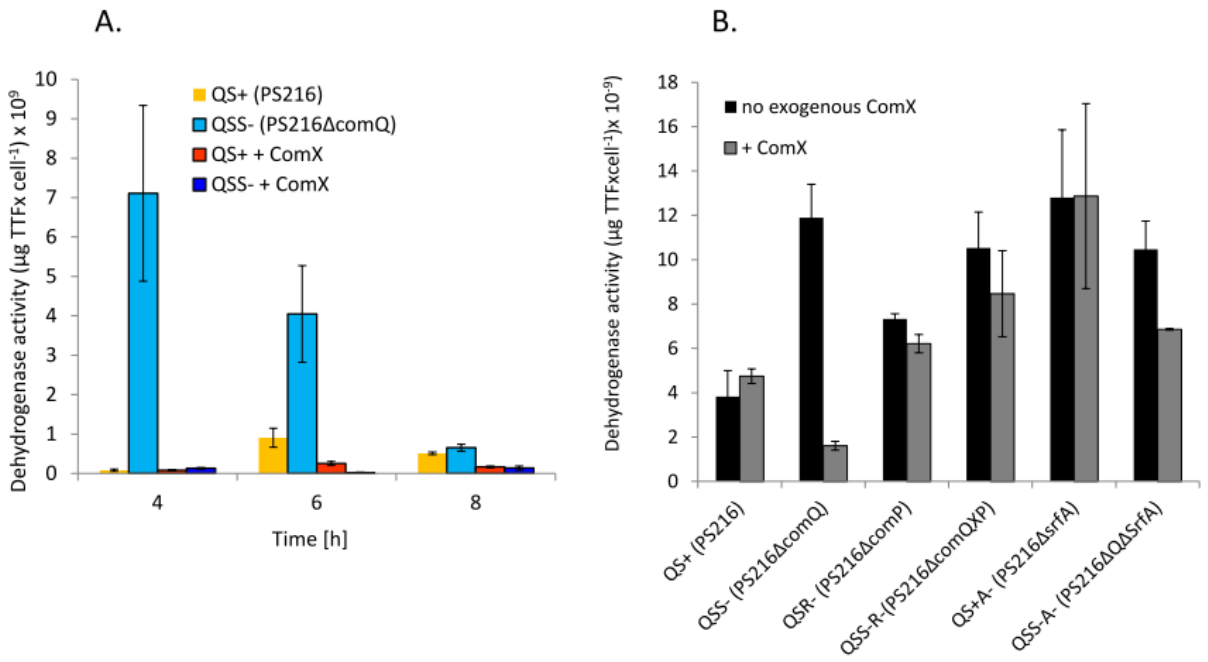


Figure 22. **Dehydrogenase activity of two B. subtilis strains: QS+ and QS mutants incubated with or without exogenous ComX**. A. The dehydrogenase activity of QS+ *B. subtilis* PS216 and QSS- PS216ΔcomQ grown with or without ComX 0.05 fraction (~10nM)) was determined after 4, 6, and 8 h of incubation. B. The dehydrogenase activity QS+ *B. subtilis* PS216 and QS mutants: signal deficient PS216ΔcomQ (QSS−) receptor-deficient PS216ΔcomP (QSR−), signal-deficient and receptor-deficient PS216ΔcomQXP (QSS−R−), surfactin-deficient PS216ΔsrfA (QS+A−), and signal-deficient and surfactin-deficient PS216ΔcomQΔsrfA (QSS−A−) strains were measured after 6 h of incubation. The same strains were also grown with 0.05 fraction (~10nM) of exogenous ComX. Data are presented as the mean with SE of biological triplicates.

Slika 22. **Aktivnost dehidrogenaze pri sevu *Bacillus subtilis* QS+ in pri mutante QSS- v prisotnosti/ odsotnosti ComX**. A. Aktivnost dehidrogenaze smo določili pri sevu QS+ *B. subtilis* PS216 in sevu QSS- PS216ΔcomQ, ki smo ju gojili z ali brez dodanega ComX (frakcija 0.05). Aktivnost smo merili po 4, 6, ali 8-urah inkubacije. B. Dehidrogenanza aktivnost pri različnih sevih: QS+ *B. subtilis* PS216; PS216ΔcomQ (QSS−), mutant z okvarjenim receptorjem PS216ΔcomP (QSR−); mutant ki ne proizvaja signala in ima okvarjen receptor PS216ΔcomQXP (QSS−R−); mutant, ki ne proizvaja surfaktina PS216ΔsrfA (QS+A−); mutant, ki ne proizvaja signala in surfaktina PS216ΔcomQΔsrfA (QSS−A−). Aktivnost smo izmerili po 6 urah inkubacije. Iste seve smo gojili brez ali z 0.05 frakcijo ComX. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z standardno napako določeno za vsako točko.

Indeed, in the absence of ComX the signal-deficient PS216ΔcomQ mutant showed a strong increase in dehydrogenase activity, which dropped dramatically and below the levels observed for the QS+ PS216 strains, if the mutant was exposed to ComX (Figure 22A). Additionally the dehydrogenase assay performed for other mutants (lacking the ComP receptor, the receptor and the signal or just the *srfA* gene) revealed that signal production and surfactin production are both required to preserve moderate primary metabolic activity. ComX could decrease this activity only in the signal-deficient strain with an intact ComP receptor, but not in strains

where either the *comP* or the *srfA* genes were missing (Figure 22B). This strongly supports the role of the ComQXP QS system in coupling the primary and secondary metabolism.

4.3 FITNESS CONSEQUENCES AND EVOLUTIONARY IMPLICATIONS OF PRIVATE LINK IN *B. subtilis* QUORUM SENSING

4.3.1 Signal deficient mutants suffer from fitness loss when facing the QS signal

We further investigated whether the increase in surfactin production and genetic competence for transformation, observed for the undomesticated PS216ΔcomQ strain in the presence of ComX (see chapter 4.2), are associated with the negative fitness effects. This would be expected because surfactin excess could cause autotoxicity or its increased production could cause metabolic burden. Additionally, in the presence of ComX, the mutant develops very large subpopulation of competent cells and it is known that competent subpopulation of cells is arrested in cell division (Haijema et al., 2001). To test both hypotheses, we monitored the growth of the signal producing *B. subtilis* PS216 and the signal-deficient PS216ΔcomQ mutant in the presence and absence of exogenously added ComX.

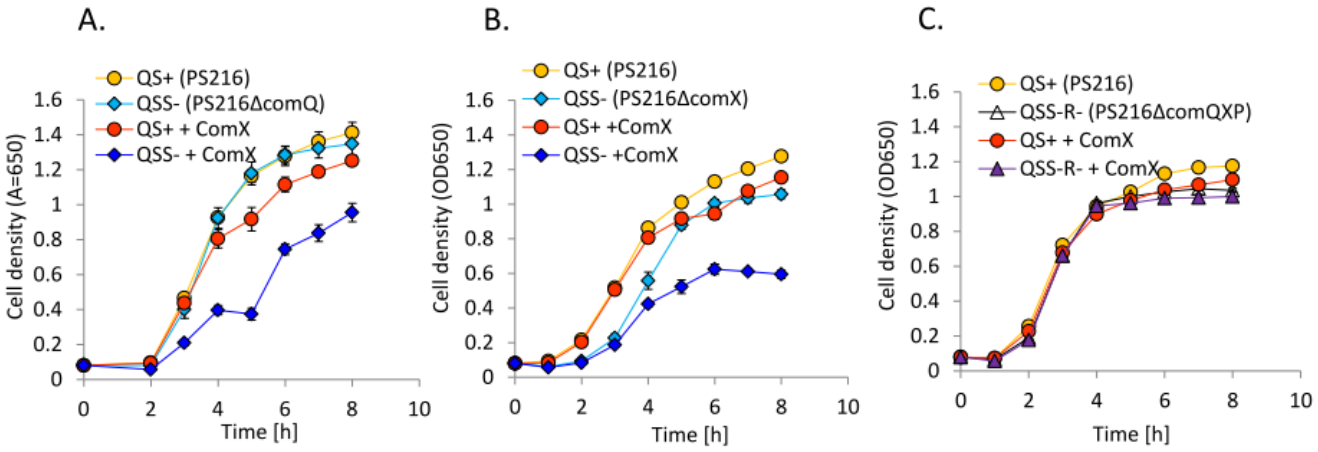


Figure 23. **Growth of QS+ and QS mutants with and without added ComX**. A. Growth of QS+ *B. subtilis* PS216 and QSS− PS216ΔcomQ without and with added ComX (0.05 fraction (~10nM)) was monitored spectrophotometrically at 650 nm. B. Growth curves of QS+ *B. subtilis* PS216 and QSS− *B. subtilis* PS216ΔcomX incubated without or with 0.05 fraction (~10nM) of ComX. C. Growth curves of QS+ *B. subtilis* PS216 and QSS−R− *B. subtilis* PS216ΔcomQXP incubated without or with 0.05 fraction (~10nM) of ComX. Data are presented as mean of biological triplicates with SE indicated for every time point in panels A-C.

Slika 23. **Rast sevov *B. subtilis* QS+ in QSS- v prisotnosti/odsotnost eksogenega ComX.** A. Rast QS+ *B. subtilis* PS216 in QSS− PS216ΔcomQ brez ali z dodanim ComX (0.05 frakcija (~10nM)) smo spremljali spektrofotometrično pri 650 nm. B. Raste krivulje QS+ *B. subtilis* PS216 in QSS− *B. subtilis* PS216ΔcomX inkubiranih brez ali z 0.05 frakcijo ComX. C. Raste krivulje QS+ *B. subtilis* PS216 in QSS−R− *B. subtilis* PS216ΔcomQXP inkubiranih brez ali z 0.05 frakcijo ComX. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

The growth curves of the signal-producing and signal-deficient monocultures were very similar in the absence of exogenously added ComX, despite the fact that the former produced ComX and surfactin in response to it (Figure 23A). In contrast, growth of the signal-deficient mutant PS216ΔcomQ was negatively affected by the presence of exogenous ComX with a 20% ($P < 0.002$) decrease in the growth rate (Figure 23A). Similarly, negative fitness effects were observed for the PS216ΔcomX, which also showed increased surfactin production (Figure 23B).

We next tested the influence of the exogenous ComX on the PS216ΔcomQXP mutant, which was both signal-deficient (due to *ΔcomQ* or *ΔcomX*) and receptor-deficient (due to *ΔcomP*). No ComX-dependent negative fitness effect was observed in the absence of the ComP receptor, confirming that the negative fitness effect was caused by specific ComX-ComP interaction (Figure 23C).

4.3.2 Surfactin is the major fitness cost paid by the signal deficient mutant

To examine the role of *srfA* in ComX-mediated growth breakdown of the mutant, we constructed the PS216ΔcomQΔsrfA double mutant, which was not able to synthesize ComX or surfactin, and monitored its growth in the presence of exogenous ComX.

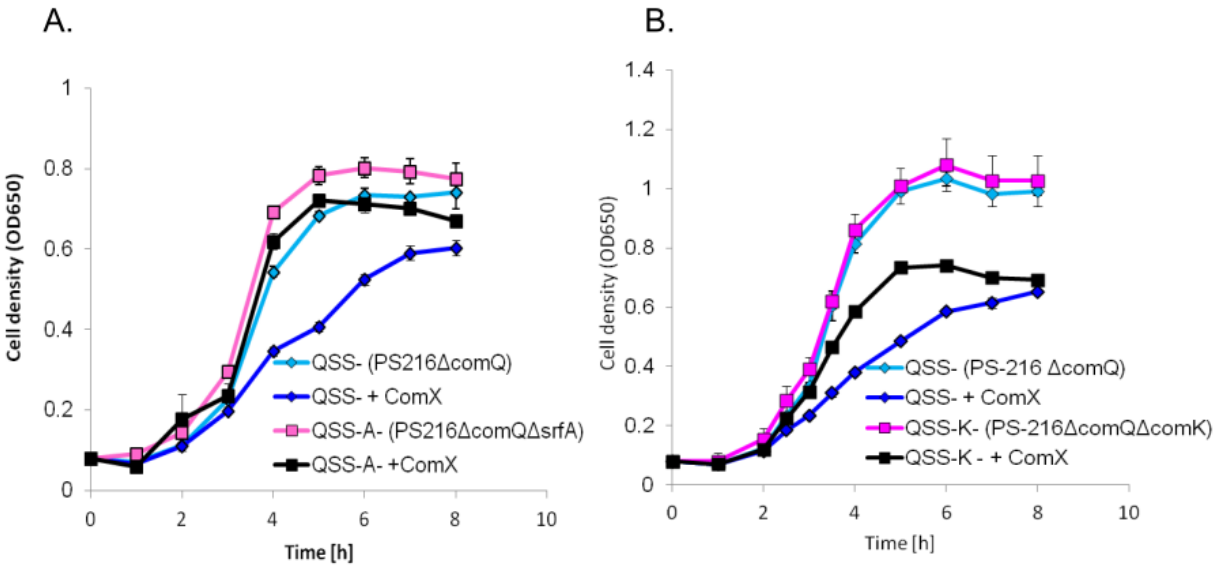


Figure 24. **Growth of QSS- mutant and double mutants (Δ*comQ*Δ*srfA* and Δ*Q*Δ*comK*) with and without added ComX**. A. Growth of *B. subtilis* PS216ΔcomQ (QSS−) and of double-mutant: QSS−A− PS216ΔcomQΔsrfA deficient in ComX and surfactin production and B. QSS−K− PS216ΔcomQΔcomK deficient in ComX and competence development; in the absence (control) and presence of ComX (0.05 fraction (~10nM)) was monitored spectro-photometrically at 650 nm. Data are presented as mean of biological triplicates with SE indicated.

Slika 24. **Rast mutant QSS- in dvojnih mutant (Δ*comQ*Δ*srfA* and Δ*Q*Δ*comK*) z ali brez dodanega ComX**. Rast *B. subtilis* PS216ΔcomQ (QSS−) in dvojnih mutant: A.QSS−A− PS216ΔcomQΔsrfA z okvarjeno funkcijo sinteze ComX in surfaktina in B. QSS−K− PS216ΔcomQΔcomK z okvarjeno funkcijo sinteze ComX in razvoja genetske competencet; brez dodanega ComX (kontrola) in v prisotnosti ComX (0.05 frakcija (~10nM)) smo spremljali sprektrofotometrično pri 650 nm. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z SN določeno za vsako točko.

In contrast to PS216ΔcomQ, no dramatic fitness effect was observed for the PS216ΔcomQΔsrfA double mutant, strongly supporting the hypothesis that increased synthesis of surfactin in the undomesticated signal-deficient mutant exerts the negative fitness effect (Figure 24A). To further confirm this we tested whether deletion of *comK* would release the QSS- from negative fitness effects in the presence of ComX. If induction of competence was a major cost the growth of the double mutant ΔcomQΔcomK should still be impaired in the presence of ComX. We observed that indeed ComX still negatively affected such mutant,

although the effect was less dramatic than for ΔcomQ (Figure 24B). This suggests that surfactin overproduction is the major fitness cost but increased competence additionally slows the mutant growth down in presence of ComX.

Finally, we examined expression of competence genes in strains with deletion in *srfA*. Since *comS* lies within the *srfA* operon, it could be that the mutation in PS216ΔcomQΔsrfA also releases the mutants from increased competence development. However, it was reported (Nakano et al., 1993) that the genetic competence for transformation is intact in the strain OKB120 (*srfA::Tn19*) which we used here as a DNA donor to disrupt the *srfA* of PS-216. To confirm this we used the *comK-gfp* fusion to compare the expression of competence gene *comK* in PS216ΔcomQ and the double mutant PS216ΔcomQΔsrfA.

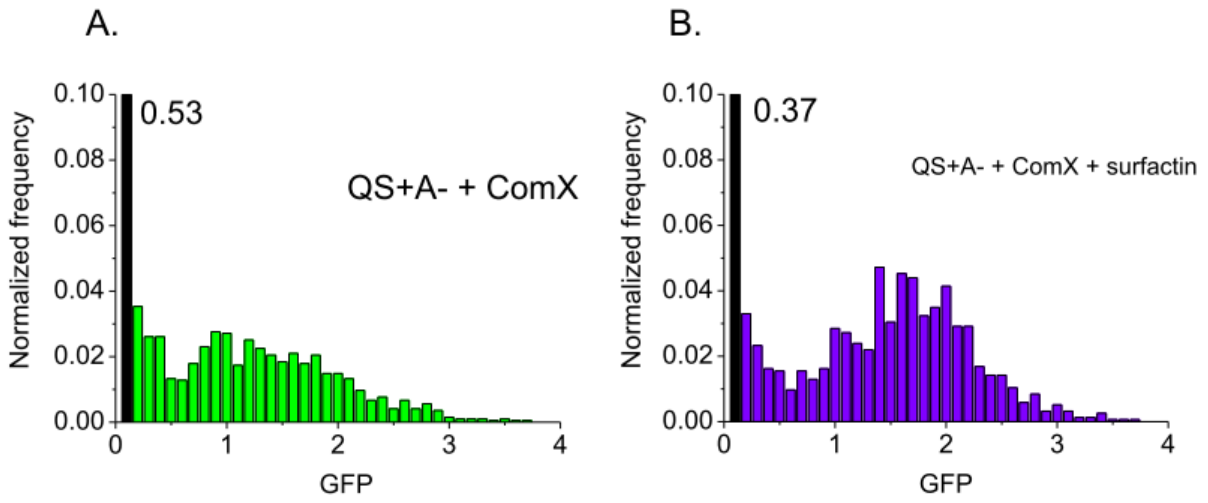


Figure 25**. Distribution of *comK-gfp* expression in the *B. subtilis* QSS-A- double mutant**. Fractions of *comK-gfp* expressing cells were determined with the use of single-cell fluorescent microscopy after 6 hours of incubation for *B. subtilis* strain QSS-A- (PS216ΔcomQΔsrfA) incubated with (A) the purified ComX and (B) with the purified ComX and surfactin. The non-induced population (on the left) is marked with black.

Slika 25**. Distribucija izražanja *comK-gfp* pri dvojni mutante QSS-A-**. Frakcije celic, ki so izražale *comK-gfp* smo določili s pomočjo fluorescenčne mikroskopije na nivoju posamezne celice po 6 urah rasti za sev *B. subtilis* QSS-A- (PS216ΔcomQΔsrfA) (A) inkubiran s ComX in (B) inkubiran s ComX in surfaktinom. Neinducirana populacija (na levi) je označena s črno.

In presence of exogenous ComX the population of *comK*-expressing cells was also increased in the double mutant (Figure 25A, compare to Figure 18B). Interestingly however, the presence of surfactin in the medium also showed an effect. Since we observed that *comK* distribution of QSS-A- grown with ComX slightly differed from QSS- grown with ComX (Figure 25A, Figure 18D) we supplemented QSS-A- medium not only with ComX but also

with surfactin. We observed that in the presence of surfactin the distribution of *comK* expression of QSS-A- changed and became very similar to the observed for QSS- grown with ComX (Figure 25B).

4.3.3 Signal-deficient mutant fails in competition with the wild type

On the basis of the results described above we hypothesized that the signal-deficient mutant PS216ΔcomQ will fail in competition with the QS wild type PS216, because it would suffer from negative fitness effects when facing the QS signal produced by the wild type.

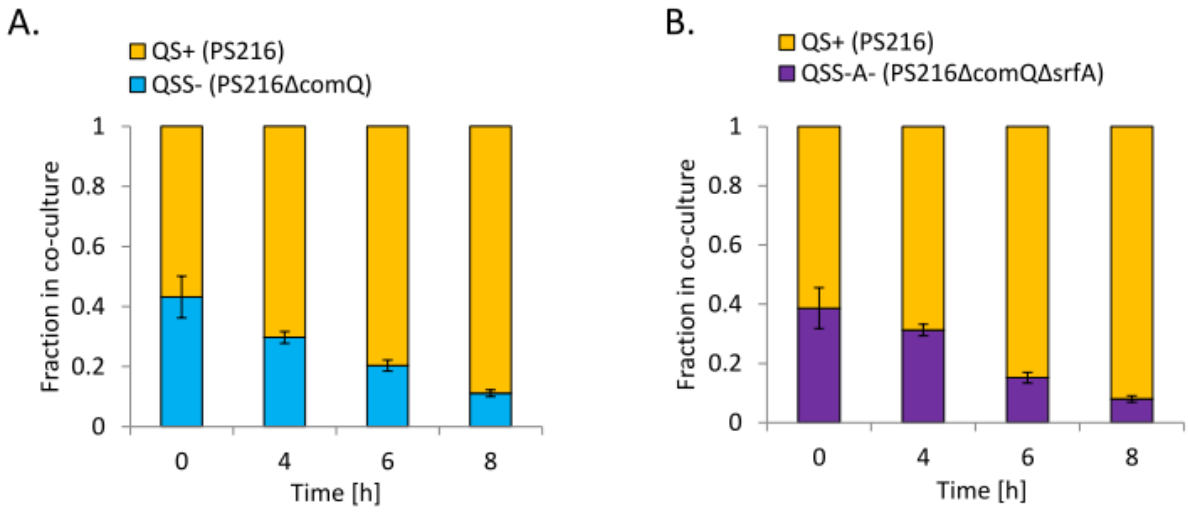


Figure 26. **Competition assay between B. subtilis QS+ and signal-null mutant (QSS-)**. Fractions of cells were determined in cocultures of (A) PS216 and PS216ΔcomQ and (B) PS216 and PS216ΔcomQΔsrfA over time by viable cell counts (CFU) using antibiotic selection. Data are presented as mean of biological triplicates with SE indicated.

Slika 26. **Testiranje kompeticije med sevom QS+ in mutantom, ki ne proizvajajo signala (QSS-)**. Frakcije celic smo določili v kokulturah (A) PS216 in PS216ΔcomQ ter (B) PS216 in PS216ΔcomQΔsrfA v različnih časovnih točkah inkubacije. Število živih celic smo določali z metodo CFU ,in selekcijo na gojišču z ustreznim anktibiotikom. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s SN določeno za vsako meritveno točko

Consistent with our hypothesis, the proportion of the signal-deficient derivative PS216ΔcomQ in a 1:1 coculture with the signal-producing *B. subtilis* PS216 was reduced to 10% ($P<0.002$) after incubation for 8 hours, indicating a significant advantage of the wild type signal-producer (Figure 26A). We next examined the fitness of the double mutant PS216ΔcomQΔsrfA that did

not show fitness loss when exposed to ComX in monoculture (Figure 24A) and surprisingly we discovered that it was also outcompeted by the wild type, comprising less than 10% ($P<0.001$) of the community after incubation for 8 h (Figure 26B).

To explore this phenomenon further we excluded surfactin expression from the system by challenging the mutant in surfactin production (PS216ΔsrfA) against the double mutant (PS216ΔcomQΔsrfA).

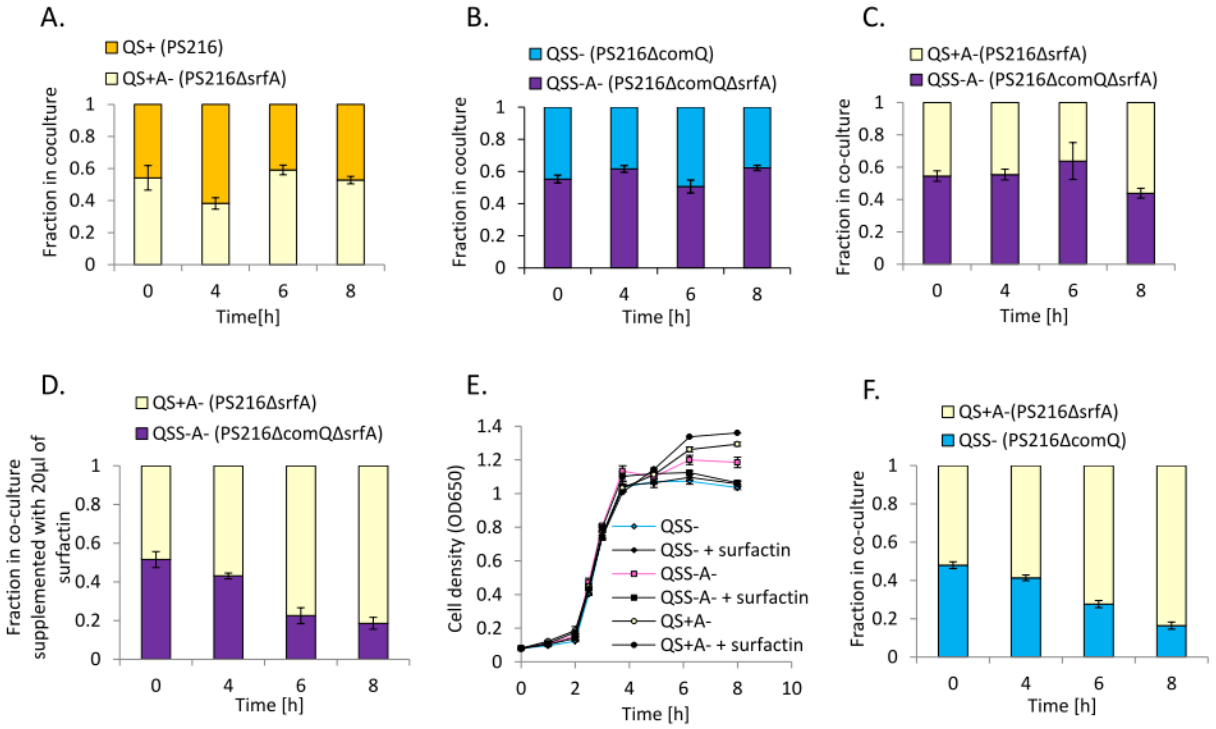


Figure 27. **Competition assay of QS+ and QS mutants in different combinations and with addition of surfactin.** Fractions of (A) PS216 and PS216ΔsrfA; (B) PS216ΔcomQ and PS216ΔcomQΔsrfA; (C) PS216ΔsrfA and PS216ΔcomQΔsrfA; and (D) PS216ΔsrfA and PS216ΔcomQΔsrfA supplemented with surfactin (20µg·ml^{-1}) in coculture over time were determined by viable cell counts (CFU) using the antibiotic selection. E. Growth curves of PS216ΔsrfA (QS+A-), PS216ΔcomQ (QSS-) and PS216ΔcomQΔsrfA (QSS-A-) incubated with or without 0.05 fraction (~10nM) of exogenous ComX. F. Fractions of PS216ΔsrfA and PS216ΔcomQ in coculture over time were determined by viable cell counts (CFU) using the antibiotic selection. Data is presented as mean of biological triplicates with standard error indicated.

Figure 27. **Kompetitivni preizkus sevov QS+ in mutant v različnih kombinacijah in z dodanim surfaktinom.** Frakcije (A) PS216 in PS216ΔsrfA; (B) PS216ΔcomQ in PS216ΔcomQΔsrfA; (C) PS216ΔsrfA in PS216ΔcomQΔsrfA; in (D) PS216ΔsrfA in PS216ΔcomQΔsrfA dopolnjeno z surfaktinom (20µg·ml^{-1}) v kukulturah, smo spremljali skozi čas preko štetja živih celic (CFU) s pomočjo selekcije na ustrezne antibiotike E. Rastne krivulje za seve PS216ΔsrfA (QS+A-), PS216ΔcomQ (QSS-) in PS216ΔcomQΔsrfA (QSS-A-) inkubirane z ali brez 0.05 frakcije eksogenega ComX. F. Frakcije PS216ΔsrfA in PS216ΔcomQ v kokulturi smo spremljali v različnih časovnih točkah rasti preko štetja živih celic (CFU) s pomočjo selekcije na ustrezne antibiotike. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev z SN določeno za vsako meritveno točko.

We also confirmed that PS216ΔsrfA did not show any changes in fitness against parental strains with the intact *srfA* gene (Figure 27A, B). We also discovered that the ratio of PS216ΔsrfA mutant and the signal-deficient double mutant PS216ΔcomQΔsrfA did not change dramatically, with 44% ±3% remaining in the coculture after incubation for 8 hours (Figure 27C). This supported the role of surfactin in the dominance of the wild type population over the double mutant. This was further confirmed when the coculture was supplemented with exogenous surfactin (final concentration 20 $\mu g \cdot ml^{-1}$), again reducing the ratio of PS216ΔcomQΔsrfA from 52% ±4% to 19% ±3% (Figure 27D). Interestingly, despite surfactin being associated with the disadvantage of the signal-deficient double mutant (PS216ΔcomQΔsrfA) in coculture, this lipopeptide antibiotic did not strongly influence growth of the double mutant in monocultures. A slight decrease in optical density was only observed in surfactin treated double mutant after 6 and 8 hours of incubation, but not during logarithmic growth (Figure 27E). This suggests that surfactin, produced by the wild type or added to the coculture predominantly indirectly contributes to fitness loss of the double mutant and that the presence of the QS proficient population is required to observe a strong negative fitness effect on the signal-deficient double mutant (PS216ΔcomQΔsrfA). However, surfactin itself also shows a minor direct negative effect on the double mutant during stationary phase, which may at least in part account for the difference in fitness during competition between the surfactin producer and the double mutant.

The question that remains is whether the fitness loss of the signal-deficient mutant PS216ΔcomQ is linked to surfactin overproduction, observed in monocultures grown in the presence of exogenous ComX. We believe this mechanism does contribute to the fitness loss of PS216ΔcomQ when challenged with QS-proficient populations. For example, in the coculture containing the surfactin deficient PS216ΔsrfA that produces ComX and the signaling mutant PS216ΔcomQ, the ratio of the latter decreased from 48% ±2% to 16% ±2% after 8 h (Figure 27E). This is consistent with the hypothesis that the surfactin overproduction adds to fitness loss of the signaling mutant.

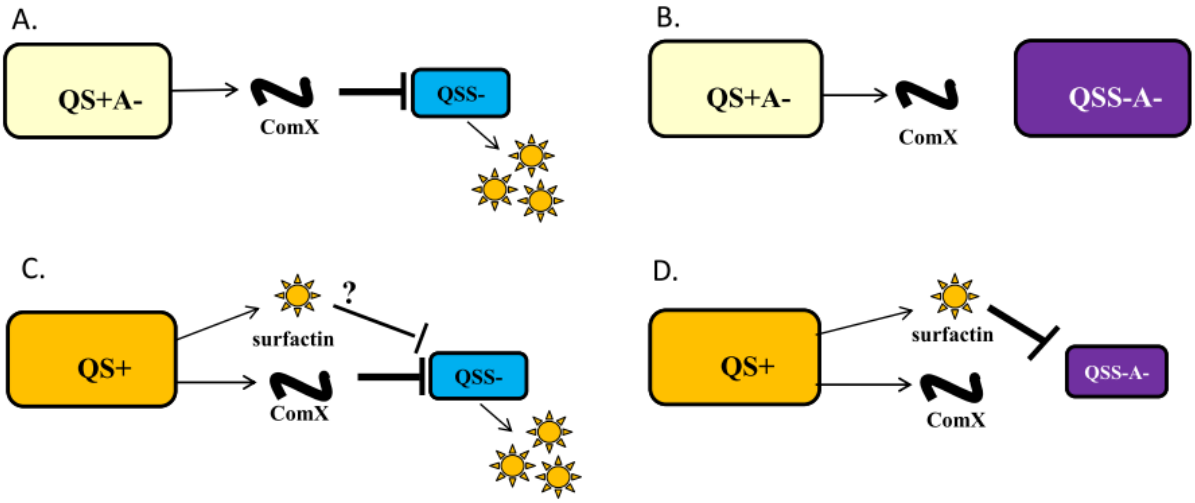


Figure 28. **Model of QSS- discrimination in cocultures with QS+ and QS+A- *B. subtilis***. Proposed strategies that discriminate signal deficient mutants in cocultures with QS proficient (QS+) and QS proficient but surfactin deficient (QS+A-) *B. subtilis*. The size of the cell indicates the relative fitness loss of the population. A. Signal deficient mutant QSS-, facing the QS signal ComX secreted by QS+A-, suffers from fitness loss associated with overproduction of surfactin. B. The signal deficient surfactin deficient double mutant QSS-A-, which does not overproduce surfactin, is released from the ComX-mediated fitness loss when competed with the signal producing but surfactin deficient QS+ A-strain. C. Signal deficient mutant suffers from fitness loss when cocultured with the QS+ strain due to ComX-mediated mechanism and possibly due to another mechanism that depends on surfactin D. The surfactin-dependent mechanism leads to the fitness loss of the QSS-A- double mutant when it is cocultured with the surfactin producing QS+ strain.

Slika 28. **Model diskriminacije QSS- v kokulturah s QS+ ali QS+A- sevi *B. subtilis*.** Predlagane strategije za diskriminacijo mutant, ki ne proizvajajo signala v kokulturah *B. subtilis* z neokvarjenim QS sistemom (QS+), sev z neokvarjenim QS sistem, ki ne proizvaja surfaktina (QS+A-) in mutant, ki ne proizvaja signala (QSS-). Velikost shematsko prikazane celice pomeni relativno znižan fitnes populacije. A. Mutante, ki ne proizvaja signala QSS- ob stiku s signalom ComX, ki ga izloča QS+A-, se zniža fitnes zaradi povečane produkcije surfaktina. B. Mutante, ki ne proizvaja signala in surfaktina QSS-A-, se znižajo metabolni stroški povečane produkcije surfaktina v prisotnosti ComX QS+ populacije v kokulturi. C. Mutante, ki ne proizvaja signala se zniža fitnes v kokulturi s QS+ zaradi povečane ekspresije surfaktina, ki jo sproži ComX in tudi zaradi direktnega vpliva surfaktina na QSS- mutanto. D. Od surfaktina odvisen mehanizem zniža fitnes dvojne mutante QSS-A- v kokulturi s sevom QS+, ki proizvaja surfaktin.

Therefore, two surfactin-dependent mechanisms may exist: a direct mechanism related to surfactin overproduction and an indirect mechanism when the competitor produces surfactin, reducing the fitness of the signaling mutant through an as yet unknown mechanism. Both mechanisms may contribute to the social selection that preserves the functionality of the quorum sensing in natural populations of *B. subtilis* (Figure 28).

4.4 COOPERATION AND CHEATING DURING BACTERIAL ,,SEX''/DNA EXCHANGE

4.4.1 ComX and surfactin serve as public goods that promote sex

B. subtilis is capable of primitive "sex", which is here envisioned as an uptake of environmental DNA by competent cells and its subsequent recombination into the chromosome of the recipient. This transformation event or "sex" may bring an evolutionary advantage to the recipient. We assumed that since ComX signal promotes competence development and surfactin triggers DNA release and thus they together enable DNA exchange between the two populations of *B.subtilis* sharing a habitat. In line with this assumption the lack of ComX or surfactin would strongly impair such exchange. We tested this in cocultures of two strains: one carrying resistance to chloramphenicol and another to spectinomycin. We prepared 1:1 cocultures of the two QS^+ strains carrying different resistance markers, the two QSS^- strains (lacking ComX) and QS^+A^- (lacking genes for surfactin synthesis) again with two different resistance markers. After 8 hours of coculturing we selected transformants for double (chloramphenicol and spectinomycin) resistance (Figure 29).

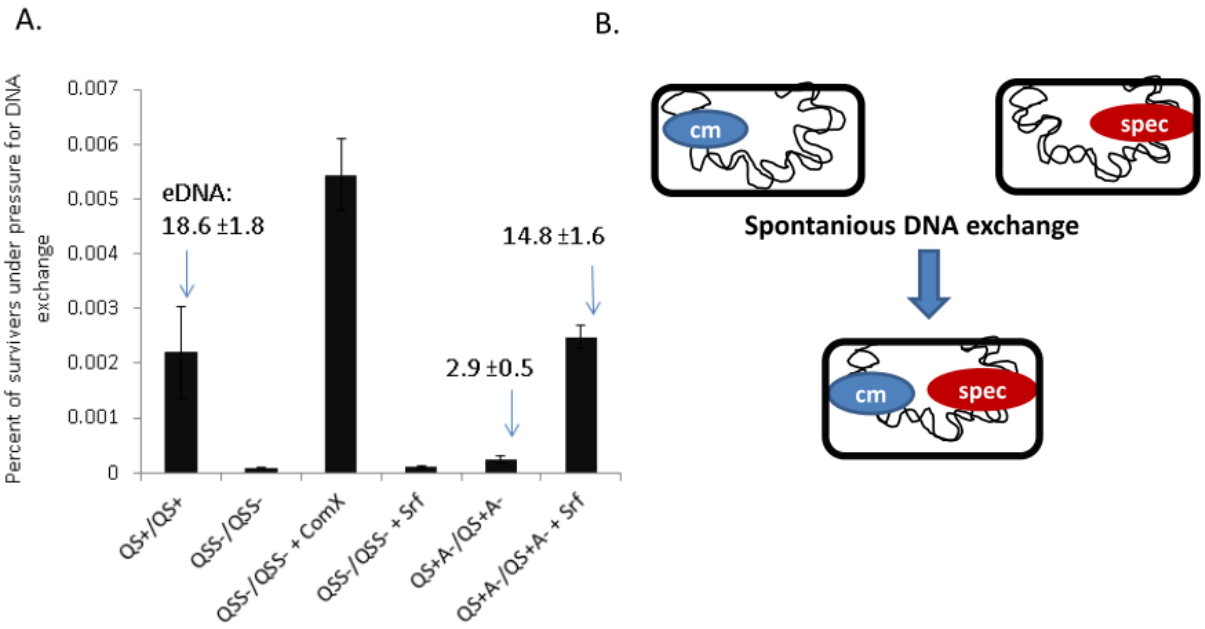


Figure 29. **DNA exchange assay in various *B. subtilis* cocultures.** Cocultures of two strains where each carried different beneficial mutation (antibiotic resistances; chloramphenicol and spectinomycin respectively) were grown for 8 hours. At this point transformants were selected for double resistance (as presented on scheme on the right). Cocultures were as follows: QS+(cm) with QS+ (spec); QSS-(cm) with QSS-(spec) without and with ComX or surfactin (20µg/ml); QS+A- (cm) with QS+A- (spec) without and with surfactin (20µg/ml). Data are presented as a mean of biological triplicates with standard error indicated

Slika 29. **Test izmenjave DNA v različnih kokulturah *B. subtilis*.**. Kokulture 1:1 sestavljene iz dveh sevov, od katerih je vsak nosil eno koristno mutacijo (rezistenco za antibitik; oziroma kroramfenikol in spektinomicin), smo gojili 8 ur in nato selekcionirali na dveh antibiotikih (kot prikazano na sliki na desni). Kokulture so bile naslednje: QS+(cm) s QS+ (spec); QSS-(cm) s QSS-(spec); in ista kokultura dopolnjena z ComX ali surfaktinom (20µg/ml); QS+A- (cm) s QS+A- (spec) in ista kultura dopolnjena s surfaktinom (20µg/ml). Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

We observed dramatically lower number of survivors in QSS^- (more than 20-fold) and QS^+A^- (more than 10-fold) double cocultures as compared to the QS^+ coculture (Figure 29A). We next tested whether ComX and surfactin can serve as public goods and whether they will improve the mutant's survival when added to the cocultures media. Surprisingly adding physiological ComX concentration (Manguson et al., 1998; Oslizlo et al., 2014), but not surfactin, to QSS^- cocultures resulted in even higher survival rate as compared to the wild type suggesting higher frequency of DNA exchange in the QSS^- coculture in the presence of ComX (Figure 2A). Surfactin also fully restored the survival rate in the double residence selection in the QS^+A^- coculture (Figure 29A). This opened a series of questions: do the mutants take

advantage of ComX and surfactin produced by QS^+ in coculture and survive better when there is selection pressure for sex providing the route to new traits?

4.4.2 QSS- in coculture with QS+ acts as a hypersexed (hypercompetent)

First we tested whether in our experimental setup, QSS- mutant can cheat in coculture with the QS+ and improve its survival under the pressure for DNA exchange using ComX produced by the wild type. We tested this in two types of cocultures: QS+ (cm^R) vs QSS- ($spec^R$) and QS+ ($spec^R$) vs QSS- (cm^R) as controls. Strains were inoculated in 1:1 ratio and after 8 hours selected for double resistance ($spec^R$, cm^R) genotype.

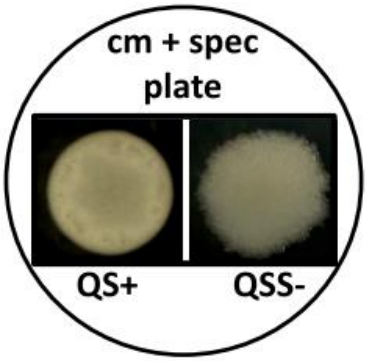


Figure 30. **Morphologies of QS+ and QSS- colonies.** Differences in morphology between QS+ and QSS- colonies were used to identify the ancestors of the transformats which arised in cocultures.

Slika 30. **Morfologija kolonij QS+ in QSS-**. Da bi določili prednike transformant, ki so nastali v kokulturah, smo izkoristili razlike v morfologiji med kolonijama QS+ in QSS-.

We took advantage of different morphology of QS+ and QSS- (Figure 30) to determine the number of the QS+ ,,offsprings" and the QSS- ,,offsprings''carrying both antibiotic resistances. We also evaluated the overall survival rate of all cells in mixed coculture and compared it with a coculture consisting of only two QS+ strains. Additionally we determined the number of QS+ and QSS- cells in mixed cocultures before the selection pressure for sex was applied.

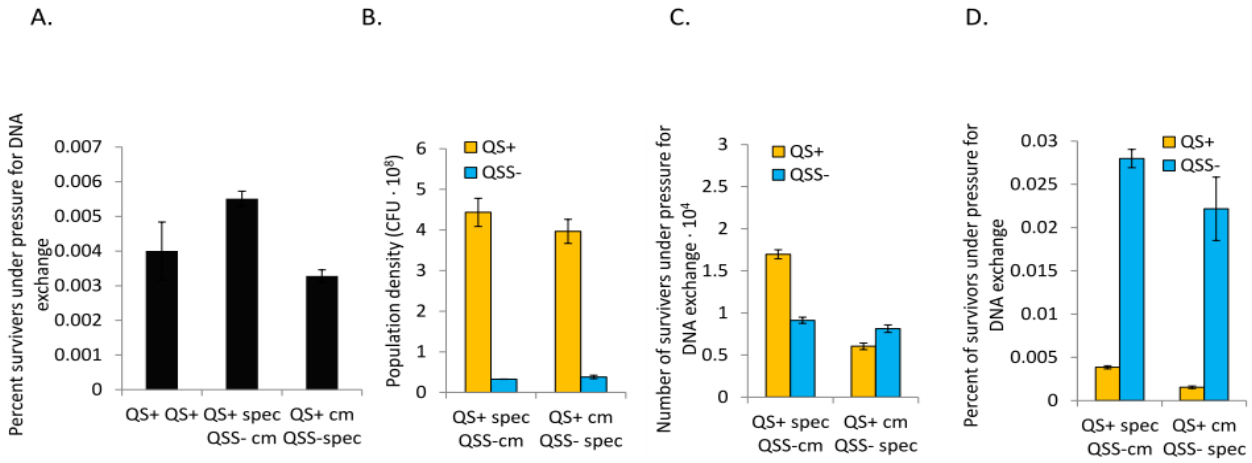


Figure 31. **Cheating under pressure for DNA-exchange in QS+ and QSS- cocultures**. A. Percent of total survivors per population after selection for transformants that exchanged the DNA, was compared between population consisted of two QS+ and between mixed populations of QS+ and QSS-. B. Final density of QS+ and QSS- in cocultures before selection for transformants was assayed. C. Number of survivors of the QS+ and QSS- genotypes in cocultures was determined. D. Frequency of transformation of QS+ and QSS- in cocultures was calculated by dividing the number of survivors that carried both cm and spec antibiotic resistances, by the number of cells before the selection was applied. Data is presented as mean of biological triplicates with standard error indicated.

Slika 31. **Goljufanje v kokulturah QS+ in QSS- pod selekcijskim pritiskom za izmenjavo DNA**. Po selekciji za transformante ki so izmenjali DNA primerljali smo procent vseh preživelih med populacijami sestavljenimi iz dveh QS+ in mešano populacijo sestavljeno is QS+ in QSS-. B. Končna gostota sevov QS+ in QSS- v kokulturah pred selekcijo za transformante. C. Število preživelih QS+ in QSS- v kokulturah smo izračunali preko delitve števila preživelih ki so nosili obe rezistence cm in spec, s celotnim številom celic pred selekcijo. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

First we observed that the presence of QSS- caused no dramatic changes in overall survival rate suggesting that the total number of DNA exchange events was similar in the pure QS+ population and the mixed QS+: QSS- populations ($P<0.08$ and $P<0.22$) (Figure 31A). As expected after 8 hours the ratio of the QSS- was dramatically reduced in both cocultures (Figure 31B). Surprisingly however, after selection for transformants carrying the double resistance the mutant did at least as good as the wild type, suggesting that by applying the pressure for DNA exchange strongly promoted the survival of the QSS- mutant (Figure 31C). In fact comparison of transformation frequency in those cocultures showed more than 5-fold higher transformability of the QSS- (Figure 31D). This led us to conclude that QSS- being more sensitive to ComX and in consequence hypercompetent, can act as hypercheaters surviving better than the wild type under the pressure for DNA-exchange. Hypercheaters as described here will therefore not only take advantage of public goods produced by the wild type, but also they will use public goods more efficiently as compared to the wild type cells. In

this line, *P. aeruginosa* siderophore-null mutants would be ordinary cheaters, because, although they can use pyoverdin produced by the wild type cells, they do not get more iron from pyoverdin-iron complex than the wild types do (according to current knowledge) (Buckling et al., 2007). In contrast, *B. subtilis* signal-null mutants are here considered hypercheaters, because in response to the same amount of QS signal (public good) provided by the wild type they can develop higher genetic competence as compared to the wild type. The hypercheating ability per se gives advantage to the mutants under selective conditions even if metabolic costs of public goods production are very low and do not significantly charge the wild type.

4.4.3. Despite its hypercomeptence QSS- cannot further increase its ratio in coculture with QS+

Can then QSS- invade the wild type QS+ population under the selection pressure for DNA exchange? We prepared a series of cocultures consisting of QS+ and QSS- double transformants in different ratios, to see whether QSS- can increase in numbers in any of those starting setups. We observed no such result for any of the starting ratios applied (Figure 32).

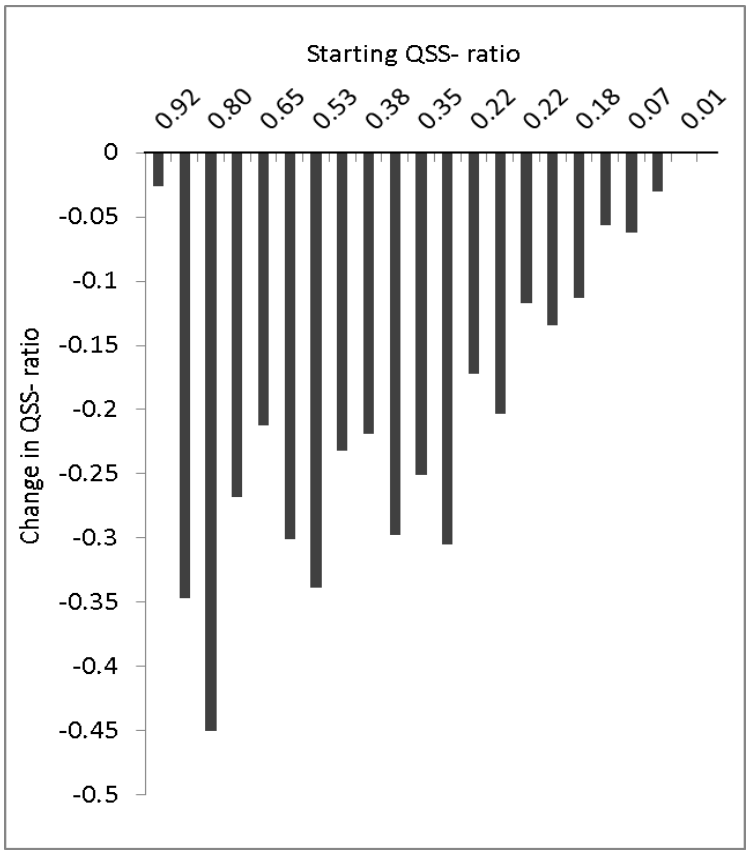


Figure 32. **Change in QSS- ratio in cocultures with QS+.** Transformants QSS- (cm, spec) were mixed with QS+ (cm, spec) in different starting ratio (x-axis) and final proportion of each strain was determined by CFU assay after 8 hours and presented as change in QSS- ratio (y-axis). Data for each biological replicate is shown.

Slika 32. **Sprememba deleža QSS- v kokulturah s QS+.** Pripravili smo mešanico transformant dveh sevov: QSS- (cm, spec) in QS+ (cm, spec); v različnih začetnih razmerjih (os x) in po 8 urah s pomočjo testa CFU smo določili končni delež vsakega od sevov. Rezultati so prikazani kot sprememba deleža QSS- (os y). Graf vsebuje vse biološke ponovitve.

QSS- ratio always decreased and this decrease was the most dramatic when QSS- ratio at the beginning of competition was from 50% to 90% (Figure 32).

4.4.4 Surfactin triggers DNA release in *B. subtilis* PS-216

Recently it was reported that *B. subtilis* 3610 actively secretes high molecular DNA during growth (Zafra et al., 2012). The authors have identified several genes involved in e-DNA

production like the QS signal ComX and its cognate receptor ComP that are known as components of ComQXPA QS system (Manguson et al., 1994; Winrauch et al., 1990).

We tested whether ComQXPA QS system regulates e-DNA secretion and if so, whether ComX regulates e-DNA secretion by specific activation of its cognate receptor ComP. We cultured signal-proficient strain QS+ (PS-216), and several mutants like: QSS- (Δ*comQ*), QSR- (Δ*comP*), QS+A- (Δ*srfA*) and extracted eDNA from filter-sterilized conditioned media of each culture.

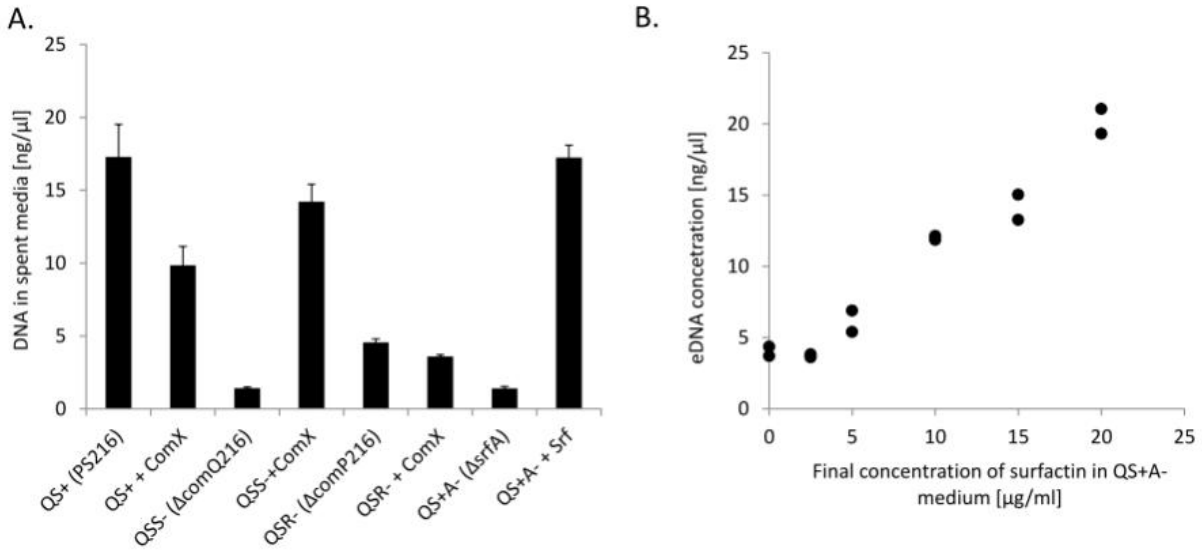


Figure 33**. ComQXPA and surfactin dependent DNA release**. Extracellular DNA was extracted after 8 hours of growth from the QS+ (PS-216), QSS- (PS-216ΔcomQ) and QSR- (PS-216ΔcomP), and from the same cultures supplemented with ComX, and from the QS+A- and the same culture supplemented with surfactin (20µg·ml^{-1}). Data is presented as mean of biological triplicates with standard error indicated. B. Extracellular DNA was extracted after 8 hours from the filter-sterilized conditioned media of QS+A- (PS-216ΔsrfA) culture supplemented with different concentrations of surfactin k (from 0 to 25 µg/ml) Data for each biological replicate is shown on graph.

Slika 33**. Od ComQXPA in surfaktina odvisno izločanje DNA**. Zunajcelična DNA je bila ekstrahirana iz izrabljenih gojišč QS+ (PS-216), QSS- (PS-216ΔcomQ) in QSR- (PS-216ΔcomP) po 8 urah rasti, iz izrabljenih gojišč enakih kultur dopolnjenih z ComX, in z izrabljenega gojišča QS+A- in iz gojišča enake kulture dopolnjene s surfaktinom (20µg·ml^{-1}). Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko. B. Zunajcelična DNA je bila ekstrahirana po 8 urah iz izrabljenega gojišča kulture QS+A- (PS-216ΔsrfA) dopolnjene z različno koncentracijo surfaktina (od 0 do 25µg/ml) Graf vsebuje podatke za vsako biološko ponovitev.

As predicted, we noticed an approx. 2-fold higher e-DNA specific absorbance (260nm) in spent media of QS+ strain than of QSS- strain supplemented with ComX, after 6 hours of

growth (T2), but n spent media of QSS- strain not chalanged with ComX the absorbance was about 10 fold lower as compared to the QS+ (Figure 33A). This effect was not dependent on RNase treatment indicating that measured absorbance was due to the DNA. Adding ComX to QSR- (PS-216ΔcomP) culture did not restore e-DNA secretion indicating that ComP receptor is neccessary for this effect (Figure 33A). We also examined the spent media of QS+A- (PS-216ΔsrfA) mutant which is not able to secrete surfactin and is impaired in e-DNA secretion (Zafra et al., 2012). We confirmed that there was very low e-DNA concentration in its spent medium as compared to the wild type strain. The e-DNA secretion in PS-216ΔsrfA mutant could be restored by supplementing the medium with surfactin (Figure 33A). Moreover the concentration of surfactin added to PS-216ΔsrfA culture linearly correlated with e-DNA detected in the culture conditioned medium after 6 hours (Figure 33B). Our results indicate that ComQXPA QS system regulates e-DNA secretion by induction of surfactin synthesis. It also indicate that surfactin may be crucial for DNA exchange between two *B. subtilis* strains and that next to ComX it may serve as a public good under the pressure for DNA exchange. This however remains to be tested.

4.5 QS RESPONSE IN PLANKTONIC AND COLONY COCULTURES OF QS+ AND QSS-

It is not known how QS functions in colony growth mode which is more common form of bacterial growth as compared to planktonic cultures. Here we compared the QS response in planktonic coculture of QS+ *B. subtilis* BD4720 (*srfA-yfp*) and QSS- *B. subtilis* BD4729 (*srfA-cfp*) that was addressed in section 4.1; with their QS response in colony coculture. It should be pointed out that we used domesticated strains that do not produce surfactin due to the *sfp*-linked mutation but are able to induce the *srfA* operon. First we compared the growth curves of both strains in planktonic growth mode and in colony.

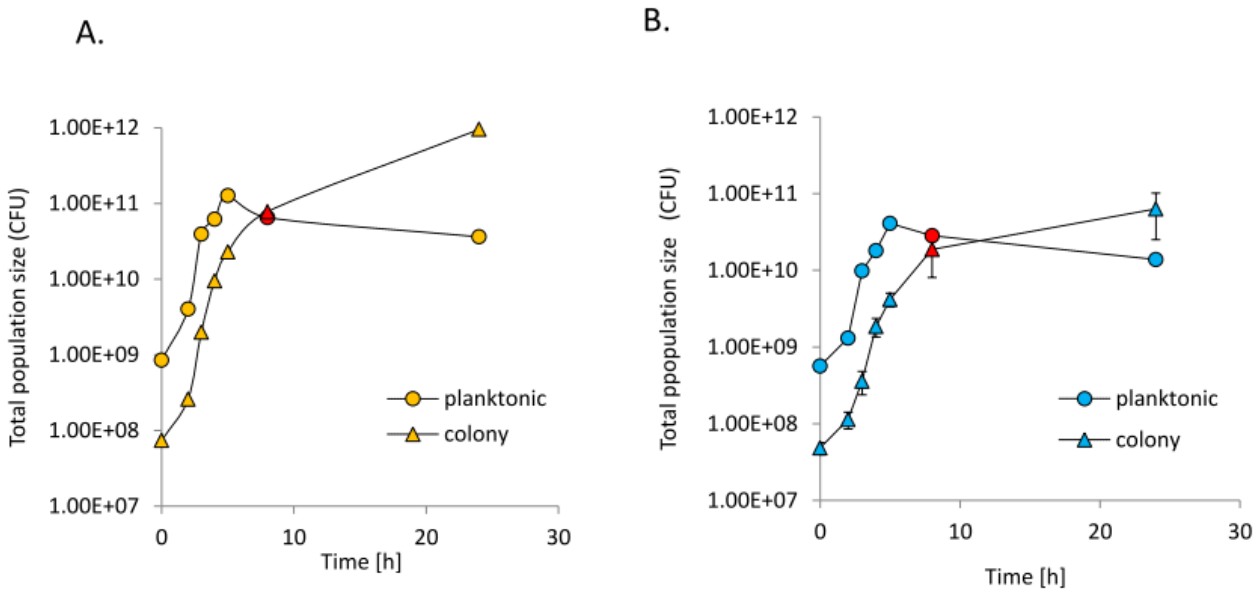


Figure 34. **Changes in QS+ and QSS- population size during growth of planktonic cocultures and colony cocultures**. A. QS+ strain *B. subtilis* BD4720 and B. QSS- strain *B. subtilis* BD4729. Differences between QS responses of the two growth modes were greatest after incubation for 8 h (marked in red). Cell counts were performed with CFU assay and the mean values and standard errors of triplicates are presented.

Slika 34. **Spremembe v velikosti populacij QS+ in QSS- v kokulturah tekom rasti v tekočem gojišču in v koloniji.** A. Sev QS+ *B. subtilis* BD4720 in B. sev QSS- *B. subtilis* BD4729. Razlika med QS odzivom v dveh pogojih rasti je bila največja po 8 urah inkubaciji (označeno z rdečo). Celice smo šteli s pomočjo testa CFU. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

The viable cell concentration of the two strains was similar in both the planktonic and colony modes (Figure 34) and the number of planktonic cells present in 10 cm^3 liquid was comparable with the numbers in colony cells on 0.2 cm^2 of solid medium. We next looked at the fluorescence in both growth modes.

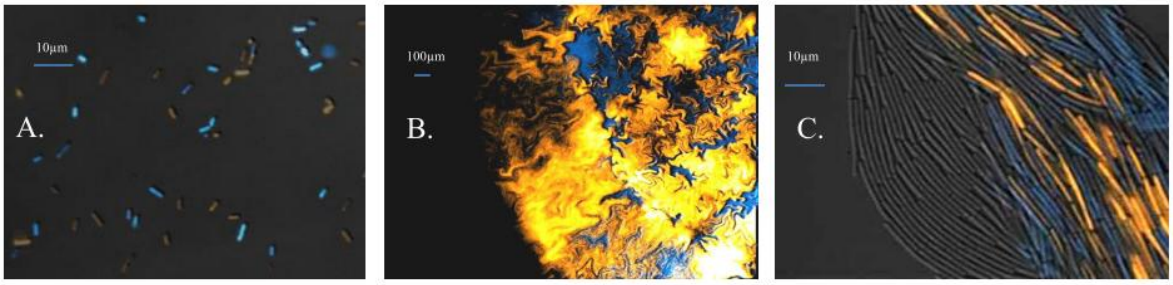


Figure 35. **Fluorescent microscopy micrographs of QS+ and QSS- planktonic and colony cocultures**. Pictures were taken after 8 hours of growth. QS+ (*B. subtilis* BD4720 *srfA-yfp*) (yellow) and QSS- mutant (*B. subtilis* BD4729; *srfA-cfp*) (blue). A. Planktonic coculture shown in cfp/yfp/DIC overlay. B. Micrograph shows an edge of the colony in cfp/yfp overlay.C. The colony arm with cfp/yfp/DIC overlay.

Slika 35. **Slika iz fluorescenčne mikroskopije prikazuje kukulture QS+ in QSS- v tokočem gojišču in v koloniji.** Slike so bile posnete po 8 urah rasti. QS+ (*B. subtilis* BD4720 *srfA-yfp*) (rumena) in QSS- mutant (*B. subtilis* BD4729; *srfA-cfp*) (modra). A. Tekoča kultura prikazana s prekrivanjem cfp/yfp/DIC. B. Slika roba kolonije prikazana z prekrivanjem cfp/yfp. C. Rame kolonije prikazano z prekrivanjem cfp/yfp/DIC.

Qualitative fluorescence microscopy indicated that the majority of planktonic cells in cocultures expressed the *srfA* gene after incubation for 8 h, and non-induced cells were rarely detected (Figure 35A). In contrast, the distribution of *srfA-yfp*-induced QS wild type cells and *srfA-cfp* induced QS mutant cells was patchy within colonies where cells had experienced physical contact during growth (Figure 35 B, C).

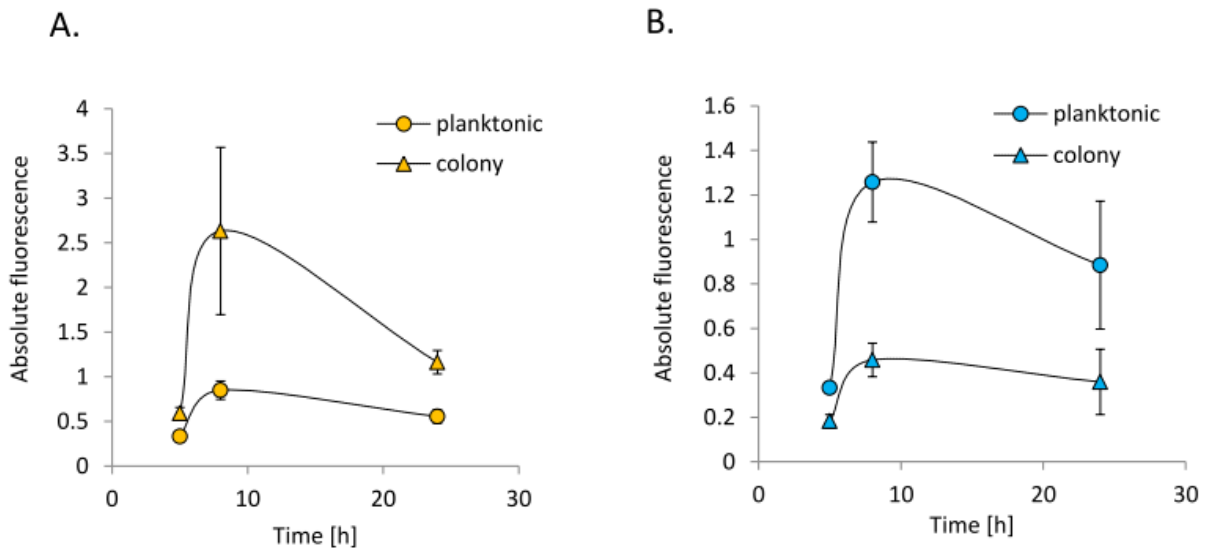


Figure 36. **A comparison of the QS response of QS+ and QSS- in the colony and planktonic coculture.** A QS+ strain *B. subtilis* BD4720. B. QSS- strain *B. subtilis* BD4729. Fluorescence units were expressed as weighted average. Data is presented as mean of biological triplicates with standard error indicated.

Slika 36. **Primerjava QS odziva QS+ in QSS- v koloniji in v tekoči kulturi.** A. Sev QS+ *B. subtilis* BD4720. B. Sev QSS- *B. subtilis* BD4729. Fluorescenčne enote predstavljajo tehtano povprečje. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

When we quantified the QS response of the QS+ in both growth modes it occurred that it was up to 3-fold greater in colony cells than in planktonic cells (Figure 36A). In contrast however, the absolute fluorescence of QSS- signal deficient mutants was significantly lower in colony populations (Figure 36B).

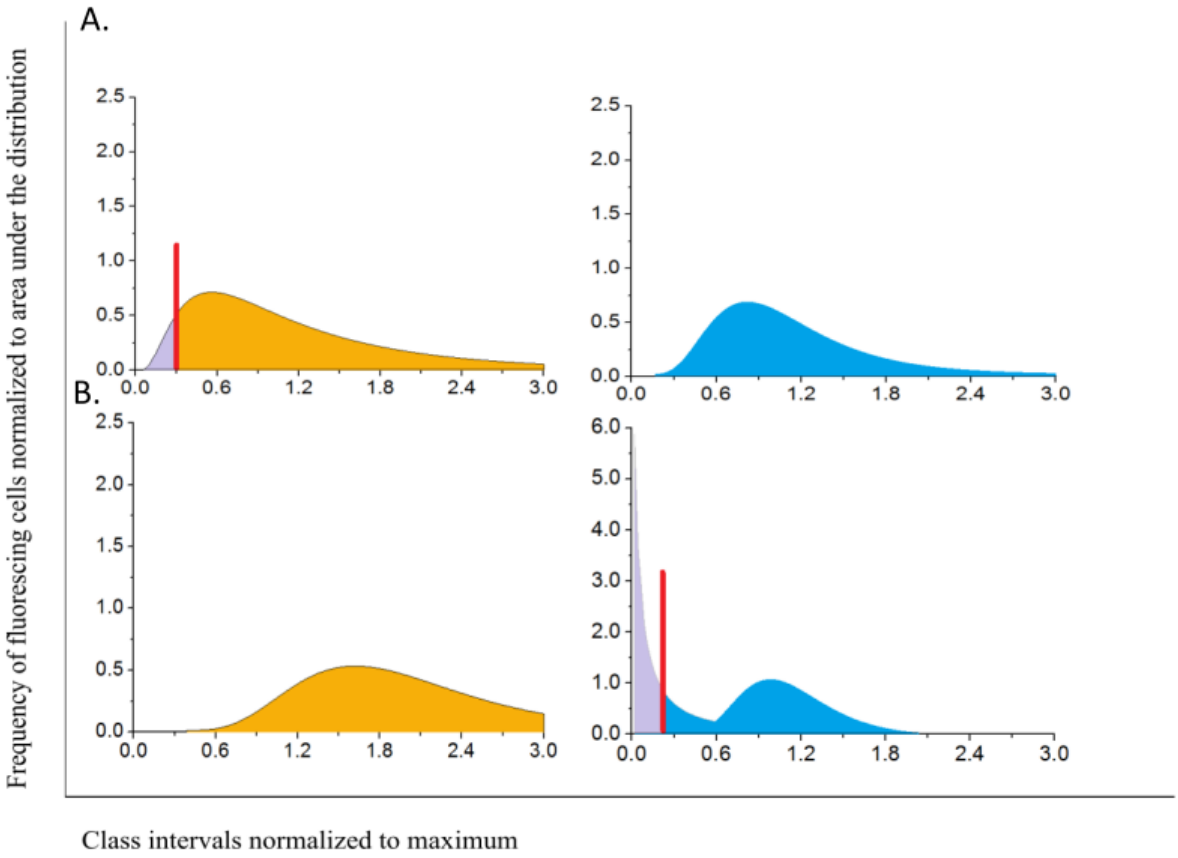


Figure 37. **A comparison of the distribution of *srfA* expression in QS+ and QSS- population grown in colony and planktonic coculture** Distribution of *srfA* expression (QS response) was presented for the QS+ *B. subtilis* BD4720 (yellow area) and the QSS- *B. subtilis* BD4729 (blue area) population in (A) planktonic coculture and in (B) colony coculture. The number of fluorescing cells (y–axes) and their fluorescence intensity (x-axes) was measured at a single cell level at the 8th hour of growth. Uninduced population (on the left) is shown in purple.

Slika 37. **Primerjava distribucije izražanja *srfA* v populaciji QS+ in QSS- v tekoči kokulturi ali v koloniji.** Distribucija izražanja *srfA* (QS odziv) prikazana za QS+ *B. subtilis* BD4720 (rumeno območje) in za QSS- *B. subtilis* BD4729 (modro območje) gojenih v (A) tekoči kokulturi in (B) v koloniji. Število fluorescenčnih celic (os y) in intenziteta njihove fluorescence (os x) sta bila izmerjena s pomočjo fluorescenčne mikroskopije na nivoju posamezne celice po 8 urah rasti. Neinducirana populacija (na levi) je označena z vijolično.

Further quantitative analysis demonstrated log-normal distribution of fluorescence in planktonic cells of both strains during coculture after induction of the QS response. . Distribution of *srfA* expression in planktonic cells of both the QS wild types and the signal deficient cells were unimodal (Figure 37A), as expected from qualitative analysis. The

variance of the distribution changed with time and was the greatest after incubation for 8h for both strains, due to the emergence of highly fluorescent cells strongly expressing *srfA-yfp* or *srfA-cfp* (Figure 37A). In contrast, distributions of *srfA* expression in colony cells differed significantly between the QS+ which were unimodal, and the QSS- population which developed two distinct subpopulations after incubation for 6 h. Both populations harbored fluorescent cells, but cells with non-induced *srfA*-fluorescence were also found in the signal deficient population (Figure 37B). This indicated bimodal expression of *srfA* among signal deficient mutants leading to a different distribution of the QS response within the population and, obviously, to a lower coherence of response in the signal deficient strain colonies. Growth mode therefore influences the behavior of the QS mutants and its decreased cumulative QS response in the colony was due to a subpopulation of uninduced cells.

4.6 INFLUENCE OF EXTERNAL FACTORS ON QUORUM SENSING

4.6.1 Influence of temperature on QS signaling

The influence of physic-chemical parameters, like temperature, on QS in not well understood. It is known that expression of *srfA* is inhibited at high temperature (Abushady et al., 2005) however it is not known whether this is due to the lack of QS signal or other repressive effects. Here we examine the influence of temperature on production and activity of ComX signal. In order to do this, we cultured signal-producer BD2833 and signal-deficient strain BD2876 at 24°C, 37°C and 51°C temperature, followed the growth curves (Figure 38) and collected the conditioned media one hour after the cultures reached stationary phase (T1).

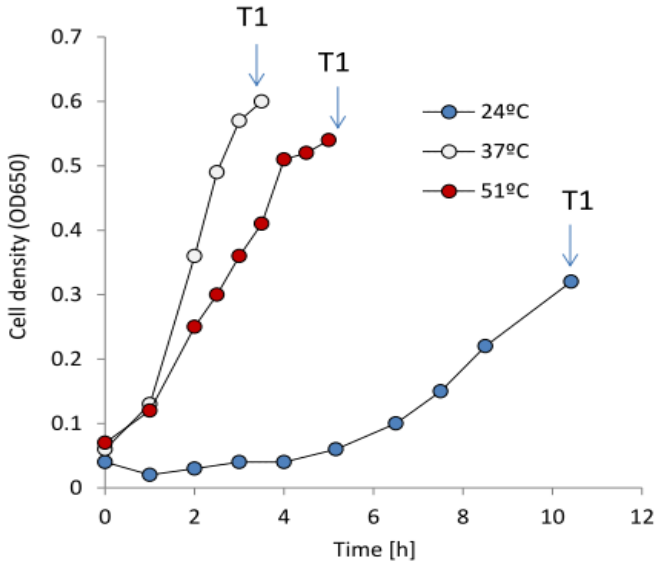


Figure 38. **Growth of *B. subtilis* BD2833 at different temperatures**. The growth was monitored spectrophotometrically at 650 nm at 24°C, 37°C and 51°C and the conditioned medium was taken one hour after reaching stationary phase (T1).

Slika 38. **Rast *B. subtilis* BD2833 pri različnih temperaturah**. Rast smo spremljali spektrofluorimetrično pri 650 nm pri 24 °C, 37 °C in 51 °C. Izrabljeno gojišče smo vzorčili eno uro po prehodu v stacionarno fazo (T1).

Conditioned media of the signal-deficient strain BD2876 grown at 24°C, 37°C and 51°C were used as the control background where equal concentrations of pure ComX signal (obtained from *E. coli* ED367) were added. We next incubated the BD2876 (*srfA-lacZ*) tester strain in the control conditioned media supplemented with ComX or in the conditioned media of the producer, and assayed its response. In addition, a set of control conditioned media (spent media) from a signal-deficient mutant BD2876 grown at different temperatures was supplemented with equal amount of ComX and frozen at -20°C. The next day the samples were thawed and used to grow the tester strain and monitor its *srfA-lacZ* expression in order to examine the influence of BD2876 background on the ComX stability.

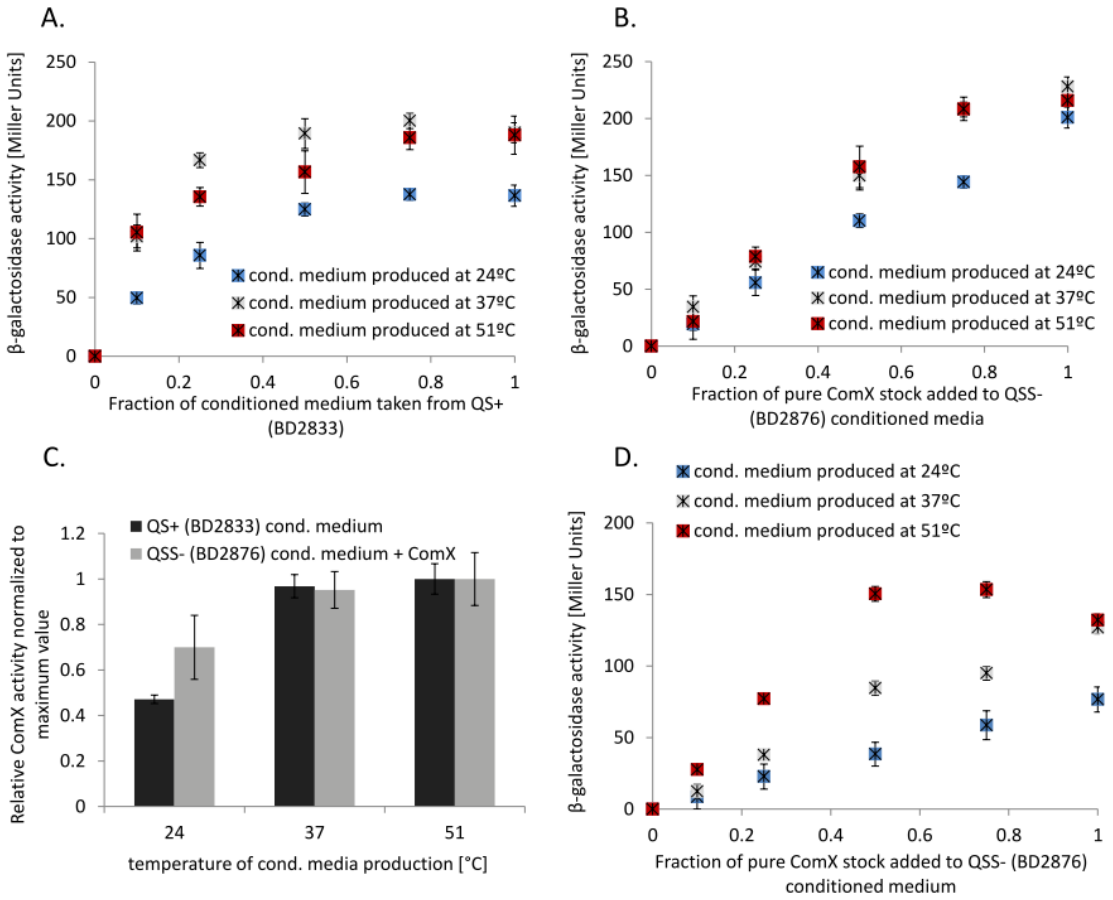


Figure 39. **Influence of growth temperature on ComX activity in conditioned medium of *B. subtilis***. The QS response of the tester strain (*B. subtilis* BD2876, *srfA-lacZ*) was monitored by β-galactosidase activity. A. Conditioned media prepared from QS+ strain (BD2833) grown in 24°C, 37°C and 51°C was mixed in ratio 1:1 with the fresh CM medium. The tester strain was then incubated in this medium until late stationary phase and tested for β-galactosidase activity. B. Conditioned media prepared from the QSS- strain (BD2876) grown in 24°C, 37°C and 51°C and supplemented with equal concentrations of pure ComX (obtained from *E. coli*) was mixed in ratio 1:1 with the fresh CM medium. The tester strain was then incubated in this medium until late stationary phase and tested for as β-galactosidase activity. C. Comparison of relative β-galactosidase activity induced by the BD2833 conditioned media and control BD2876 conditioned media prepared from strains grown at different temperature and supplemented with ComX. D. Conditioned media was prepared from the QSS- strain (BD2876) grown in 24°C, 37°C and 51°C, supplemented with equal concentrations of pure ComX and frozen at -20°C. Next day it was thawed and mixed in ratio 1:1 with the fresh CM medium. The tester strain was then incubated in this medium until late stationary phase and its response was determined as β-galactosidase activity. Data is presented as mean of biological triplicates with standard error indicated.

Slika 39. **Vpliv temperature rasti na aktivnost ComX in na izrabljeno gojišče *B. subtilis***. QS odziv testerskega seva (*B. subtilis* BD2876, *srfA-lacZ*) smo spremljali preko testa aktivnosti β-galaktozidaze: A. Pripravili smo 1:1 mešanico izrabljenega gojišča ki je bilo pridobljeno iz standardno napakova QS+ (BD2833) gojenega v 24°C, 37°C in 51°C in svežega gojišča CM. Testerski sev je bil nato inkubiran do stacjonarne faze in testiran na aktivnost β-galaktozidaze. B. Pripravili smo mešanico izrabljenega gojišča ki je bilo pridobljeno iz standardno napakova QSS- (BD2876) gojenega v 24°C, 37°C in 51°C in nato dopolnjeno z enakimi

koncentracijami očiščenega ComX (pridobljen iz *E.coli* in svežega gojišča CM. Testerski sev je bil nato inkubiran do stacionarne faze in testiran na aktivnost β-galaktozidaze. C. Primerjava relativne aktivnosti β-galaktozidaze, ki jo je induciralo izrabljeno gojišče BD2833 in kontrola BD2876 dopolnjena z ComX. D. Izbrabljeno gojišče pridobljeno iz QSS- (BD2876) gojenega v 24°C, 37°C in 51°C, je bilo dopolnjeno z enakimi koncentracijami očiščenega ComX in nato zmrznjeno na -20°C. Nasledni dan smo vzorce odtajili in zmešali v razmerju 1:1 s svežim gojiščem CM. Testerski sev je bil nato inkubiran do stacionarne faze in testiran na aktivnost β-galaktozidaze. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

Although conditioned media of the producer strain grown at 3 different temperatures differed in ComX activity (Figure 39A), similar differences in srfA-lacZ expression were observed in control samples supplemented with ComX (Figure 39B) suggesting that temperature dependent differences in activity are due to different, temperature-depended backgrounds and not due to lack of ComX. We observed about 30% ($P<0.00005$) lower activity of ComX in 23°C background as compared to 37°C (Figure 39B,C) and 51°C and almost 50% lower activity of 24°C-derived conditioned medium of the producer as compared to 37°C and 51°C (Figure 39A,C).
Interestingly, the effect of the background on ComX activity after freezing (-20°C) and thawing back, was significant. Again, the lowest activity of the QS signal was observed for 24°C sample, 2-fold higher ($P<1\cdot10^{-6}$) activity was observed for 37°C but almost 4-fold higher activity for 51°C derived background as compared to what??? Suggesting that conditioned medium produced at high temperature contains components that stabilize ComX activity (Figure 39D).

4.6.2 Influence of salinity on QS

Although *B. subtilis* is known for its resistance against salt stress it is not known how high salinity influences cell-cell signaling in this bacterium. We examined this by using ComQXPA QS system. *B. subtilis* strain BD2833 (*srfA-lacZ*) was grown in CM medium supplemented with 4% (w/v), 8% (w/v) NaCl and in the control medium (not supplemented with NaCl) and QS signal production and QS response were assayed.

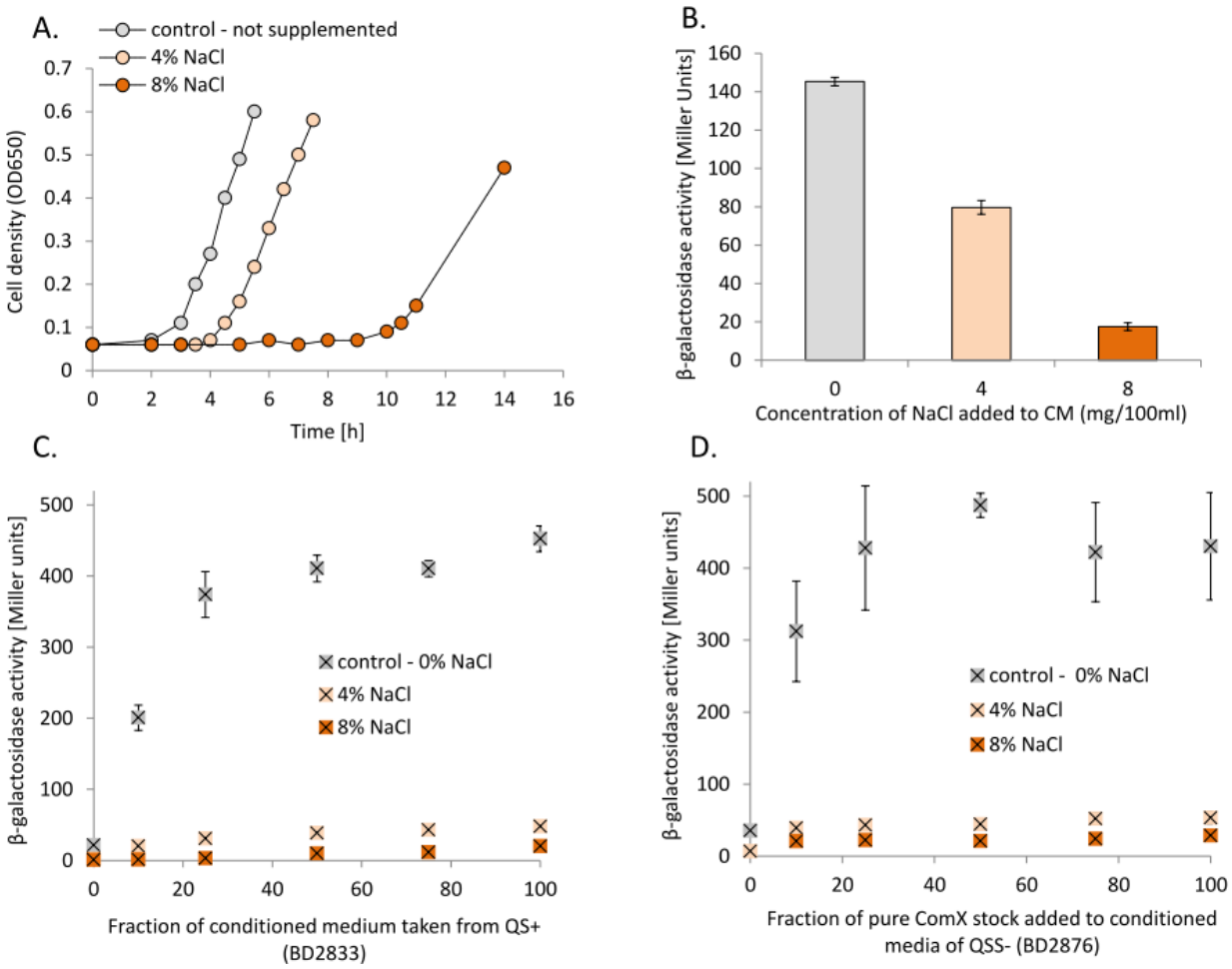


Figure 40. **Influence of salt concentration on growth and QS functions of *B. subtilis*.** .A. Growth of *B. subtilis* BD2833 was monitored spectrophotometrically at 650 nm in control CM medium (not supplemented with NaCl) and in media supplemented with 4% (w/v) and 8% (w/v) NaCl. The conditioned medium was taken one hour after the cultures reached stationary phase (T1). B. Expression of *srfA-lacZ* of BD2833 strain was monitored by β-galactosidase activity in control CM medium and in media supplemented with 4% and 8% concentration of NaCl. C. The QS response of the tester strain (*B. subtilis* BD2876, *srfA-lacZ*) to conditioned media of the BD2833 strain grown in different NaCl concentrations: control medium and media supplemented with 4% NaCl and 8% NaCl, was tested by β-galactosidase activity. D. Purified ComX was added in equal concentrations to conditioned media produced by QSS- BD2876 in control medium and media supplemented with 4% NaCl and 8% NaCl. Data is presented as mean of biological triplicates with standard error indicated.

Slika 40. **Vpliv koncentracije soli na rast in funkcije QS pri *B. subtilis*.** A. Rast *B. subtilis* BD2833 smo spremljali spektrofluorometrično pri 650 nm v kontrolnem gojišču CM in v CM gojišču z 4% (w/v) in 8% (w/v) NaCl. Izrabljeno gojišče smo odvzeli eno uro po prehodu v stacionarno fazo (T1). B. Izražanje *srfA-lacZ* pri BD2833 gojenim v gojišču CM in v CM z 4% in 8% NaCl smo spremljali s testom aktivnosti β- galatozidaze.C. QS odziv testerskega seva (*B. subtilis* BD2876, *srfA-lacZ*) na izrabljeno gojišče seva BD2833, ki smo ga gojili pri različnih koncentracijah NaCl. D. Očiščen ComX smo dodali v enakih koncentracijah v izrabljeno gojišče seva QSS- BD2876 pridobljeno iz kulture vzgojene v kontrolnem gojišču, gojišču z 4% NaCl in 8% NaCl. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

We observed that with increased salinity of the medium the lag phase also increased, however the growth rates in different salt concentrations were very similar (Figure 40A). We collected the cells in T1 and measured the *srfA-lacZ* expression. We discovered a negative influence of increasing concentration of NaCl on expression of QS-regulated gene *srfA*. The *srfA-lacZ* expression was almost 2-fold lower in 4% NaCl ($P<1\cdot10^{-6}$) and over 8-fold lower in 8% NaCl ($P<1\cdot10^{-6}$) as compared to the control (Figure 40B). We further examined whether these effects are due to different concentrations of ComX in the medium or rather due to ComX-independent regulation of *srfA* gene expression at different salinity.

We sampled conditioned media of ComX-producing strain (BD2833) grown in different salinities and also the conditioned media of the signal-deficient strain (BD2876) were used as control background similarly as in previous experiments (see section 4.5.1). Next the conditioned media were added to the tester strain BD2876 (*srfA-lacZ*) and its QS response to those media was assay by β-galactosidase. We discovered similar differences in ComX activity in conditioned media derived from the ComX producer as in control samples suggesting that observed differences in activity are due to different, salinity-depended backgrounds rather than due to different concentrations of ComX signal (Figure 40 C, D).

We further examined whether the same effects will be observed in different growth modes for example in floating biofilm. We sampled the cells after 48 hours of growth on Petri dishes without shaking.

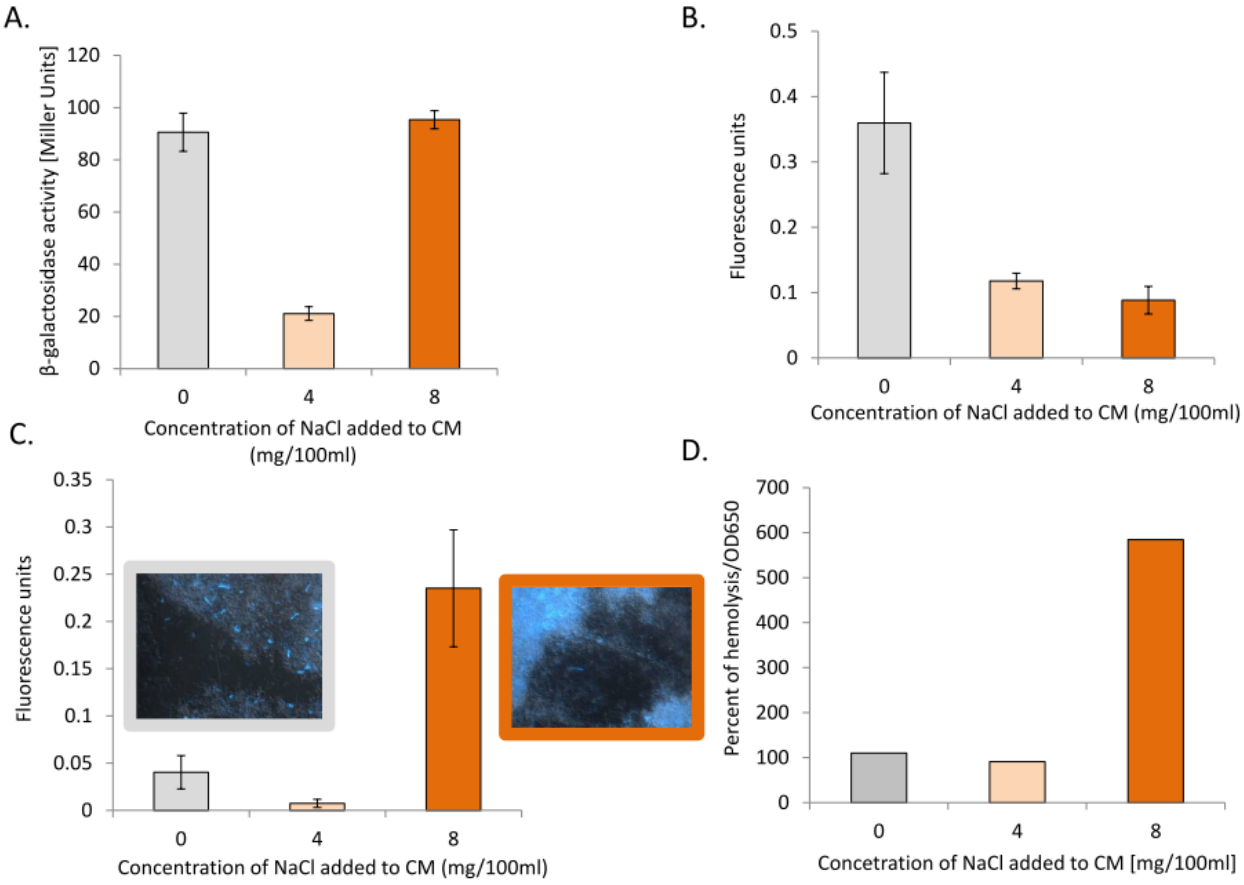


Figure 41. **Comparison of NaCl effects *srfA* expression in planktonic culture and biofilms of *B. subtilis***. A. Expression of *srfA-lacZ* of BD2833 strain was measured after 48 hours of growth in pellicle biofilm and it was monitored by β-galactosidase activity in control CM medium (not supplemented with NaCl) and in media supplemented with 4% and 8% concentration of NaCl. B. Expression of *srfA-cfp* of PS-216 strain grown in control CM medium and in media supplemented with 4% and 8% concentration of NaCl was measured in T1. C. Expression of *srfA-cfp* of PS216 was measured after 48 hours of growth in pellicle biofilm in control CM medium and in media supplemented with 4% and 8% concentration of NaCl. Corresponding fluorescence microscopy micrographs of biofilms grown in control medium and in 8% NaCl are presented. Data is presented as mean of biological triplicates with standard error indicated. D. Hemolytic activity of biosurfactants in conditioned media produced by PS216 pellicles grown in control medium, 4% and 8% NaCl.

Slika 41. **Primerjava učinkov NaCl na izražanje *srfA* v tekočih kulturah in v biofilmih *B. subtilis***. A. Izražanje *srfA-lacZ* pri BD2833 je bilo izmerjeno po 48 urah rasti v biofilmu v kontrolnem gojišču CM (ne dopolnjeno z NaCl) in v gojišču z 4% in 8% NaCl s pomočjo testa aktivnosti β-galaktozidaze. B. Izražanje *srfA-cfp* pri PS-216 gojenim v kontrolnem gojišču CM in v gojišču z 4% in 8% NaCl je bilo izmerjeno v času T1. C. Izražanje *srfA-cfp* pri PS216 je bilo izmerjeno po 48 urah rasti v biofilmu v kontrolnem gojišču CM in v gojišču z 4% in 8% NaCl. Prikazani sta tudi ustrezni sliki fluorescenčne mikroskopije za biofilm ki je nastal v kontrolnem CM in v 8% NaCl. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko. D. Hemolitična aktivnost biosurfaktantov v izrabljenih gojiščih biofilmov PS216 nastalih v kontrolnem CM in v gojišču z 4% in 8% NaCl.

Surprisingly the result was unexpected and the expression of *srfA* in 8% NaCl was as high as in the control CM medium (Figure 41A). This indicated that in high salt concentration *srfA* gene undergoes different regulation in dispersed culture and in biofilm. We further examined whether the same medium-dependent regulation effects can be observed for the undomesticated *B. subtilis* PS-216 (*srfA-cfp*).

Here again, in planktonic culture the expression of *srfA-cfp* decreased with increasing salt concentration and it was more than 3-fold lower ($P<0.03$) in 4% NaCl and more than 4-fold lower ($P<0.03$) in 8% NaCl, as compared to the control (Figure 41B). Surprisingly in biofilm very strong *srfA* induction was observed in CM with 8% NaCl and the expression of *srfA-cfp* was 5 times higher ($P<0.055$) as compared to biofilms grown in the control CM medium (Figure 41C). It was also observed that there were less *srfA-cfp* expressing cells in the control biofilm, as compared to the one grown at high salt (Figure 41B). Using hemolytic assay we conformed that there was also more surfactin in the CM with 8% NaCl used for PS-216 biofilm growth as compared to the control (Figure 41D). This opened an interesting question about the role of surfactin in pellicle biofilms grown in the presence of high salt.

The first possible explanation could be that increased surfactin production in 8% NaCl is beneficial for *B. subtilis* and it increases its fitness. In order to test this hypothesis we compared optical density (after resuspention of cells) and dry biomass of biofilms of the PS-216 (QS+) and the PS-216ΔcomQ (QSS-) which does not produce ComX necessary for surfactin expression and 216ΔcomQ supplemented with ComX signal, that restores surfactin production.

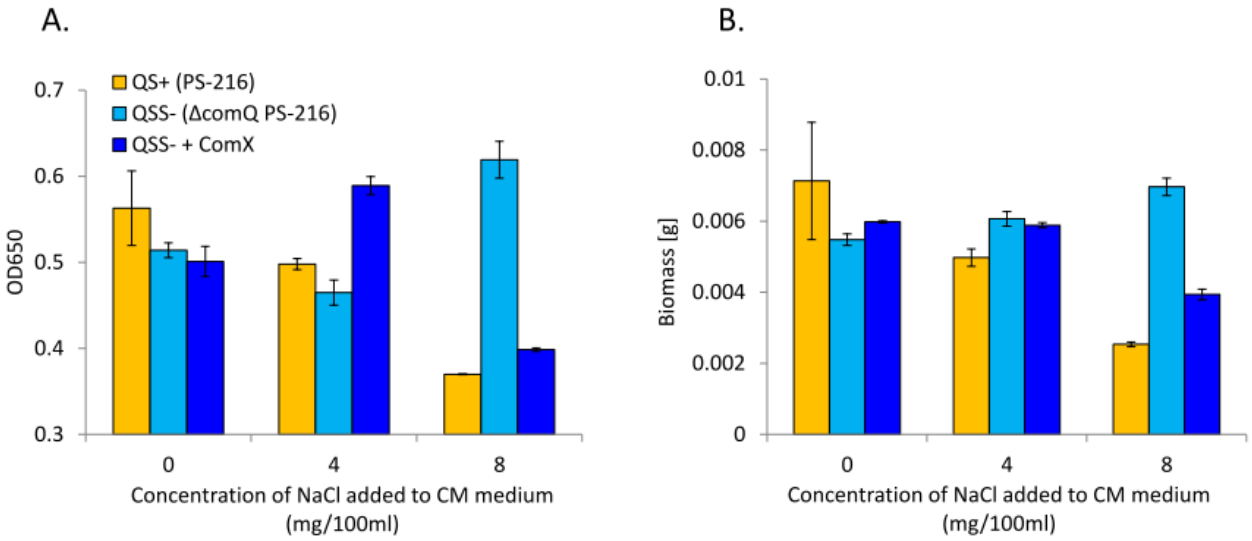


Figure 42. **Cell density and biomass of QS+ and QSS- biofilms created in different salt concentrations.** Optical densities measured after homogenization (A) and dry biomass (B) of biofilms formed by the PS216 (QS+), ΔComQ216 (QSS-) and ΔComQ216 supplemented with ComX. Biofilms were grown for 48h in control CM medium (not supplemented with NaCl) and in CM supplemented with 4 and 8% NaCl. Data is presented as mean of biological triplicates with standard error indicated.

Slika 42. **Celična gostota in biomasa biofilmov QS+ in QSS- nastalih v različnh koncentracijah soli**. Optična gostota je bila izmerjena po homogenizaciji (A) in suha biomasa (B) biofilmov nastalih iz PS216 (QS+), ΔComQ216 (QSS-) in ΔComQ216 dopolnjenem z ComX. Biofilmi smo gojili 48 ur v kontrolnem gojišču CM (ne dopolnjeno z NaCl) in v gojišču dopolnjenem s 4% in 8% NaCl. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

First, we discovered that in the case of QS+ biofilm and QSS- supplemented with ComX, OD650 and dry biomass was decreasing with increasing salinity (Figure 42). In contrast QSS- mutant did not show this negative fitness effect and its dry biomass was the same in all concentrations of NaCl tested and the OD650 even increased at 8% NaCl (Figure 42). This suggested that ComX-dependent production of surfactin may negatively influence the fitness of *B. subtilis* at high salinity. However analysis of QSS-A- (Δ*comQ*Δ*srfA*) fitness grown in 8% NaCl in presence and absence of ComX is needed to confirm this hypothesis. Other fitness traits should be examined in the future (for example long term survival) to explain why increased surfactin production takes place in 8% NaCl despite negative effects on fitness observed after 48 hours.

4.6.3 Influence of surfactin on ComX sensing

It is known that in undomesticated *B. subtilis* ComX induces production of surfactin. It was proposed that in biofilm surfactin acts as a paracrine signal and induces production of extracellular matrix (EPS) in a subpopulation of cells (Lopez et al., 2009a). It was also shown that EPS producers and surfactin producers represent different subpopulations of cells and proposed that EPS producers lose their sensitivity to ComX and stop making surfactin due to physical barrier of the EPS (Lopez et al., 2009a). However, it was never tested how the presence of surfactin itself influences ComX sensing. Here we used domesticated tester strain (BD2876) which does not secrete surfactin nor ComX and we incubated it with equal concentration of pure ComX (obtained from *E. coli* ED367) and different concentrations of surfactin (Sigma). BSA was used as control for unspecific effects on ComX sensing.

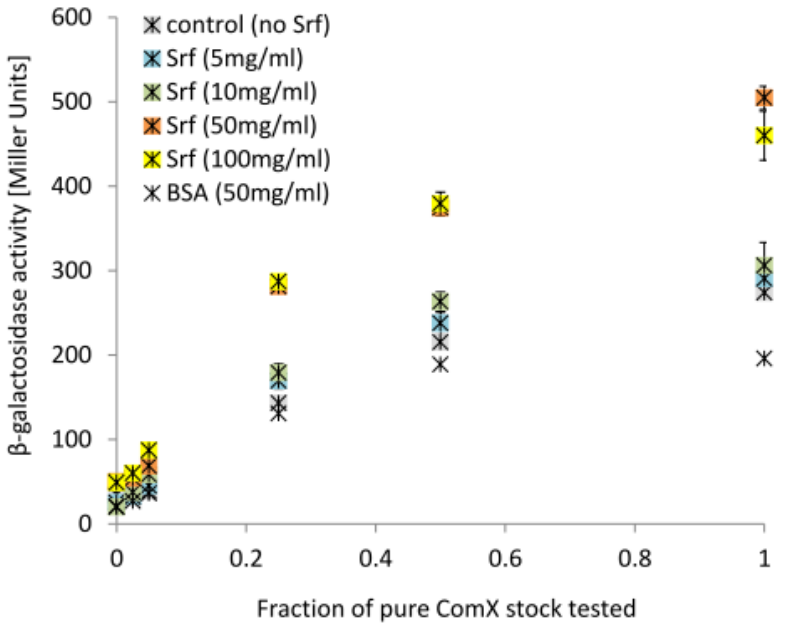


Figure 43. **Influence of surfactin on ComX sensing by QSS- tester strain**. The QS response of the tester strain BD2876 (*srfA-lacZ*) was monitored by β-galactosidase activity. Tester strain was supplemented with different fractions of ComX (purified from *E. coli*) and additionally with different concentrations of surfactin (commercial sample) or with BSA (50mg·ml^{-1}) as the control. Data is presented as mean of biological triplicates with standard error indicated.

Slika 43. **Vpliv surfaktina na zaznavanje ComX pri testerskim sevu QSS-.** QS odziv testerskega seva BD2876 (*srfA-lacZ*) smo spremljali preko testa aktivnosti β-galaktozidaze. Tester je bil dopolnjen z različnimi frakcijami ComX (očiščenim iz *E. coli*) in dodatno z različnimi koncentracijami surfaktina (komercialni surfaktin) in z BSA (50mg·ml^{-1}) kot s kontrolo. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

We discovered that the addition of surfactin in concentration 50mg/ml significantly increased sensitivity of the tester strain to ComX signal (Figure 43). The response of the tester was nearly 2-fold stronger ($P<1\cdot10^{-6}$**)** in the presence of surfactin (calculated from the difference obtained for linear area of activity/fraction function). Further increase of surfactin concentration did not change the response of the tester to ComX (Figure 43). This result indicates that surfactin can positively influence sensing of ComX in *B. subtilis* which is in contrast to current knowledge on surfactin acting as negative paracrine signal downregulating its own expression (Lopez at al., 2009a).

4.7 DIVERSITY OF QS LANGUAGES, PLANT GROWTH PROMOTING AND BIOCONTROL PROPERTIES OF *Bacillus* spp. ISOLATES FROM TOMATO RIZOPLANE

Most work on *B. subtilis* ecology has been performed by studying spores isolated from soil (Mandic-Mulec and Prosser, 2012). We used a set of *B. subtilis* isolates and close relatives isolated from tomato rhizoplane, to address the genetic and functional diversity of spore-formers. The strains were isolated by group of Prof. Maja Rupnik at National Laboratory for Health, Environment and Food (Maribor, Slovenia) from rhizoplane of tomato plants from two home vegetable gardens located in North-west Slovenia. Altogether 20 isolates phenotypically resembling colonies of *B. subtilis* were obtained from 21 different tomato plants (assigned as T isolates, see chapter 3.1.6). Sequencing of 16S rRNA confirmed that they all belong to the *Bacillus* genus. Next, strains were further identified on the basis of the *gyrA* gene as *B. subtilis* (13 strains), *B. licheniformis* (4), *B. amyloliquefaciens* (1), *B. pumilus* (1) and *B. megaterium* (1) (chapter 3.1.6). Next *comQ* sequences were amplified as described by Stefanic & Mandic-Mulec (2009). Sequence analysis showed that *comQ* genes were highly polymorphic and fall into three clusters: 168, RS-D-2/NAF4 and RO-H-1/RO-B-2; with only 65-70% identity at the nucleotide level between clusters. This confirmed previous results obtained for soil isolates (Ansaldi et al., 2002; Stefanic & Mandic-Mulec, 2009)

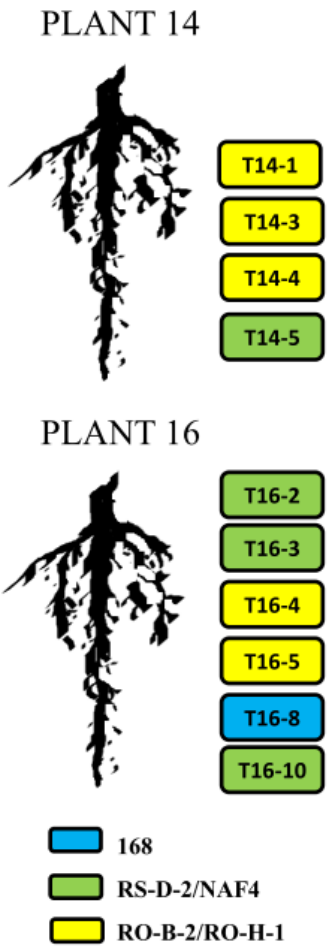
4.7.1 Pherotype specificity of the *comQXP* QS loci

All *Bacillus* spp. isolates obtained from the tomato rhizoplane were tested for their activation of QS response of 6 tester strains representing different pherotypes (languages). Tester strains were marked with *srfA-lacZ* fusion and their QS response to conditioned media of the isolates could be monitored with β-galactosidase test as *srfA* expression is activated in response to ComX. Specific producer strains were used as positive controls.

Table 1. **Specific activation of the QS response by conditioned media of *Bacillus* spp.** The specificity of conditioned media was measured using tester strains able to detect one of the four previously determined pherotypes through activation of the *srfA-lacZ* (first pherotype: 168, second pherotype: RS-D-2 and NAF4, third pherotype: RO-B-2 and RO-H-1, and fourth pherotype: RO-E-2). Strains were grown in CM (competence medium) and the conditioned media were sampled in T1. Tester strains were then inoculated (1:50) into conditioned medium mixed with an equal volume of fresh CM medium, grown for 16 hours and assayed for β-galactosidase activity. Symbols: ++, strong response, similar to positive control; +, moderate response, approximately 50% of the positive-control response; +/−, weak but reproducible response; −, no activation.

Preglednica 1. **Specifična aktivacija QS odziva preko izrabljenih gojišč *Bacillus* spp.**
Specifičnost izrabljenih gojišč smo določili s pomočjo testerskih sevov, ki lahko zaznavajo eden od štirih ferotipov preko aktivacije *srfA-lacZ* (prvi ferotip: 168, drugi ferotpi: RS-D-2 in NAF4, tretji ferotip: RO-B-2 in RO-H-1, in šetrti ferotip: RO-E-2). Seve smo gojili v gojišču CM in v času T1 smo vzorčili izrabljeno gojišče. Testerske seve smo nacepili (1:50) v 1:1 mešanico izrabljenega gojišča s svežim gojiščem CM, gojili 16 ur in nato testirali aktivnost β-galaktozidaze. Simboli: ++, močen odziv, podoben pozitivni kontroli; +, srednji odziv, približno 50% odziva pozitivne kontrole; +/−, slab odziv; −, ni aktivacije.

Pherotype group and isolate from which conditioned media was obtained	**B-galactosidase activity of tester strain**					
168	**168**	**RS-D-2**	**NAF 4**	**RO-B-2**	**RO-H-1**	**RO-E-2**
B. subtilis T16-8	++	-	-	+/-	+/-	+/-
B. subtilis T21-2	++	+/-	+/-	+/-	+/-	+
RS-D-2/NAF4						
B. subtilis T12-1	-	++	++	+/-	-	+
B. subtilis T14-5	-	++	-	+/-	-	+/-
B. subtilis T16-2	-	++	++	+	+/-	+
B. subtilis T16-3	-	+	-	+/-	-	+/-
B. subtilis T16-10	-	++	++	+/-	+/-	+/-
B. subtilis T17-1	-	++	-	+/-	+/-	+
RO-B-2/RO-H-1						
B. subtilis T14-1	-	-	-	++	++	+/-
B. subtilis T14-3	-	-	-	++	++	+
B. subtilis T14-4	-	-	-	++	++	+
B. subtilis T16-4	-	-	-	++	++	+/-
B. subtilis T16-5	-	-	-	++	++	+
none						
B. licheniformis T15-1	-	-	-	-	-	+
B. licheniformis T16-6	-	-	-	-	-	-
B. amyloliquefaciens T16-7	-	-	-	-	-	-
B. megaterium T19-1	-	-	-	-	-	-
B. pumilus T24-5	-	-	-	-	-	-
B. licheniformis T26-2	-	-	-	-	-	+/-
B. licheniformis T31-1	+/-	-	-	-	-	+/-



On the basis of strong and moderate activation responses, 15 out of 21 strains could be placed within three pherotypes (Table 1). These specificities were in line with clustering based on comQ sequences similarity. Strains T16-8 and T21-2 belonged to 168 pherotypes. Strains T12-1, T14.5, T16-2, T16-3, T16-10 and T17-1 activated RSD2 tester strain. Three of these isolates (T12-1, T16-2 and T16-10) caused strong response of NAF4 tester strain, however remaining strains did not. Strains T14-1, T14-3, T14-3, T16-4 and T16-5 induced strong response of tester RO-H-1 and RO-B-2. All strains which induced strong response of the tester (marked as „++'') belonged to *B. subtilis* species. Strain T15-1 representing *B. licheniformis* induced moderate response of RO-E-2 tester. Also cross-talk could be observed with testers outside the pherotype („+'' or „+/- ''), but it was never as strong as specific response. Again it was confirmed that within a single plant various QS specificities can be found.

4. 7. 2 Diversity of potential biocontrol properties within *B. subtilis* pherotypes and plants

As both surfactin production (D'Souza et al., 1994; Comella & Grossman, 2005) and indirectly also biofilm formation (Lopez et al., 2009a) are under ComQXPA QS control we examined the variability of these traits within the rhizoplane collection of *B. subtilis* isolates. We were particularly interested whether this diversity is evident also within a pherotype. We used the *B. subtilis* BD2833 strain (Tortosa et al., 2001), which is deficient in surfactin production and biofilm formation (BD2883 derives from 168 described in McLoon et al., 2011) as a negative control. In addition the *B. subtilis* GB03 strain was used as a positive control for biofilm formation (Beauregard et al., 2013).

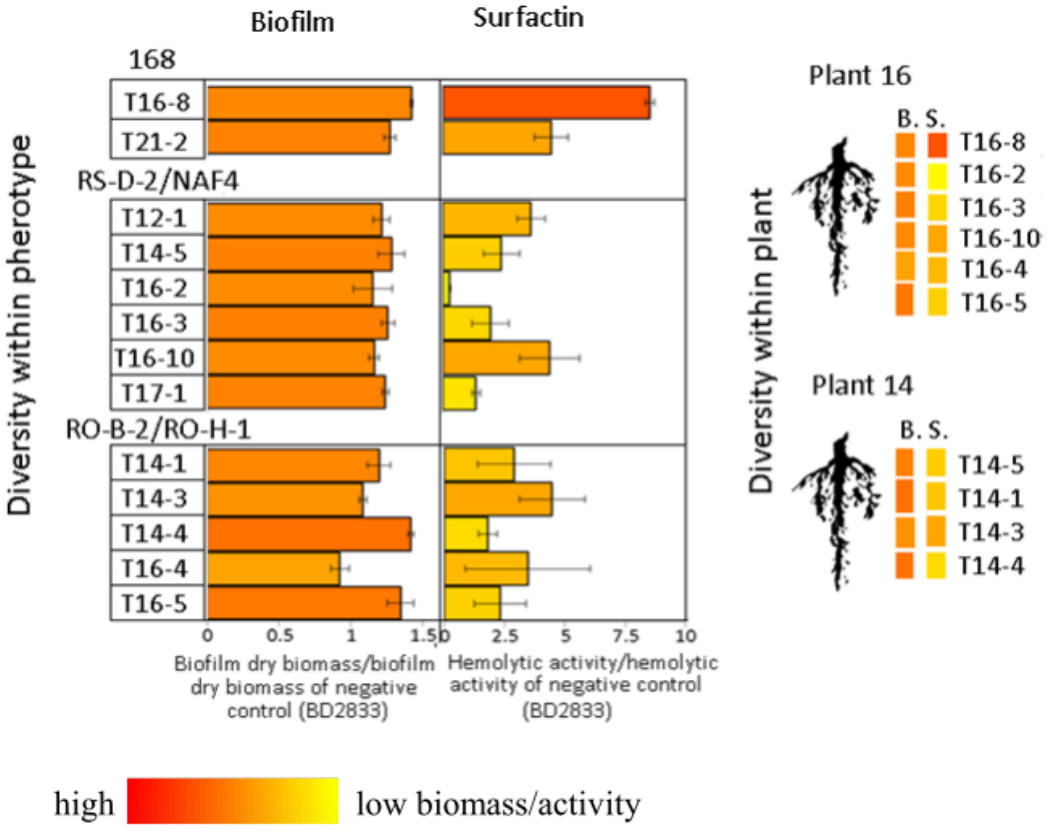


Figure 44. **Biofilm biomass and surfactin activity of *B. subtilis* isolates from tomato rhizoplane**. Biofilms were harvested and their dry mass was determined. The dry mass of each biofilm was then devided by the dry mass of biofilm formed by negative control BD2833. Conditioned media produced during biofilms growth were filter-sterylized and the presence of biosurfactants was determined by heamolytic test. Strain BD2833 which is deficient in surfactin production was used as negative control. Percent of haemolysis obtained for each conditioned medium was devided by the value obtained for negative control. Data represent average of three independent replicates with SE indicated. To better show the diversity colums on the graph were marekd with RGB intensities that directly correspond to quantitative values measured for each trait (as shown below the table). The colors representing biofilm dry biomass (B.) and hemolytic activity (S.) were also used to demonstrate the diversity at the plant level (on the right).

Slika 44. **Biomasa biofilmov in aktivnost surfaktina izolatov *B. subtilis* iz rizoplana paradižnika.** Določili smo suho maso biofilmov. Suha masa vsakega biofilma je bila nato deljena s suho maso biofilma negativne kontrole BD2833. Izrabljeno gojišče ki je nastalo med. tvorbo biofilmov, je bilo sterilizirano preko filtracije in prisotnost biosurfaktantov je bila določena s pomočjo kemolitičnega testa. Sev BD2833 ki ne producira surfaktina, je bil uporabljen kot negativna kontrola. Procent kemolize določen za vsako izrabljeno gojišče je bil nato deljen s rezultatom negativne kontrole. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko. Za boljši prikaz raznolikosti uborabili smo intensiteto RGB, ki direktno ustreza kvantitativim vrednostiom izmerjim za vsako lastnost (kot je prikazano pod preglednico). Barve ki ustrezajo suhi biomasi biofilmov (B.) in kemolitični aktivnosti (S.) smo uporabili tudi za prikaz diversitete na nivoju posamezne rastline (na desni).

We observed that all but one *B. subtilis* isolates produced pellicle biofilms with higher biomass as compared to negative control and that 3 isolates (T16-8, T14-4 and T16-5) produced larger biofilm biomass as compared to the positive control (Figure 44). Within one pherotype strains differed in both tested traits: biofilm formation and surfactin production (Figure 44). For example, within the pherotype RO-H-1/RO-B-2 one strain formed a copious biofilm and another was comparable to a negative control (Figure 44). The diversity of surfactin production was also very pronounced and within each pherotype we could find very strong biosurfactant producers (up to 8 times more as compared to the control) but also strains which showed very weak surfactin activity (measured by hemolytic activity) or no such activity at all (Figure 44). Similar diversity could be observed when strains were grouped in respect to the plant they were isolated from. For example, each plant (plant 14 and plant 16) contained a very strong surfactant producer (T14-3 and T16-8 respectively), a moderate producer or even a non-producer (T16-4) (Figure 44).

In order to confirm that the hemolytic assay measured surfactin-specific activity of the conditioned media produced by rhizoplane isolates, we inactivated *srfA* gene in 5 randomly chosen isolates: T16-8, T16-10, T16-4, T16-5 and T16-2 and compared the hemolytic activity of the mutants with their ancestors.

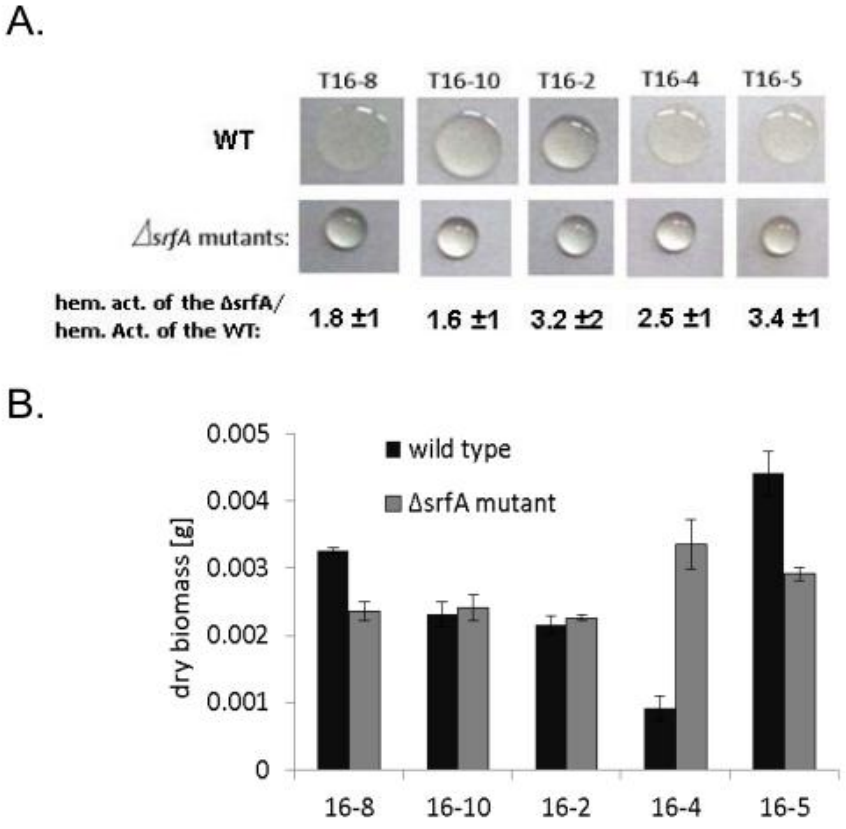


Figure 45. **Effects of surfactin deletion in 5 different *B. subtilis* isolates from tomato rhizoplane**. A. Pictures of 30 µl droplets of conditioned media taken after 48 hours from pellicles grown in MSN medium at 28°C. Below droplets of conditioned media of T16-8, T16-2, T16-10, T16-4 and T16-5 isogenic Δ*srfA* mutants are presented. The numbers below respresent percents of hemolytic activity measured in conditioned medium of isogenic Δ*srfA* mutants as compared to its ancestor wild type strain. B. Biofilms were harvested and their dry mass was determined. Data represent average of three independent replicates (independent experiments) with SE indicated.

Slika 45. **Učinek delecije surfaktina v 5 različnih izolatih *B. subtilis* iz rizoplana paradižnika.** A. Slike 30 µl kapljic izrablejnega gojišča, pridobljenega po 48 urah iz peliklov gojenih v gojišču MSN pri 28°C . Od spodaj kapljice izrabljenega gojišča izogenih Δ*srfA* mutant T16-8, T16-2, T16-10, T16-4 in T16-5. Številke od spodaj predstavljajo procent kemolitične aktivnosti izmerjene v izrabljenih gojiščih Δ*srfA* mutant v primerjavi z ustreznimi divjimi tipi. B. Suha masa biofilmov. Podatki predstavljajo povprečje najmanj treh bioloških ponovitev s standardno napako določeno za vsako točko.

All 5 Δ*srfA* mutants showed over 95% decrease of hemolytic activity (Figure 45A). Therefore hemolytic activity of wild rhizoplane isolates was surfactin dependent. Moreover, the drop

collapse test (Jain et al., 1991) which is an alternative, qualitative method of surfactin detection correlated with the hemolytic assay (Figure 45A).
Next we tested the effects of surfactin deletion on biofilm biomass production. Interestingly, not all strains were affected equally. In the T16-8 and T16-5 strains surfactin deficiency decreased biofilm biomass by 28% ($P<0.02$) and 34% ($P<0.02$) respectively while the T16-2 and T16-10 mutants formed comparable biofilms to the wild type ancestors. Surprisingly, the T16-4-Δ*srfA* mutant had 3.5 fold ($P<0.02$) larger biomass than the parental strain (Figure 45B). Therefore, the regulatory role of surfactin in biofilm formation might be strikingly different among natural isolates of *B. subtilis* and it will be interesting to identify genetic differences behind this observation in the future.

4.7.3 PGPR properties within *B. subtilis* pherotypes

It is known that bacteria can directly promote the growth of plant by various secretions (Lopez-Bucio et al., 2007; Kloepper et al., 1980; Molam et al., 1984). We therefore tested whether secreted molecules of the rhizoplane isolates can influence the growth of plant roots and leaves of the model plant *Arabidopsis thaliana*. Bacteria were inoculated on MS solid medium (see methods) app. 5 cm away from the *A. thaliana* seedlings and the plant biomass versus control (no bacteria inoculated) was measured after 10 days.

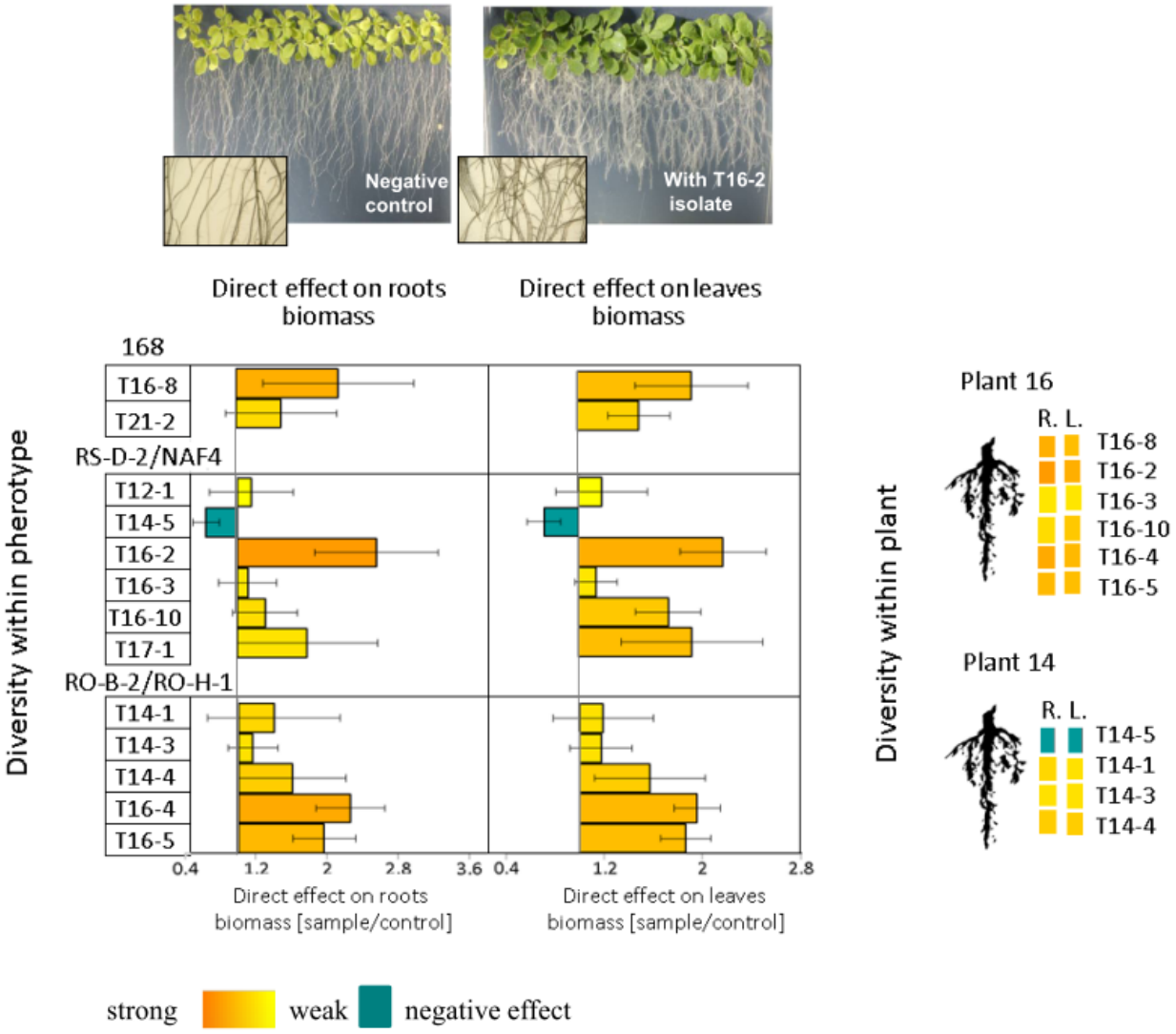


Figure 46. Influence of *B. subtilis* isolates from tomato rhizoplane on *A. thaliana* roots and leaves biomass. Plants were grown for 14 days in app. 5 cm distance from the bacterial inoculums. Positive effect of *B. subtilis* isolate presence on growth of the model plant is shown at the pictures above. Final biomas of roots and leaves were then devided by biomas of control plants (roots and leaves) that were grown on sterile medium. To better show the diversity colums on the graph were marekd with RGB intensities that directly correspond to quantitative values measured for each trait (as shown below the table). The colors representing effect on roots biomass (R.) and leaves biomass (L.) were also used to demonstrate the diversity at the plant level (on the right).

Slika 46. Vpliv izolatov B. subtilis iz rizoplana paradižnika na biomaso korenin in listi *A. thaliana*. Rastline smo gojili 14 dni približno 5 cm stran od inokoluma bakterije. Pozitivni učinek prisotnosti izolata *B. subtilis* na rast modelne rastline, je prikazan na sliki zgoraj. Končna biomasa korenin in listi smo nato delili s biomaso kontrolnih rastlin (korenine in listje) ki so rastle na sterilnim gojišču. Za boljši prikaz raznolikosti uborabili smo intensiteto RGB, ki direktno ustreza kvantitativim vrednostiom izmerjim za vsako lastnost (kot je prikazano pod preglednico). Barve ki ustrezajo vplivu na biomaso korenin (R.) in biomaso listov (L.) smo uporabili tudi za prikaz diversitete na nivoju posamezne rastline (na desni).

Isolates sharing a pherotype or being derived from the rhizoplane of a single plant had different effects on roots and leaves biomass (Table 2, Figure 46, Table 3). For example, two strains of the same pherotype RS-D-2/NAF4, namely T16-2 and T16-4, increased the biomass of roots ($P<0.06$ and $P<0.002$, respectively) and leaves ($P<0.03$ and $P<0.006$, respectively) two fold, while the strain T16-3 of the same pherotype had no effect on the plant biomass (Figure 46).

Similar diversity was found among isolates from one plant, with plant 16 isolates giving very strong PGP effect or no effect; and isolates from plant 14 showing weak positive effects, no effects, or even negative effects on plant biomass (Figure 46).

In addition we analyzed three phenotypic traits that can influence plant growth: production of indole-3-acetic acid (IAA) (Lopez-Bucio et al., 2007) or siderophores (Kloepper et al., 1980) and the ability to solubilize phosphate (Molla et al., 1984).

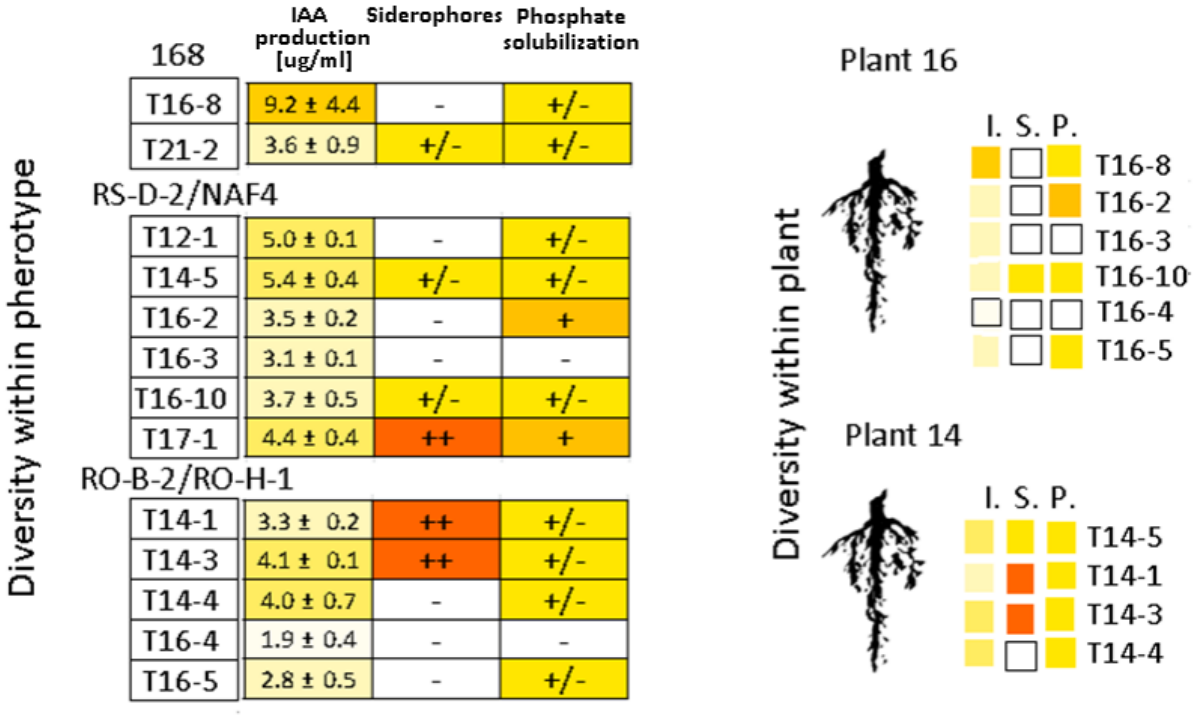


Figure 47. Comparison of potential PGPR traits: IAA production, siderophores production and phosphate solubilization. Symbols: ++, strong effect (minimally 2-fold, or in case of biofilms – significantly higher than positive control), +, moderate effect (significantly lower as compared to maximal effect observed); +/−, weak but reproducible effect, - no different from negative control. To better show the diversity we used RGB intensity that directly corresponds to quantitative values measured for each trait (as shown below the table). The colors representing IAA concentration (I.), siderophores (S.) and phosphate solubilization (P.) were also used to demonstrate the diversity at the plant level (on the right).

Slika 47. Primerjava potencialnih PGP lastnosti: produkcija IAA, produkcija sideroforjev in raztapljanje fosfatov. . Simboli: ++, močen učinek (najmanj 2-krat, ali v primeru biofilmov –značilno več kot pozitivna kontrola), +, srednji učinek (značilno manj kot maksimalni učinek); +/−, slab ampak ponovljiv učinek, - ni različno od negativne kontrole. Za boljši prikaz raznolikosti uporabili smo intensiteto RGB, ki direktno ustreza kvantitativim vrednostiom izmerjim za vsako lastnost (kot je prikazano pod preglednico). Barve ki ustrezajo koncentraciji IAA (I.), siderovorjem (S.) in raztapljanju fosfatov (P.) smo uporabili tudi za prikaz diversitete na nivoju posamezne rastline (na desni).

The same traits (IAA production, siderophores production and phosphate solubilization) and direct effects on roots and leaves biomass were also examined in other *Bacillus* spp. isolates that were obtained from the tomato rhizoplane.

Table 2. **Data on other *Bacillus* spp. rhizoplane isolates: effects on roots and leaves biomass and indole-3-acetic acid production (IAA) and qualitative estimation of siderophores production and phosphate solubilization.** Symbols: ++, strong effect (app. 2-fold), +, moderate effect (significantly lower as compared to maximal effect observed); +/−, weak but reproducible effect, - no different from negative control.

Preglednica 2. Podatke o drugih izolatih *Bacillus* spp. iz rizoplana: vpliv na rast korenin in listi in produkcija indol 3-ocetne kisline (IAA) in kvalitativna ocena sideroforjev in raztapljanja fosfatov. Simboli: ++, močen učinek (približno 2-kratni), +, srednji učinek (značilno manj kot maksimalni učinek); +/−, slab ali ponovljiv učinek, - ni različno od negativne kontrole.

Strain	Species	direct effect on roots biomass [sample/control]	direct effect on leaves biomass [sample/control]	IAA production [µg/ml]	siderophores	phosphate solubilization
T15-1	*B. licheniformis*	1 ± 0.3	1 ± 0.2	4.0 ± 0.6	+/-	+
T16-6	*B. licheniformis*	2.1 ± 0.7	1.6 ± 0.3	3.3 ± 0.5	+/-	+/-
T16-7	*B. amyloliquefaciens*	0.9 ± 0.2	0.9 ± 0.1	5.5 ± 0.2	-	+/-
T19-1	*B.megaterium*	2.5 ± 0.4	2.1 ± 0.4	25.3 ± 0.8	++	+
T24-5	*B. pumilus*	0.8 ± 0.3	0.9 ± 0.1	3.1 ± 0.4	+/-	+
T26-2	*B. licheniformis*	1.3 ± 0.4	1.3 ± 0.3	5.2 ± 0.1	-	++
T31-1	*B. licheniformis*	0.7 ± 0.2	0.7 ± 0.1	3.8 ± 0.2	-	+
6051	*B.subtilis*	1.2 ± 0.4	1 ± 0.2	2.8 ± 0.4	+/-	+
GB03	*B.subtilis*			7.5 ± 2.3	-	+
FZB42	*B. amyloliquefaciens*			3.8 ± 0.4	+/-	+

These properties were also variable within a pherotype and there was no correlation between plant growth promotion measured directly and these traits. For example strain T16-4 which had strong positive effect on plant biomass, was negative in all PGP traits tested (IAA, siderophores and phosphate solubilisation) (Figure 46, Figure 47). Similarly strains T16-10 and T16-7 were positive in two out of three PGP traits but did not promote the growth of roots (Figure 46, Figure 47, Table 2). This indicates that direct effects on plant growth cannot be easily predicted only by testing the established PGP traits. In addition to PGP bacteria we also found inhibitors of plant growth: T14-5 (*B. subtilis*, pherotype RS-D-2/NAF-4) and T31-1 (*B. licheniformis*) induced 30% decrease in root's ($P<0.08$ and $P<0.05$, respectively) and leave's ($P<0.09$ and $P<0.06$, respectively) biomass. Additionally, we isolated an interesting PGP candidate from plant 19, *B. megaterium* T19-1, which produced app. 5 times higher concentration of IAA of 25µg/ml, compared to other strains, showed very strong production of siderophores and also strongly (2-fold) promoted the growth of roots ($P<.004$) and leaves ($P<.002$) of *A. thaliana* (Table 2).

We also analysed production of extracellular degrading enzymes that could be useful in fighting against plant pathogens and/or in direct inhibition of plant pathogen *Ralstonia solanacearum* (Figure 48).

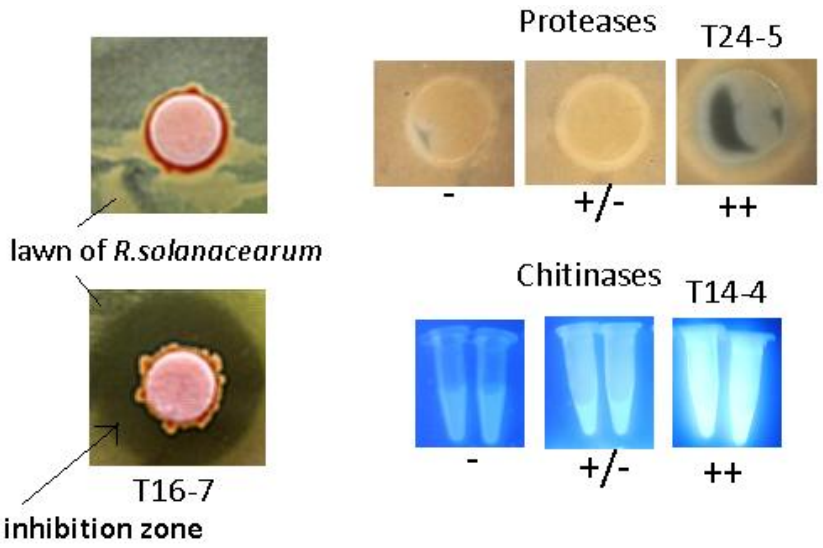


Figure 48. **Qualitative detection of *R. solanacearum* inhibition zone on King's B medium; proteases and chitinases.** Concentrations of chitinases were also measured quantitatively using spectrofluorimetry.

Slika 48. **Kvalitativna detekcija cone inhibicije *R. solanacearum* na trdnem gojišču King's B; produkcije proteaz in hitinaz.** Koncentracije hitinaz so bile izmerjene kvantitiativno oziroma spektrofluorimetrično.

We observed that the effects on plant pathogen varied between strains and strain T16-7created the largest and very clear halos around the inoculums inhibiting the growth of R. solanacearum (Figure 48), similarly to positive controls GB03 and FZB42.*B. pumilus* (T24-5) formed the largest zone of proteolysis (Figure 48). Majority of the isolates showed chitinase activity with the highest activity measured for the strains T14-4 (*B. subtilis*) and T16-7 (*B. amyloliquefaciens*) (Figure 48).

5 DISCUSSION

5.1 INTRACELLULAR LINK BETWEEN QS SIGNALING AND QS RESPONSE IN *B. subtilis*

The canonical model of quorum sensing assumes that QS signals after being secreted from cells act extracellularly by activating their cognate receptors thereby inducing QS response (Miller & Bassler, 2001). In line with this model lack of signal production results in loss of receptor activation function unless exogenous QS signal is provided. Here we asked if signal-null mutants are capable of normal QS response when the signal is being produced by signal-proficient wild population. We therefore used ComQXPA QS system of *B. subtilis* to evaluate the intracellular codependence of two essential QS functions: signaling and response. We show that loss of signaling function results in overly responsive QS system. This can be observed by comparing QS response dynamics of the QS+ and QSS- strains in coculture, where they are facing the same concentration of QS signal. It can be also observed by comparing absolute QS response of QS+ and QSS- in monocultures when medium is saturated with exogenously added signal purified from *E. coli*. The accurate QS response therefore requires synthesis of ComX signal, its continuous secretion from the cell or both. Whatever is the case, the results show that QS response is linked to signal production and its ComQ-dependent modification at the posttranslational level. The repressive effect of signal production on QS response may depend on physical interactions between ComQ and ComX or even ComQ, ComX, and ComP. Another possibility is that QS+ and QSS- differ in number of ComP receptors in the extracellular membrane. In fact proximal arrangement of genes (like ComQXP) may lead to unexpected gene expression dynamics where for example the expression of one upstream gene inhibits the transcription of downstream gene. This phenomenon has been described as a promoter occlusion (Bateman & Paule, 1988; Zafar et al., 2014). The localization of ComP promoter have not yet been confirmed but it was predicted to be positioned in the terminal part of *comX* [deletion of ComX in fact inactivates ComP as mentioned by Solomon et al., (1995) and as we also observed in this work when we attempted to delete the whole *comX* gene]. A precise understanding of these interactions awaits further study.In domesticated Δ*comQ* strains the increased QS response was visible at the level of *srfA* transcription, which dramatically increased in the presence of ComX. A small decrease in *srfA* expression in the presence of exogenous ComX observed for the domesticated signal-proficient strain suggests negative feedback regulation as an intrinsic property of the ComQXPA system.

We have also analyzed QS response of QSS- and QS+ in cocultures of different ratios. By decreasing the ratio of QS+ in coculture we also decreased the concentration of available QS signal in this coculture. This enabled us to compare the QS response of QSS- and QS+ to

different concentrations of QS signal. Travisano and Velicer (2004) predicted that the presence of QSS- in coculture with the wild type would result in lower concentration of QS signal and as result in lower QS response of both populations. The authors have proposed that the decreased QS response could promote QS stability by minimilizing metabolic burden of the wild type response and limiting the risk of invasion by signal-null mutants (Travisano & Velicer, 2004). However, we observed that the QS response in cocultures containing only 20% of the QS+ was not affected by the presence of the QSS-. This may be due to ,,overload'' of the ComX signal. In other words, the QS+ appears to prodce ComX in significant excess and already 1/5 of its final concentration in the media is enough to induce maximal QS reponse of the population. By analogy, Reymont and colleagues (2012) compared the efficiency of host infection by *B. thuringiensis* mixed in different ratios with a mutant that does not produce the key Cry toxins. It occurred that even cocultures containing only 30% of the wild population and 70% of *cry*$^{-}$ mutants caused the same level of host mortality as observed for the wild type monocultures (Reymont et al., 2012). This means that the *B. thurengiensis* wild type produced enough toxins to be shared with the mutant and retained maximal toxicity. Analogously, *B. subtilis* QS+ makes enough QS signal to be shared with the QSS- mutants while retaining maximal level of the QS response.

5.2 METABOLIC CONSEQUENCES OF PRIVATE LINK BEWTEEN QS SIGNALING AND RESPONSE IN *B. subtilis*

What are the consequences of overly responsive QS system of the signal-null mutant? To answer this question the most accurately we addressed it using the genetic background of undomesticated strain PS-216 (Stefanic & Mandic-Mulec, 2009; Durrett et al., 2013). Unlike BD2833 the isolate is not domesticated and preserves many QS-regulated social traits that were lost by the BD2833 during domestication process (McLoon et al., 2011). One of these traits is surfactin production.

In fact the over-response effect in undomesticated QSS- PS216ΔcomQ mutants was also detectable at the level of surfactin synthesis, which was significantly higher than in QS+ controls. In addition, we observed that a higher fraction of QSS- cells activated *srfA* gene transcription in the presence of ComX than in the undomesticated QS+ population. Bimodal expression of *srfA* in undomesticated strains was reported previously (Lopez et al., 2009a) and was suggested to lead to phenotypic differentiation of the *Bacillus* population and a division of labor that provide fitness benefits (Lopez & Kolter, 2009). Our results show that the private link between signal production and QS response also has consequences for bimodal expression of *srfA* in the *B. subtilis* population. Recently it was reported that in salmonella, which shows

bimodal expression of virulence, the existence of an avirulent subpopulation is essential for the fitness and evolutionary stability of virulence (Diard et al., 2013).

Since *srfA* and *comS* are under control of the same promoter (D'Souza et al., 1994; Hamoen et al., 1995) it is not surprising that over-response of the mutant also manifested at the level of *comK* expression and in higher competence for transformation of signal-null mutants grown in presence of ComX. This was also observed in coculture where both QS+ and QSS- were facing the same, linearly increasing concentration of ComX. This proves that increased competence of QSS- manifests not only in terms of saturation of the medium with ComX at the beginning of growth, but also in more ,,natural'' conditions, where ComX concentration increases gradually, being produced by the wild type population. Therefore increased response of the signal-null mutant resulted in at least two aberrations in secondary metabolism of *B. subtilis* – increased production of surfactin and up-regulation of competence machinery.

We also examined the consequences of changed QS response for primary metabolic activity of *B. subtilis*, which we accessed by classical dehydrogenase activity test. It is interesting that despite the QS+ and QSS- growth in pure culture did not differ, the dehydrogenase activity of the mutant was dramatically higher as compared to that of the wild type. This suggest that when not producing QS signal the mutant is much more metabolically active as compared to QS+, while growing with the same rate and reaching the same final density as the wild type. Further measurement of ATP-level could be performed in the future to confirm that the mutant indeed produces more chemical energy as compared to the wild type. In the same line, signal-null mutant, making more ATP as compared to the wild type would have more phosphate reserve for ComP histidine kinase phosphorylation, which could be an alternative explanation for QS response outburst in QSS- when ComX is provided.

Interestingly analogical changes in primary metabolism were observed in signal-null mutants of *Streptomyces chattanoogensis* (Du et al., 2011) and *Burkholderia thalengensis* (Chandler et al., 2009) where increased respiration rates of signal-null mutants were indicated. We have also observed increased metabolic activity of surfactin-null mutant suggesting that signal deletion can indirectly cause this effect. In fact in the presence of ComX, when signal-null mutant overly produces surfactin, its dehydrogenase activity drops dramatically reaching lower level as observed for the wild type. Interestingly, it was already noticed by de Roubin and colleagues (1989) that increased levels of surfactin production in *B. subtilis* correlated with decreased activity of citric acid cycle. All these data suggest that QS and surfactin may be crucially important for preserving balance between primary and secondary metabolism.

5.3 FITNESS CONSEQUENCES AND EVOLUTIONARY IMPLICATIONS OF PRIVATE LINK IN *B. subtilis* QUORUM SENSING

Under our experimental conditions neither ComX signal production, nor surfactin production were charged with any obvious fitness costs as the growth of the QS+ wild type was very similar to that of the signal-null mutant when no exogenous signal was added. The question whether one should even expect fitness costs of QS signal production is rather controversial. For example in its commentary Haas (2006) wrote that since AHLs production is metabolically cheap (only 0.01% of the total cellular amount of ATP), signal-null mutants should not have fitness advantage over signal producers. The opposite would be true for receptor-null mutants, because the QS response is much more metabolically costly (at least 5% of the total energy supply) (Haas, 2006). On the other hand a year later Diggle and colleagues (2007a) predicted and experimentally demonstrated that not only QS response but also production of AHL QS signals stands for metabolic costs. In addition it was assumed that among QS signals, AHLs produced by gram negative bacteria can be app. 20 times metabolically ,,cheaper'' as compared to more specific peptide signals of gram positive bacteria (Keller & Surette, 2006). Fitness costs of peptide signal production were also experimentally demonstrated in competition experiments between wild type and signal-null mutants of *Streptococcus pneumonia* (Yang et al., 2010). Those however were measured in coculture of QS+ and QSS- and there is no data on how the strains performed in monocultures (Yang et al., 2010).

At any rate we saw no such costs in monocultures of QS+ and QSS- of *B. subtilis* which analogically to *S. pneumonia* uses the peptide QS signal, produced from ribosomally synthesized polypeptide precursor which undergoes cleavage and modification (Knutsen et al., 2004). However, this changed dramatically when signal was added to the *comQ* mutant, decreasing the fitness of the mutant. Therefore, generating a higher proportion of surfactin producers may have significantly contributed to the fitness loss of the mutant.

It was shown that in *P. aeruginosa* production of rhamnolipid biosurfactant is limited to the stationary phase and thus free of fitness charges, but release of biosurfactant production from QS control (using an inducible promoter for its induction) resulted in its imprudent production and fitness loss (Xavier et al., 2011). In *B. subtilis*, surfactin is also produced during the stationary phase, but in signal-deficient mutants this prudency is lost due to overproduction of surfactin.

Despite elevated levels of surfactin secretion, QSS- showed increased expression of *comK* in the population and increased genetic competence in presence of ComX, however our data

suggest that surfactin rather than competence was mainly responsible for negative fitness effects of ComX on signal-null mutant. This is rather surprising because competent cells are arrested in DNA replication and cell division (Haijema et al., 2001) and an increase in *comK*-expressing subpopulation from less than 10% to nearly 50% could easily explain decrease in QSS- growth rate in the presence of ComX. These results show therefore, that it is hard to predict which secondary metabolic activity is more expensive for the cell to over-perform. In addition other surfactin-depend mechanism can be involved here. Our results also show that surfactin itself, when produced by the competitor, is indirectly involved in the fitness loss of the signaling mutant. This mechanism negatively affects the signal-deficient mutants even if they are released from the ComX-dependent disadvantage of surfactin overproduction (e.g., in the double-mutant PS216ΔcomQΔsrfA). This is consistent with current knowledge that surfactin serves as a signaling molecule that triggers cannibalism in the *B. subtilis* population (Gonzalez-Pastor et al., 2003; Lopez et al., 2009b). The advantage of the surfactin-deficient mutant (PS216ΔsrfA) over the double-mutant PS216ΔcomQΔsrfA in the presence of exogenously provided surfactin suggests that the QS+A− and QSS−A− populations differ in response to surfactin. This may translate, for example, into generation of higher numbers of cannibals in the QS+ population than in the QSS− population and provide a testable hypothesis that, however, awaits further study.

Signal-deficient mutants are common among certain bacterial species such as *P. aeruginosa* (Rumbaugh et al., 1999; Denervaud et al., 2004), but they have not been detected among more than 60 strains of *B. subtilis* isolated from various environments (Ansaldi et al., 2002; Stefanic & Mandic-Mulec, 2009; Tortosa et al., 2001; Stefanic et al., 2012). Our data offer an explanation for this observation. The private regulatory link between signaling and response may therefore mean that signal-deficient mutants are rapidly lost from native populations of *B. subtilis* simply because making signal stands for selfish benefits.

Finally, how did the pleiotropic constraint on signal loss arise in *B. subtilis*? Probably, the private effect evolved to improve the regulatory performance of the QS system, which then also helped ensure its evolutionary stability. Whatever the situation, it is clear that signal production now plays an important role in regulating secondary metabolism and other traits related to the response of the population to surfactin. Majority of studies addressing molecular basis of *B. subtilis* QS (including this study), were performed on strains representing „168'' QS specificity group (pherotype) (Manguson et al., 1994; Pozzi et al., 1996; Schneider et al., 2002; Hameon et al., 2003; Oslizlo et al., 2014). It would be interesting to examine the existence of a similar signaling-response intracellular link in other pherotypes of *B. subtilis* (Stefanic & Mandic-Mulec, 2009) and in ComQXP-like QS systems of other *Firmucutes* phylum (Dogsa et al., 2014). We believe that the link that ensures prudent QS response to self-produced QS molecules was likely to appear at the early stages of signal-receptor co-

evolution (Steiger et al., 2011). Eventually in natural habitats, where cells grow in tightly packed agglomerations, signal producers are likely to encounter very high local concentrations of self-produced molecules and therefore sensitivity to these molecules requires tuning mechanisms. Negative coupling of signal production with the response seems perfect regulatory solution. Following this logic, it would be interesting to test whether increased production of ComX leads to decreased sensitivity to exogenous ComX. Signaling-response link, when emerged as an integral feature of the *comQXP* locus, probably provided dramatic fitness advantage to prudent responders and therefore it could easily be fixed before pherotypes diversification or HGT transitions of the locus to other species. Alternatively, if the signaling-response linked negative feedback exists in other pherotypes, it could be selected for several times independently.

Moreover it was also shown that there are constrains on signaling in *Vibrio fisheri* (Chong et al., 2013). Signal-null mutants of this species similarly as in our case are outcompeted by the wild type, indicated that also in *V. fisheri* signal production is directly beneficial. For some reasons the *luxI* gene encoding for QS signal remains stable after experimental evolution in rich medium, which is in opposite to *luxR* encoding for QS receptor. This suggests that in species mentioned above signaling is evolutionary more stable than QS response and that in *V. fisheri* the expression of the *luxI* gene and activity of its product represents an unknown selective advantage to the cell (Chong et al., 2013). Interestingly it appears that most *P. aeruginosa* QS mutants isolated from natural environment happen to be receptor-null mutants, and not signal-null mutants (Heurlier et al., 2006). Since signals most likely evolved from metabolic byproducts (Steiger et al., 2011), they may be ,,nested'' in the metabolic pathways so deeply, that deletion of signaling function results in negative pleiotropic effects that are costly to the cell. Evolutionary stability of sharing signals by bacteria could be then explained by direct benefits to producers.

An interesting question that follows from our discovery is - would the ComP receptor deletion influence signaling function similarly as signaling defect influences the function of ComP? Our preliminary data show that this is not the case and that receptor-null Δ*comP* mutant produces a similar amount of active ComX as the wild type and shows similar ComX-production dynamics as the wild type. Eventually, following the metabolic economy of QS – imprudent signalling is not likely to constrain costly QS response – it could still pay-off to the cell to avoid the response and overproduce QS signals; but costly QS response can efficiently constrain signaling function.

5.4 COOPERATION AND CHEATING DURING GENETIC EXCHANGE WHERE ComX AND SURFACTIN SERVE AS PUBLIC GOODS

The major role of competence in evolution of bacteria remains under debate. Competence in *Legionella pneumophila* promotes repair of the DNA damage (Charpentier et al., 2011) although experiments with *Haematophilus influence* and *Bacillus subtilis* rejected this hypothesis (Redfield, 1993). *E. coli* can benefit from eating exogenous DNA (Redfiled, 2001; Finkel & Kolter, 2001). Competent cells, since they undergo growth arrest, can also benefit from being more resistant to growth-targeting antibiotics that will mostly harm actively growing non-competent cells (Johnsen et al., 2009). It was also shown that competence for transformation, by increasing genetic variation can increase the rate of bacterial adaptation to novel environments (Baltrus et al., 2008) and is therefore beneficial. This suggests that recombination in bacteria may have similar evolutionary role, that has been proposed many times for eukaryotic sex (Weismann, 1889; Muller, 1932; Burt, 2000), and which is to increase genetic variation for natural selection.

At any rate, genes encoding for recombination through natural competence are present in genomes of many unrelated species, so benefits of carrying these genes are very likely to exist. Those may depend on a species, because there are no universal rules of competence development, for example: *Neisseria gonorrhoeae* is constitutively competent exhibiting very high recombination rates in nature (Haubolt et al., 1998) while *Bacillus subtilis* develops competence only in presence of specific signals, competent state is restricted to only few hours of stationary phase and it is probably very rare in nature (Haubolt et al., 1998; Roberts & Cohan, 1995). Signaling determines competence development in several species and we demonstrate on *B. subtilis* model that such signals may serve as public goods - tickets to gain ,,free'' access to the competence state –a private good. How then signals production remains evolutionary stable and resist invasion by signal-null mutants?

First, we discovered that not only ComX, but also surfactin released in response to ComX, are both important in the process of DNA-exchange between strains, with the letter promoting the DNA release to the conditioned medium. We did not further investigate how surfactin increases the concentration of e-DNA, therefore we do not have enough data to support previous conclusions (Zafra et al., 2012) of ComQXP QS-dependent DNA release being an active process instead of a consequence of cell lysis. In general it is not well understood where DNA for natural transformation of bacteria usually comes from. For example in *Streptococcus pneumonia* competence development is coupled with predatory behavior towards non-

competent siblings (Steinmoen et al., 2002). On contrary, type IV secretion system for active secretion of chromosomal DNA was identified in *Neisseria gonorrhoeae* (Hamilton et al., 2005). Finally, it was documented that even short fragments of ancient (43 000 years old) and degraded DNA can be taken up by naturally competent *Acinetobacter baylyi* (Overballe-Petersen et al., 2013).

Whatever is the mechanism in *B. subtilis*, our data indicate that surfactin plays an important role in cooperative DNA-exchange, acting as a public good and in fact promoting the release of another public good – the DNA, just in time. Almost a decade ago the microarray analysis revealed that *comS* (competence) and *srfAA* (surfactin) genes are the two main targets of ComQXPA (Comella & Grossman, 2005). This result is logical because *comS* is located within *srfAA* operon and the expression of those two is coupled (Comella & Grossman, 2005; D'Souza et al., 1994; Hamoen et al., 1995). Here we provide and explanation for this coupling, which is important role of surfactin in DNA release. Such coupling of DNA-uptake and DNA-release is well established in *S. pneumonia* (Steinmoen et al., 2002), a species which is similar to *B. subtilis* in other sexual aspects – for example in both species only part of the population becomes competent, competence in restricted to few hours of stationary phase and distinct competence- regulating signal-receptor pairs have evolved by diversifying selection, so that signals of one group cannot induce competence of another group. *S. pneumonia* like *B. subtilis*, produces competence-inducing peptide signal and then competent cells release another compound that leads to release of the DNA from non-competent siblings (Steinmoen et al., 2002). It remains to be discovered whether competent cells and DNA donors in *B. subtilis* are, by analogy to *S. pneumonia*, different subpopulations of cells.

Here we discovered that when the signal-null mutants (QSS-) are present in the wild population (QS+) and there is a selection pressure for the DNA-exchange, the QSS- by sensing ComX, can not only develop competence, but also, due to being hypersensitive to the signal (Oslizlo et al., 2014), it becomes overly competent hypercheat. To our knowledge it is the first example when cheater due to link between signaling (public good production) and response (leading to private good access) becomes more greedy/efficient public good user as compared to the wild type. This also means that in the moment when recombination pays off, signal production is not an ESS.

Recombination however is a time-limited and after it, the descendent genotypes compete against each other carrying the genetic background of their ancestors modified only by genes acquired by the recombination event. Our data show that despite short-term advantage of QSS-, it cannot further invade even when inoculated in advantageous starting ratio. This is because the hypersensitive signal-null mutant, in not only hypercompetent but it is also surfactin hyperproducer (Oslizlo et al., 2014) so it is both hypercheater and hypercooperator.

The trade-offs of hypercomptence are therefore too high and QSS- strategy is not stable, but it is rather a short window of opportunity.

In fact our study brings support for Fitness-Associated Recombination hypothesis of Eshel & Feldman (1970). The hypothesis assumes that recombination frequency should be negatively associated with fitness, because this would increase probability of breaking bad combination of genes and creating better one resulting in increase of more fit genotypes in the population. To reach such association, genes that control recombination should more often appear in a company of advantageous genes (Eshel & Feldman, 1970) or they should be directly advantageous (Dawkins, 1976). This exactly is the case for competence-signal-encoding genes (*comQ, comX*) of *Bacillus subtilis* (Oslizlo et al., 2014), where the lack of these genes decreases fitness and dramatic increases the recombination rate in presence of wild population, indicating that evolutionary stability of recombination in *B. subtilis* species can be at least partly explain by FAR effects.

So is this important for *B. subtilis* and other species that may carry similar QS locus that modulates similar traits (Dogsa et al., 2014)? *ComQXP* QS locus accounts for highly variable and vastly-evolving as compared to the rest of the genome, and loss-of-signaling function mutations were likely to occur during its evolutionary history. We propose that consequence of such mutation would create a hyperrecombinator population, which would locally increase the concentration of surfactin, increasing also the DNA release in local neighborhood. An intake of signal-encoding DNA from the wild type would rescue the mutant from fitness costs of surfactin overproduction. Therefore also during the recombination selection acts against the mutant because higher competence of the mutant and its high surfactin release would promote the DNA flow from the WT to the mutant promoting the reversion of QSS-. In addition signal-null hyper-recombinators which are under high selective pressure for reversion could play a significant role in evolution of QS diversity in *B. subtilis*, however this hypothesis awaits further studies.

5.5 QS RESPONSE IN PLANKTONIC AND COLONY COCULTURES OF QS+ AND QSS-

Most bacteria live and consequently also communicate in solid growth mode. The spatial distributions of cells in planktonic cultures and surface attached colonies are very different (Mikkelsen et al., 2007) with large distances between cells in dispersed cultures and close physical contact between cells in the colony. However, the majority of experiments exploring QS responses were performed in stirred planktonic cultures.

In our experimental This may be due to higher signal concentrations in colonies, but colony growth may generate other conditions, e.g. chemical-physical gradients and direct cell to cell

contact, that may influence the QS response setting, the number of planktonic cells present in 10 cm^3 liquid was comparable with the number of colony cells on 0.2 cm^2 of solid medium. Conell and colleagues (2010) studied QS in closed 3D picolitre-scale microcavities and proposed that the QS response is determined by at least three parameters: cell density, diffusion rate and population size. We therefore expected that the substantially higher cell density and presumably lower diffusion rate in colonies would lead to a higher QS response in wild type cells than in planktonic cocultures. This was supported by a three-fold greater cumulative QS response of QS wild types in colonies (Mikkelsen et al., 2007). In addition, it is possible that other factors specific for colony growth mode may play a role. For example, intercellular connections between *B. subtilis* cells in biofilms enable the exchange of cytoplasmic molecules (Dubey & Ben-Yahuda, 2011) and the activity of the signalling molecules after secretion can be altered by many environmental factors as well as interactions with other molecules (Decho et al., 2010). Small peptide signals like ComX pheromone tend to interact with charged molecules and are likely influenced by physical, chemical and biological environmental factors (Li & Tian, 2012). It is therefore possible that close contact between cells in the colony provides a stable environment for the hydrophobic pheromones facilitating its intercellular movement without release into the environment, thereby reducing the activity loss. Interestingly, the signal produced in the mixed colony by the wild type did not appear to be adequately shared with QS mutants. Indeed, cumulative *srfA* expression of the mutant population in the colony was half that of the planktonic culture, despite similar population sizes.

Expression of the *srfA* fluorescent reporter followed a log-normal distribution for all populations studied, except the QS mutant in the colony coculture. An uninduced subpopulation of QS signal-deficient cells was identified in the colony and it reduced the cumulative *srfA* expression of the signal-deficient population. This suggests that similarly to enzymes or extracellular are not entirely „public'' in tightly packed communities, signals also do not diffuse far away from QS+ after secretion. Similar was shown for gram positive bacteria *S. pneumoniae* where cells that can use the secreted signals produced by neighbors but are unable to produce it themselves benefit through signal theft in mass action environments but less so at low diffusivity (Yang et al., 2010). In the same line, Friman et al., (2013) noticed that in conditions where QS was important for *P. aeruginosa* to resist predation, signal negative mutants could not exploit signals produced by the wild type in biofilm and their fitness was reduced. However, the authors did not exclude the possibility that pleiotropic effects of signal-deficiency could contribute to decreased fitness of signal-null mutants (Friman et al., 2013). For example in *B. subtilis* overly responsive QS system stands for pleiotropic effect of being signal deficient. In contrast, in the colony, probably due to limited diffusion, hypersensitivity of QSS- could not be measured with the techniques applied.

It would be expected that the response of QSS- is still strongly increased locally, at the areas of close contact with QS+ cells. Confocal microscopy or fractioning of colony layers using microtome, could help to confirm this prediction in the future.

Ours and other recent results (Yang et al., 2010) imply that the diffusion of the QS peptide signal is significantly limited in colonies and this should be taken into account when theoretically or experimentally addressing QS-regulated social behaviours of microbes.

5.6 INFLUENCE OF ENVIRONMENTAL CONDITIONS ON QUORUM SENSING

5.6.1 Temperature

It is known that *B. subtilis* mainly resides in the upper layer of soil where it experiences a wide variety of environmental challenges. At the same time knowledge on how QS operates in different physical conditions like temperature or salinity, remain scarce. It is known for example that AHLs of gram negative bacteria are in general sensitive to high temperature and their production is abolished at 37°C. In hyperthermal environment peptide based signaling offers an advantage to communicating gram positive bacteria, because their signals display high thermostability (Montgomery et al., 2013). Consistently it was shown that ComX is stable in high temperature of 90°C (Manguson, 1994). Here we accessed the secreted concentration of ComX until T1 (one hour after reaching stationary phase) at three different temperatures, where growth rates of *B. subtilis* significantly differed. As previously reported the growth rate at low temperature (24°C) was significantly slower, which is in line with down-regulated primary metabolic functions of *B. subtilis* at low temperature (Buddle et al., 2005). We saw no effect of temperature on ComX production, however there were significant, temperature related „background' effects on ComX activity and even stronger effects on its activity after freezing (-20°C) and thawing. This indicates that at low temperature (24°C), despite similar concentration of ComX being produced, the conditioned medium cannot induce *srfA* expression in the tester to the same level as either the 37°C or 51°C derived conditioned medium. What are other factors in the medium that influence *srfA* expression and that might be influenced by temperature? ComA activity and in consequence *srfA* expression can be affected by other components secreted to the medium, like Phr peptides (Auchtung et al., 2006). In fact it was shown that oligopeptide ABC transporters involved in import of Phr peptides to the cell are up-regulated during so-called cold shock (Buddle et al., 2005). Since Phr peptides by inhibiting Rap proteins positively affect *srfA* expression (Auchtung et al., 2006), it could be that these peptides are more actively transported into the cells grown at low temperature (15-16°C), and consequently the conditioned medium derived

from low temperature cultures contains less of them and its potential for ComA activation is lower.

Another observation was significant effect on ComX activity after freezing, in the 51°C-derived conditioned medium of *B. subtilis*. This indicated that in 51°C bacterium secretes molecules that dramatically stabilize activity of ComX during freezing. We speculate that this is a side-effect of heat-shock specific proteins which then may serve as ComX-stabilizers during freezing/refreezing, preventing its binding to a storage vessel. This might be similar to BSA, which is added to pure protein solutions in standard protein storage protocols. The question is, whether this is important and should we bother? In fact it is not likely that in natural habitat *B. subtilis* faces very high temperatures (51°C) immediately followed by freezing/refreezing, so we conclude that ComX is stabilized by high-temperature secretions by chance and that this mechanism is not a selected adaptation. More interesting question is why at 51°C ComX is still being produced, despite abolished *srfA* transcription? One possibility is that it may be involved in regulation of other traits expressed at 51°C in density dependent manner. The role of ComX in high temperature (if any) therefore remains to be discovered.

5.6.2 Salinity

Influence of salinity on QS of *B. subtilis* has not been addressed before. Surprisingly we discovered that bacteria adapted differently to salt stress when grown in planktonic culture and in biofilm. Briefly in planktonic culture, *srfA* synthesis is abolished again despite similar amounts of QS signal produced in 0% and 8% NaCl. On the other hand in the biofilm *srfA* expression in 8% was the same (in domesticated strain) or significantly higher (in undomesticated strain) as compared to CM medium not supplemented with additional salt. It was reported that transcription of *srfA* requires DNA binding by PerR (Hayashi et al., 2005). This regulon is related to oxidative stress that leads to decreased DNA-binding activity of PerR, and consequently inhibition of *srfA* transcription (Hayashi et al., 2005). Since salt stress results also in low PerR, this could also result in lack of *srfA* induction.

Why is this not the case in biofilm? Different osmotic pressure Cells in biofilms face increased osmotic pressure even in low salt medium and may thus differently respond to salt stress than planktonic cells. Surprisingly our results show that increased surfactin expression in 8% NaCl negatively influences biofilm biomass. This opens an interesting question on the role of surfactin in adaptation to salt stress in biofilm.

5.6.3 Surfactin

Surfactin synthesis is one of the major responses induced by ComX signal (Comella & Grosmann, 2005) and it was shown that besides its antimicrobial properties it serves as a

signal that induces biofilm formation and cannibalism (Lopez et al., 2009a; Lopez et al., 2009b). It was also shown that in biofilm surfactin negatively regulates its own production (Lopez et al., 2009a). We discovered that in planktonic culture the effects of surfactin on *srfA* gene expression are positive. It occurs that presence of surfactin increases response of the tester strain to ComX. There are several possible explanations for this effect and one could be that surfactin as biosurfactant simply improves solubility of ComX in the medium making it more accessible for bacteria. In fact ComX with its hydrophilic amino acid tail and large hydrophobic tail is expected to form micelles at high concentrations and surfactin could prevent this effect. Another possibility is that surfactin, since it can be incorporated into *B. subtilis* membrane and form small pores (Lopez et al., 2009b), it may change membrane properties and influence ComP-ComX interaction. Whatever is the case our result shows that a molecule, which is produces in response to ComX signal, can potentiate ComX sensing. The dynamics of ComQXPA QS response were measured in domesticated strains (Schneider et al., 2002), which do not produce surfactin due to mutation in *sfp* gene (McLoon et al., 2011). This dynamics may be completely different in a wild isolate, which secretes surfactin. In gram negative bacteria QS response shows a very sharp induction pattern because QS signals like AHL act as autoinductors and induce their own expression in so called autocatalytic loop (Miller & Bassler, 2001). In *B. subtilis*, surfactin could play an analogical role and potentiate a pattern of QS response typical for gram negative bacteria.

5.7 DIVERSITY OF QS LANGUAGES, PLANT GROWTH PROMOTING AND BIOCONTROL PROPERTIES OF *Bacillus* spp. ISOLATES FROM TOMATO RIZOPLANE

B. subtilis strains that persist in soil are classified to four distinct QS groups - pherotypes (Ansaldi et al., 2002, Stefanic & Mandic-Mulec, 2009). As plant rhizosphere was proposed the main habitat of *B. subtilis* vegetative existence (Norris & Wolf, 1961), we asked here, whether the pherotype diversity is also found among rhizoplane isolates.

We confirmed that different QS groups (pherotypes) can coexist on roots of a single plant. This was shown by *comQ* sequence analysis and by specific induction of QS response. Despite low number of *B. subtilis* isolates per plant (6 isolates from plant 16 and 4 isolates from plant 14), we found a comparable diversity, manifested in three pherotypes, which was previously observed for soil millimeter scale (Stefanic & Mandic-Mulec, 2009), confirming that *comQXP* diversity is widespread and easy to find. This again brings up the fundamental question on adaptive role of this diversity, which is also found in other gram positive bacteria, including *Staphylococcus aureus, Streptococcus pneumoniae* (Pozzi et al., 1996; Ji et al., 1997; Whatmore et al., 1999), and *B. cereus* (Slamti & Lerecius, 2005). Stefanic et al. (2012) found

that most but not all *B. subtilis* strains of the same ecotype belong to the same pherotype and proposed that pherotypes may at least in part be under ecological selection. Rhizoplane strains were not analyzed for ecotype association but their high phenotypic diversity (e.g. variability in surfactin production and other biocontrol properties) suggests that many of them may be ecologically distinct. Interestingly, isogenic strains of *S. pneumoniae* of distinct pherotypes differed in their ability to form biofilms, possibly due to pherotype-dependent strength of QS signaling (Carrolo et al., 2014). Here we found no correlation between QS type (pherotype) and the expression of a QS-regulated trait – surfactin production. This is in contrast to observation by Carrolo and colleagues (2014), who noticed such interrelationship, but it should be stressed that they used isogenic strains which only differed in pherotypes. We used wild isolates of different genetic backgrounds that may additionally influence the surfactin synthesis and secretion and dominate over pherotype association.

Strong variability in surfactin production among rhizoplane isolates, even among isolates of a single plant, may also influence social life of this species. Since surfactin is secreted and probably shared between neighboring *B. subtilis* strains, it may, under certain conditions, serve as a public good (West et al., 2007). Differences in surfactin production could also result in disproportions in metabolic investment and fitness among strains (Oslizlo et al., 2014) allowing weak surfactin producers to benefit from strong producers when plant pathogen invades and needs to be opposed. Coexistence of social and less social phenotypes was previously found in neighboring strains of *Myxococcus xanthus* (Kreamer & Velicer, 2014) or *Pseudomonas aeruginosa* (Wilder et al., 2011). In case of *M. xanthus* it was shown that ,,social'' strains can promote the persistence of less social isolates without negative effects on the social's fitness, but sometimes when highly abundant, the less social strains can decrease fitness of the whole community (Kreamer & Velicer, 2014). It was recently suggested that spatial segregation facilitates the evolution of cooperation in *B subtilis* biofilms (van Gestel et al, 2014). Therefore, high spatial segregation of different pherotypes might help to stabilize cooperative traits and public good production, like surfactin. Consequently selection for cooperation my lead to assortment and diversity of pherotypes even at small distances. In addition it was recently discovered that *B. subtilis* colonizes hyphae of *Aspergilus niger* where surfactin expression is down regulated by these fungi (Benoit et al., 2014). Could then the decreased level of surfactin secretion in certain *B. subtilis* strains (like T16-2 orT14-4) be an adaptation to peaceful coexistence with fungi, which are also common rhizoplane inhabitants?

It is not known how presence of poor surfactin producers influences the performance of the whole *B. subtilis* community in fighting plant pathogens and it is an interesting problem of sociomicrobiology which could be addressed in the future. It is also worth noting that some of the rhizoplane isolates (T16-10 or T16-2) produce surfactin even if it does not contributes to biofilm biomass under laboratory conditions as they do when lack of this lipoptide entails a dramatic decrease in biofilm biomass, as found for strains T16-8 and T16-5. It is known that

surfactin serves as a paracrine signal for biofilm formation (Lopez et al., 2009a). Our results, however, indicate that surfactin role in biofilm formation may be more complex and strain-specific. This result also further supports previous assumptions about high genetic and phenotypic diversity of strains sharing a pherotype.

We observed only moderate diversity at the level of biofilm formation. Although we did not look into biofilm-related gene expression, our results are in line with previous data showing that genetic diversity within *B. subtilis* is especially high with respect to antibiotic-related genes (like surfactin) and low with respect to biofilm-related genes (Earl et al., 2007). The reason for the later may be associated with attached growth being essential for rhizocompetence and probably represents a competitive advantage in rhizoplane.
While our strain isolation strategy does not allow us to speculate on the original spatial distribution among the *Bacillus* spp. isolates on roots, the results confirm that different pherotypes reside on roots of a single plant. So does the QS diversity within root-associated *B. subtilis* matter for the plant?
Eldar (2011) and Stefanic and colleagues (2012) proposed that ratios of pherotypes continuously cycle in nature due to induction of costly products released by high-frequency pherotype and temporary advantage of the low frequency pherotype. Therefore the diversity of pherotypes could be naturally selected by means of social conflict based on release of costly products (Eldar et al., 2011, Stefanic et al., 2012. However, since members of one pherotype dramatically differ in QS-regulated biocontrol traits, direct effects on plant growth and other potential PGP behaviors (IAA secretion, production of siderophores or phosphate solubilization), pherotype diversity would not threaten the biocontrol function of the *Bacillus* community – eventually each pherotype contains a strong surfactin producers, or/and direct plant-growth promoters. Therefore, the diversity of pherotypes on plant roots may promote coexistence of different strains and thus positively influence the biocontol potential of this species.

The idea to use microbes as biocontrol agents emerged many years ago and many studies screening for PGP properties of rhizosphere and rhizoplane isolates were performed. Some strains were patented and its commercial use is steadily increasing (Maheshvari, 2011). Although it is known that diverse community of microbes determines the plant health, PGPR-based preparations are still based on monocultures (Maheshvari, 2011). There were several successful attempts of applying multispecies formulations (Raupah & Kloepper 1998; Singh et al., 1999), but sometimes, simply due to interspecies competition, the effects can be just opposite to the expectations (Chiarini et al., 1998; de Boer et al., 1999). In fact in terms of sharing a niche, one should rather expect the competition not a synergy between microbial species (Foster & Bell, 2012). It is therefore important to bring more attention to intraspecies

genetic and phenotypic diversity in the rhizosphere and rhizoplane, where next to strong indirect competition, more cooperation, especially within a pherotype would be predicted. Testing biocontrol properties or plant-growth promotion effects of mixed *B. subtilis* communities should be the next step to answer this question.

We show here that *B. subtilis* residing on roots differ in QS pherotypes, potential PGPR traits and the ability to influence the growth of *A. thaliana*. We believe this study opens new interesting questions about the role of strain diversity in arms race between *B. subtilis* and plant pathogens, or in interactions with host plant. We also believe that applying diverse strains of one genus or species carrying diverse biocontrol properties should be considered as an alternative to non-monoculture-based biocontrol agents design.

Finally, why do we find such high diversity within the *B. subtilis* species anywhere we sample (soil, desert, rhizoplane)? In fact *B. subtilis* is not an exception in highly diversified microbial world. New molecular methodologies such as metagenomic sequencing revealed remarkable genomic plasticity within bacterial species that can be ~100% identical in 16S rRNA but as little as 40% conserved in total gene content (reviewed in Cordero & Polz, 2014).

Recent analysis of whole genomes and carbon source utilization patterns of very closely related *B. subtilis* strains (99,4% average nucleotide identity in core genome), revealed that they were ecologically distinct, which indicates that ecological diversification within a species can be very rapid evolutionary process (Kopac et al., 2014).

Intraspecific competition for resources is one of the most powerful forces shaping evolution and in an environment providing multiple ecological niches, such as a static liquid culture or a biofilm, competition can lead to selection for variants that are better suited to colonize these alternative niches (reviewed in Hibbing et al., 2013). In fact evolution experiments show that diversity can readily emerge from genetically uniform population and produce new genotypes each with its own niche preferance (Rainey & Travisano, 1998). A non-transitive interaction network resembling rock-paper-scissors game (A dominates species B, which out-competes C, which in turn out-competes A) can be another diversifying force. (Kerr et al., 2002). Also evolutionary arms race of bacteria and their viral predators can induce and maintain intraspecies diversity (Marston et al., 2010) for example generating negative frequency-dependent selection (Rodriguez-Valera et al., 2009). Cordero and Polz (2014) have recently attempted to explain intraspecific genetic diversity in the light of evolutionary ecology. They have proposed that highly conserved genes within the species are most likely to encode for core metabolic functions and should be habitat-independent, or they will encode for habitat specific functions and segregate the ecological populations. On the other hand, genes of medium or low frequencies within the species (those that are strain specific) will probably be the signatures of local interactions ecology (like competition or predation) which create negative frequency-dependent selection (Cordero & Polz, 2014).

High throughput genome studies of global and local scale collections of *B. subtilis*, combined with powerful evolution experiments, will enable to uncover the forces that shape the striking social diversity of this bacterium (e.g. in pherotypes, in biopesticide and other plant- microbe interaction traits etc).

5.8 CONCLUSIONS

- QS signal production in *B. subtilis* is linked to regulation of QS response, so that deletion of signaling function results in overly responsive QS system in the presence of the signal ComX provided by the wild type cells in coculture or added to the mutant.

- Overresponsive QS system in signal null mutant manifests in increased surfactin production and competence development the presence of the signal.

- The QS signal ComX can serve as public good in conditions where genetic exchange between strains can guarantee better survival of the population. In this condition signal-null mutant becomes hypercompetent in the presence of ComX and can act as hypercheater.

- Signal-null mutants cannot compete with wild type population because increased QS response of the mutant to the signal produced by the wild type is associated with fitness costs that decrease the frequency (ratio) of the mutant in coculture with the wild type.

- QS response of the wild type and signal-null mutants differs in planktonic culture and colony. When grown together QS response of the wild type is stronger in the colony and the opposite is true for the mutant,- The mutant shows bimodal distribution of QS response in the colony with part of the population not responding to the QS signal and only cells bordering wild type population get induced.

- Conditioned medium produced by *B. subtilis* in low temperature (24°C), negatively influences the activity of ComX QS signal

- In high salt concentration (8%) QS response of *B. subtilis* is down-regulated in planktonic culture, but it can be up-regulated in biofilm. This manifests in different expression of *srfA* gene in planktonic culture and in biofilm.

- Different QS pherotypes can exist within a rhizoplane of a single plant. There is remarkable intraspecies diversity of potential biocontrol traits (like biofilm formation or surfactin production) and PGP traits within *B. subtilis* isolated from rhizoplane of a single plant.

6 SUMMARY

Bacterial use QS to induce expression of genes that often encode for costly traits important in social struggle for survival. These traits are also of human interest due to their significance in pathogenesis, transformation and evolving antibiotic resistance, proteolysis or finally biocontrol. Evolutionary stability of QS has been addressed several times and phenotypes and fitness of QS deficient mutants was examined. Signal-null mutants were also addressed and the only considered phenotypic effect of a signal deletion was the failure to synthesize the QS signal. Here we show that coevolution of signal and receptor in *B. subtilis* resulted in intracellular coupling of signaling and response, leading to increased response of signal-null mutants and therefore constrains on signaling. Due to these constrains mutants are unable to compete with the wild population. In addition mutants showed increased primary metabolic activity in the absence of the signal but decreased primary metabolism in its presence, emphasizing the link between QS and metabolic balance in the cell. Finally, fitness loss of the signal-deficient mutant is caused by the second, surfactin-linked mechanism that acts even in the absence of metabolic constraint. This work shows novel mechanism of QS regulation in gram positive bacteria and indicates that sharing QS signals with others may be evolutionary stable simply due to direct benefits of signaling. Despite selfish benefits of making the signal, we also show that ComX production can benefit others allowing them to develop competence and exchange genes in terms when new genotypes have better chance of survival. In such situation mutants can cheat being overly competent. However, this does not last for long because they also pay costs of surfactin production. Finally, our results indicate that signals in colony may not be fully shared, suggesting another possible explanation for stability of signaling in *B. subtilis.* We show that QS can be affected by environmental conditions like temperature and salinity, which opens an interesting question like on the role of increased QS response in biofilms grown in high salt concentration. Finally we show here that *B. subtilis* strains residing on roots differ in QS pherotypes, potential PGPR traits and ability to influence the growth of plants. We believe this opens new interesting questions about the role of this diversity in arms race between *B. subtilis* and plant pathogens, or on its potential role in interaction with host plant. We also believe that applying diverse strains of one species should be considered as an alternative direction for non-monoculture-based biocontrol agents design.

7 POVZETEK

Že desetletja je znano, da bakterije niso povsem neodvisna enocelična bitja, katerih edini »cilj« je neprekinjena rast in delitev. Mikrobi namreč živijo v združbah, kjer kolektivno sprejemajo odločitve, ki so pomembne za preživetje (Tomasz, 1965; Nealson in sod., 1970). Tako kot pri večceličnih organizmih je tudi pri enoceličnih bakterijah pomembno, da med sabo komunicirajo in tako uskladijo odzive. Mikrobi komunicirajo preko sistema za zaznavanje kvoruma (ang. quorum sensing; QS). V tem procesu, mikrobi med rastjo konstantno izločajo majhne molekule, ki se lahko kopičijo v bližini celic. Ko signali dosežejo kritično koncentracijo, lahko aktivirajo njim specifične receptorje, kar sproži ekspresijo določenih genov (Waters & Bassler, 2005; Platt & Fuqua, 2010). Visoka lokalna koncentracija signalnih molekul je večinoma povezana z visoko celično gostoto, vendar tudi ena sama celica, ujeta v dovolj majhnem prostoru, lahko zazna kritično koncentracijo QS signalov, kar sproži QS odziv (Carnes in sod., 2010). Velja tudi, da kljub visoki lokalni gostoti, signalne molekule lahko izgubijo aktivnost (zaradi visoke temperature ali neprimernega pH), kar prepreči indukcijo QS odziva. Torej v procesu imenovanim ,,zaznavanje kvoruma" celice zaznavajo lokalno koncentracijo QS signalov, ti pa se lahko kopičijo in ostanejo aktivni v različnih okoljskih pogojih (Platt & Fuqua, 2010).

Raziskave so pokazale, da je QS lahko pomemben za preživetje bakterij (Diggle in sod., 2007a; Carnes in sod., 2010). V takem primeru QS signali in molekule, ki so del QS odziva in se izlivajo iz celice, služijo kot skupne dobrine. Te dobrine si celice-proizvajalke delijo, vendar jih lahko izkoriščajo tudi celice, ki jih ne proizvajajo a so v bližini (Diggle in sod., 2007a; Xavier in sod., 2011; Yang in sod., 2010). Take celice imenujemo tudi ,,nepoštene" mutante ki so lahko prisotni v naravnih populacijah nekaterih vrst bakterij, kot na primer pri bakteriji *Pseudomonas aeruginosa* (Rumbaugh in sod., 1999; Denervaud in sod., 2004). Zanimivo je, da takšne mutante lahko v laboratorijskih pogojih prerastejo populacije divjega tipa, ker prihranijo metabolne stroške sinteze dobrin in se na ta račun hitreje delijo (Diggle in sod., 2007a; Yang in sod., 2010). Torej eno od ključnih vprašanj iz področja evolucije komunikacije in socialnih interakcij je: »Kako se lahko QS ohranja v naravnih populacijah in kaj prepreči invazijo nepoštenih mutant-goljufov?«

Večina raziskav usmerjenih v to problematiko se osredotoča na skupne dobrine, ki se izlivajo iz celice. Med temi so na primer encimi ali biosurfaktanti. (Drescher in sod., 2014; Xavier in sod., 2011; van Gestel in sod., 2014). Predvidevamo, da te dobrine predstavljajo večji presnovni strošek v primerjavi s QS signali, (Haas, 2006). Kljub temu so lahko QS signali, predvsem peptidi, ki jih proizvajajo gram pozitivne bakterije metabolni strošek (Keller & Surette, 2006; Yang in sod., 2010) in skupna dobrina, še posebej v pogojih, kjer pozitivno vplivajo na fitnes celic v populaciji (West in sod., 2006). Kot so pokazali je produkcija QS

signalov, kot tudi produkcija sekundarnih metabolitov, ki so uravnavani z QS v nevarnosti, da jo tisti, ki ne prispevajo k sintezi signalnih molekul »goljufajo« in zato tudi prerastejo (Yang in sod., 2010).

ComX je komponenta ComQXPA QS sistema gram pozitivne bakterije *Bacillus subtilis* (Manguson in sod., 1994; Schneider in sod., 2002). ComX se sintetizira iz petinpetdesetih amino kislin dolgega prepeptida, ki ima na triptofanskem ostanku vezan izoprenoid. Za modifikacijo in procesiranje ComX zadošča protein ComQ, ki verjetno sodeluje tudi pri izolčanju peptida iz celice (Schneider in sod., 2002; Ansaldi in sod., 2002). Ko feromon doseže kritično koncentracijo, se ta veže na receptor ComP, ki je vezan na celično membrano, kar sproži njegovo avtofosforilacijo. Fosforiliran ComP nato fosforilira odzivni regulator ComA, ki sproži QS odziv (Weinrauch in sod., 1990). Fosforiliran ComA neposredno regulira izražanje številnih genov, med drugim tudi operona *srfAA*, ki je ključen za sintezo surfaktina (lipopeptidnega antibiotika) in za razvoj kompetence za transformacijo (Dubnau, 1991; Comella & Grosmann., 2005). Surfaktin lahko pomaga *B. subtilis* v neposredni konkurenci z drugimi vrstami bakterij, kot na primer *Pseudomonas syringae* (Bais in sod., 2004). Genetska kompetenca za transformacijo lahko v določenih pogojih prispeva k preživetju populacije (Johnsen in sod., 2009). Ali je ComQXPA QS sistem nujen za preživetje *B. subtilis* ? Če je to res, kako ta bakterija prepreči invazijo celic goljufov? To so bazična vprašanja in naslavljajo le del neznanega o medcelični komunikaciji.

Mikrobi obraščajo površine in planktonski način življenja je manj razširjen v mikrobnem svetu (Costerton in sod., 1995). Kljub temu je večina raziskav zaznavanja kvoruma bila narejena na stresanih in zato homogenih planktonskih kulturah. V kolonijah in v biofilmih so razdalje med posameznimi celicami neprimerljivo manjše, poleg tega pa so za pritrjene oblike življenja značilni gradienti fizikalno kemičnih parametrov: kisika, pH, hranil (Decho in sod., 2010). Dinamika zaznavanje kvoruma in sama difuzija signalnih molekul v biofilmih niso poznani) Poleg tega zelo malo vemo o povezavah med dejavniki okolja in zaznavanjem kvoruma: kako vplivajo ekstremna slanost, pH, temperatura na zaznavanje kvoruma. Na primer QS signali gram negativnih bakterij, acil homoserin laktoni (ang. acyl homoserine lactone; AHL), so občutljivi na visoko temperaturo, saj se stopnja njihove produkcije ob zvišani temperaturi zniža (Latour in sod., 2007; Tait in sod., 2010). Po drugi strani pa so peptidni signali gram pozitivnih bakterij temperaturno stabilni (Manguson in sod., 1994). Vendar ne vemo ali temperatura vpliva na izlivanje signalov.

Znano je, da je *B. subtilis* rizobakterija in lahko posredno ali neposredno spodbuja rast rastlin (Earl in sod., 2005). Na primer, sinteza surfaktina je uravnavana s QS, ta lipopeptidni antibiotik pa negativno vpliva na rastlinski patogen *P. syringae* (Bais in sod., 2004). Surfaktin je tudi pomemben kot signal za kolonizacijo korenin (Zeriouh in sod., 2014). Zanimivo je, da

najdemo znotraj QS sistemov ComQXPA visok polimorfizem, ki zagotavlja, da peptidi ene komunikacijske skupine (ferotipa) predvsem aktivirajo sisteme istega ferotipa. Medtem ko signal ne more aktivirati QS sistem drugega ferotipa oziroma druge ,,jezikovne" skupine (Ansaldi in sod., 2002). Ta polimorfizem so odkrili tudi pri izolatih *B. subtilis* izoliranih iz majhnega talnega vzorca, kar kaže, da lahko sobivajo (Stefanic & Mandic-Mulec, 2009), vendar ni znano, ali je ta polimorfizem prisoten tudi med izolati, ki jih najdemo v rizosferi ene rastline.

V tej doktorski disertaciji smo preučevali regulacijo in stabilnost zaznavanja kvoruma; delovanje le tega v pogojih planktonskega (stresana kultura) ali pritrjenega (kolonija); vpliv dejavnikov okolja na medcelično signaliziranje. Poleg tega smo tudi preučevali raznolikost QS sistema ComQXPA pri sevih izoliranih iz rizosfere paradižnika. Te seve smo tudi natančno okarakterizirali kot potencialne vzpodbujevalce/zaviralce rasti korenin in celo kot zaščitnike pred patogeni. Rezultati bodo prispevali k boljšemu razumevanju socialne mikrobiologije in poznavanju mehanizmov, ki ohranjajo medcelično komunikacijo evolucijsko stabilno in neobčutljivo na pojav goljufov, ki ne sintetizirajo signalov.

V nalogi smo postavili naslednje hipoteze:

- dinamika QS odziva mutant, ki ne proizvajajo QS signala QSS- *(*Δ*comQ;* Δ*comX*) je drugačna kot dinamika odziva divjega tipa, v pogojih, kjer obe populaciji izpostavimo enaki koncentraciji signala
- prisotnost mutant QSS- v populacij *B. subtilis* bo vplivala na QS odziv te populacije
- ComX signal je lahko skupna dobrina, ki pozitivno pripeva k preživetju *B. subtilis*, ki je izpostavljen selekcijskemu pritisku
- način rasti in dejavniki okolja (temperatura in slanost) lahko vplivajo na QS
- v rizosferi ene rastline lahko najdemo različne ferotipe oz. jezikovne skupine ComQXPA

Da bi preverili ali se dinamika QS odziva divjega tipa QS+ in mutanta QSS- razlikujeta, smo sledili QS odziv na nivoju posamezne celice z uporabo fluorescenčne mikroskopije. Zato smo uporabili udomačene seve QS+ in QSS- označene z fluorescenčnimi markerji oziroma *srfA-yfp* in *srfA-cfp* (QS odziv sproži ekspresijo gena *srfA* v celotni populacij udomačenih sevov *B. sutbilis*). Meritve fluorescence obeh sevov tekom rasti v kokulturi so potrdile, da je dinamika QS odziva v QS+ in QSS- drugačna in da QSS- v kokulturi s QS+ prej doseže svoj maksimalni QS odziv. Da bi bil rezultat bolj zanesljiv smo uporabili tudi kontrolne seve, z zamenjanima fluorescenčnima markerjema (QS+: *srfA-cfp* in QSS-: *srfA-yfp*). Kontrolni poskus je pripeljal do enakih zaključkov. Za nadaljnje raziskave smo uporabili ComX signal, ki smo ga heterologno izrazili v *E. coli* in nato očistili z metodo tekočinske kromatografije

visoke ločljivosti (ang. high performance liquid chromatography – HPLC). Monokulture QS+ in QSS- smo izpostavili enaki koncentraciji očiščenega peptida ComX ter spremljali QS odziv preko dveh neodvisnih metod: spektrofluorometrije (tukaj smo uporabili seve QS+: *srfA-yfp* in QSS-: *srfA-yfp* ter QS+: *srfA-cfp* in QSS-: *srfA-cfp*) in testa aktivnosti beta-galaktozidaze (z uporabo sevov QS+: *srfA-lacZ* in QSS-: *srfA-lacZ*). Ti eksperimenti so pokazali, da mutacija v genu *comQ*, ki odgovarja za procesiranje signalne molekule, povzroči povečan odgovor na signal ComX.

Prav tako smo pripravili kokulture sestavljene is QS+ in QSS- v različnih razmerjih. Predpostavili smo, da se bo z naraščajočim deležem QSS- mutante koncentracija dostopnega QS signala v izrabljenem gojišču kokulture znižala. To smo tudi potrdili preko testiranja izrabljenih gojišč teh kokultur s pomočjo testerskega seva QSS- (*srfA-lacZ*). Opazili smo, da kljub temu, da je kokultura sestavljena v 80 % QSS- mutant, izrabljeno gojišče lahko še vedno vsebuje kritično koncentracijo ComX. Torej 20 % QS+ celic v populaciji sintetizira dovolj ComX za maksimalni QS odziva testerskega seva. Dodatne analize QS odziva, QS+ in QSS- v kokulturah, so pokazale, da lahko prisotnost QSS- negativno vpliva na QS odziv celotne populacije zaradi znižane koncentracije signala, ampak samo takrat, ko je QSS- prisoten v zelo visokim deležu (>80%).

Glede na to, da smo pokazali da mutacija v genu *comQ* poveča občutljivost mutant na signal ComX nas je zanimalo kakšne so metabolične posledice povečanega odziva mutant. Nadaljevali smo raziskave z uporabo neudomačenega seva *B. subtilis* PS216, ki je bil pridobljen iz tal nabrežja Save (Stefanic & Mandic-Mulec, 2009). Ta v nasprotju od udomačenih sevov (na primer BD2833 ali BD2876), pod vplivom ComX izloča surfaktin. Zanimalo nas je ali bo sev PS216 QSS- v prisotnosti signala izločil več surfaktina v primerjavi z divjim tipom, kar je logična predpostavka glede na predhodne raziskave z udomačenimi sevi. Tako smo očistili in z HPLC kvantificirali surfaktin iz izrabljenih gojišč obeh sevov in ugotovili, da QSS- sev v prisotnosti ComX sintetizira bistveno več surfaktina, kot QS+ sev. Zanimivo je, da pri neudomačenih sevih povečana koncentracija surfaktina ne korelira z povečanim izražanjem gena *srfA* v celotni populaciji. Še vedno odzivna populacija QSS- v prisotnosti ComX predstavlja večji del celotne populacije v primerjavi z odzivno populacijo divjega tipa v prisotnosti ComX. . Povečan odziv na signal ComX signalne mutante smo prav tako zasledili preko analize genetske kompetence za transformacijo. Prav tako smo opazili, da je mutant QSS- v kokulturi s QS+ je bistveno bolj kompetenten (pridobili smo 6 do 16-krat več transformant; frekvenca transformacije je bila tudi odvisna od tipa DNA, ki smo jo uporabili za transformacijo). S fluorescenčno mikroskopijo smo potrdili, da v populaciji mutant z dodanim ComX polovica vseh celic izraža gen *comK*, ki je glavni regulator kompetence, medtem ko isti gen izraža samo 5 do 10% celic divjega tipa.

Ugotovili smo tudi, da ima mutant QSS- znižan fitnes v kokulturi z divjim tipom ali v prisotnosti ComX. Predpostavili smo, da je za znižan fitnesa odgovorno prekomerno izražanje

vsaj ene od dveh lastnosti: sinteze surfaktina oz razvoja genetske kompetence za transformacijo. Izkazalo se je, da inaktivacija kompetenčnih genov ne prepreči negativnega učinka ComX na fitnesmutante, medtem ko inaktivacija *srfAA* operona skoraj docela prepreči negativen učinek na fitnes. Torej je sinteza surfaktina glavni fitnes strošek in vzrok poraza QSS- v tekmovanju s QS+ v kokulturi. Da bi ta zaključek potrdili, smo izpostavili dvojni mutant QSS-A- tekmovanju s QS+. Pričakovali smo, da se po 8 urah razmerje med sevi ne bo spremenilo, vendar je QS+ sev prerasel QSS- sev. Raziskava prvič dokaže, da protein ComQ, ki je del sistema za zaznavanje gostote, deluje kot "feedback" represor, ki zniža odziv celic na signal divjega tipa in posledično so mutante, ki signala ne sintetizirajo zaradi mutacije v *comQ* „kaznovane" s prekomerno odzivnostjo na signal. To povečano odzivnost plačajo tudi s povečano občutljivostjo na lipopeptidni antibiotik surfaktin.

V naslednjem delu naloge smo želeli pokazat, da je lahko signal ComX skupna dobrina, ki jo celica-producent deli z drugimi v populaciji. To pozitivno vpliva na fitnes mutanta. Ker je ComX ključen za razvoj kompetence in sprejem DNA, ima mutant, ki ne proizvaja ComX *(*Δ*comQ*) nižjo sposobnost sprejema tuje DNA. Ob selekcijskih pogojih, ki zahtevajo sposobnost izmenjave DNA (na primer sprejem genov za antibiotično rezistenco) ima tak mutant bistveno nižji fitnes v primerjavi z divjim tipom. To smo pokazali z uporabo kultur QS+ ali QSS- ki so bile označene z rezistenco na različna antiiotika (kloramfenikol ali spektinomicin). Ob selekcijskem pritisku za izmenjavo DNA in tvorbo novih genotipov (dvojna rezistenca: kloramfenicol+spektinomicin) je bilo preživetje QS+ boljše, ker je v planktonski kokulturi dveh sevov prišlo do izmenjave DNA med populacijama.

Zanimivo je, da je mutant Δ*comQ* prekomerno občutljiv na signal ComX (Oslizlo in sod., 2014) in v prisotnosti ComX tudi prekomerno kompetenten za transformacijo. Torej so signalne mutante v prisotnosti divjega tipa, ki producira signal, supergoljufi. V kokulturi z divjim tipom mutant sprejme selekcijski marker v večjem deležu kot divji tip. Prednost mutanta pa ni dolgega veka, saj povečana občutlijvost na ComX pomeni tudi supersodelovanje v obliki povečane produkcije surfaktina in povečane občutljivosti na ta lipopetid (Oslizlo in sod., 2014). Ugotovili smo tudi, da surfaktin spodbudi izločanje DNA v zunajcelični prostor, kar dodatno prispeva k skupnim dobrinam. Raziskava torej prvič pokaže, da je goljufanje na nivoju ComX komunikacije le kratkoročno koristno in da je kooperativna izmenjava DNA med partnerji lahko stabilna evolucijska strategija.

V naslednjem delu naloge smo primerjali medcelično komunikacijo *B. subtilis* pri dveh načinih rasti: planktonsko rast v stresanih kulturah v tekočem gojiču ter pritrjeno rast, ki je značilna za bakterijske kolonije. Zat preverjanje teh vprašanj smo uporabili udomačene seve *B. subtilis* z fluorescenčnimi markerji (QS+: *srfA-yfp* in QSS-: *srfA-cfp*). Sledili smo rast in QS odziv obeh sevov v kokulturah in ugotovili, da je QS odziv divjega tipa močnejši v koloniji in obratno, da je QS odziv mutant močnejši v tekoči kulturi. S pomočjo fluorescence smo izmerili izražanje *srfA* na nivoju posamezne celice in nato s pomočjo programa Origin

Pro izrisali distribucije izražanja *srfA* v vseh populacijah. Ugotovili smo, da se v mešani koloniji QS+ in QSS- distribucija izražanja *srfA* seva QSS- bistveno razlikuje od QS+ in predstavlja bimodalno porazdelitev. Sklepali smo, da je v koloniji najverjetneje difuzija signala ComX omejena, kar povzroči znižan odziv mutante, v primerjavi z tekočo kokulturo, kjer je ComX homogeno razporejen med celice QS+ in QSS-.
Preučevali smo tudi vpliv temperature in slanosti na medcelično komunikacijo pri *B. subtilis*. Zanimalo nas je ali visoka temperatura (51 °C) pozitivno vpliva na akumulacijo signala ComX v gojišču (Dogsa in sod., neobjavljeno delo). Seve QS+ in QSS- smo gojili pri treh različnih temperaturah (24 °C, 37 °C in 51 °C). Izrabljeno gojišče smo vzorčili v času T1. Izrabljeno gojišče seva QSS- nam je služilo kot kontrola vpliva ozadja, kamor smo dodali enako koncentracijo očiščenega ComX (pridobljenega iz *E. coli*). Vzorce gojišča QS+ in kontrolne vzorce QSS- z dodanim ComX smo nato inkubirali s testerskim sevom (*srfA-lacZ*) ter spremljali njegov odziv preko testa aktivnosti beta-galaktozidaze. Ugotovili smo, da visoka temperatura ne vpliva na akumulacijo ComX v gojišču, temveč da gojišče, v katerem *je B. subtilis* rasel pri nizki temperaturi (24 °C) sproži nižji QS odziv v primerjavi z ostalimi gojišči, ki smo jih pridobili z gojenjem bakterije *B. subtilis* pri višjih temperaturah (37 °C in 51 °C). Kontrolni poskus je pokazal, da znižana koncentracija ComX v gojišču ni vzrok znižane ComX-specifične aktivnosti tega gojišča, ampak da je aktivnost ComX znižana zaradi ozadja (ostale snovi, ki jih *B. subtilis* izloča v temperaturi 24 °C).
Preučevali smo tudi vpliv slanosti na sintezo ComX in na QS odziv. Seve QS+ in QSS- smo gojili v gojišču CM v treh nastavitvah: CM brez dodanega NaCl, CM z 4% NaCl in CM z 8% NaCl. Enako kot pri temperaturnemu eksperimentu, smo z upoštevanjem vpliva ozadja, izmerili aktivnost ComX v izrabljenih gojiščih. Ugotovili smo, da ni značilnega učinka slanosti na produkcijo ComX. Izkazalo se je, da visoka koncentracija NaCl negativno vpliva na izražanje gena *srfA*. Nasproten fenotip smo opazili v biofilmu, kjer je visoka koncentracija NaCl (8 %) spodbudila izražanje *srfA* in sintezo surfaktina, kar smo izmerili s hemolitičnim testom. Zanimivo pa je, da biomasa biofilma QS+ pada z naraščajočo slanostjo, kar se ne zgodi v primeru biofilma iz celic QSS- . Če gojišča na katerem gojimo QSS- sev dopolnimo še s ComX, bo masa biofilma QSS- manjša z naraščajočo slanostjo, podobno kot smo opazili za biofilme seva QS+. Ta rezultat kaže, da povečana produkcija surfaktina pri visoki slanosti negativno vpliva na biomaso biofilma. Rezultati odpirajo zanimivo vprašanje, vezana na vlogo surfaktina pri adaptaciji na visoko slanost oziroma zaradi slanosti povišan osmotski pritisk.

V zadnjem delu naloge smo analizirali genetske in fenotipske lastnosti več kot dvajsetih sevov iz rodu *Bacillus*, ki so bili izolirani iz rizosfere paradižnika. S pomočjo testerskih sevov različnih ferotipov (168; RS-D-2/NAF4; RO-H-1/RO-B-2; RO-E-2), ki nosijo fuzijo *srfA-lacZ*, smo preučevali specifičnost izrabljenih gojišč teh izolatov za indukcijo QS odziva. Ugotovili smo, da lahko znotraj rizosfere ene rastline sobivajo sevi vsaj treh različnih

ferotipov. Prav tako smo primerjali biomaso biofilma določenih sevov, sposobnost sinteze surfaktina in neposredni učinek na rast modelne rastline *A. thaliana*. Ugotovili smo, da so si sevi zelo različni v naštetih fenotipskih lastnostih in da znotraj ene rastline sobivajo sevi, ki kot taki (v čisti kulturi) spodbujajo ali zavirajo rast rastline. Analizirali smo tudi številne lastnosti, za katere verjamemo, da posredno ali neposredno pospešujejo rast rastlin: izločanje rastlinskega hormona ali sideroforjev, raztapljanje fosfata ali izločanje razgradnih encimov. Med izolati smo identificirali enega iz rodu *Bacillus megaterium*, ki izloča visoke koncentracije rastlinskega hormona, ki za več kot 2-krat poveča biomaso korenin in listov. V sodelovanju s kolegi iz Nacionalnega inštituta za biologijo smo preverjali učinek izbranih izolatov proti rastlinskemu patogenu *R. solanacearum* in tako identificirali izolat iz rizosfere, ki zavira rast patogenov enako učinkovito kot komercialni biopesticid *B. subtilis* GB03 (uporabljen kot pozitivna kontrola).

Na osnovi pridobljenih rezultatov smo sklepali:

- pri *B. subtilis* je produkcija QS signala povezana z regulacijo odziva na ta signal, tako da okvara funkcije signaliziranja pripelje do povečane občutljivosti na ta signal. Posledično v prisotnosti ComX ali populacije divjega tipa, ki prispeva ComX, QSS- mutant kaže povečano sintezo surfaktina ter povečan razvoj genetske kompetence

- prisotnost QSS- mutant lahko negativno vpliva na QS odziv populacije, vendar samo takrat, ko je mutant prisoten v zelo visokem deležu (več kot 80 %)

- signal ComX je lahko skupna dobrina v selektivnih pogojih, ko izmenjava DNA med sevi zagotovi boljše preživetje populacije, ki je DNA (in s tem rezistenčni marker) sprejela. V takšnih pogojih preveč občutljivi mutant razvije povečano kompetenco za transformacijo in se lahko obnaša kot »hipergoljuf«

- mutante, ki ne proizvajajo QS signala so občutljive za surfaktin in ga v prisotnosti ComX prekomerno sintetizirajo, kar zniža njihov fitnes- Zato v takih, neselektivnih pogojih mutante QSS- ne morejo uspešno tekmovati z divjim tipom,

- QS odziva divjega tipa in QSS- mutant sta različna v tekoči kulturi in v koloniji. V kokulturah kolonije je QS odziv divjega tipa višji kot ga izmerimo v stresanih kulturah. Obratno velja za mutanta, ki v koloniji pokaže izrazito bimodalno distribucijo QS odziva, saj se del populacije, ki ni v direktnem stiku z divjim tipom (QS+) ne odziva na ComX. ki ga QS+ proizvaja

- izrabljeno gojišče proizvedeno v nizki temperaturi rasti (24 °C) negativno vpliva na aktivnost signala ComX - pri visoki koncentraciji NaCl (8 %) je QS odziv *B. subtilis* v stresani kulturi znižan, v biofilmu pa povečan

- različni ferotipi ComQXPA QS sistema lahko sobivajo v rizosferi ene rastline. V rizosferi ene rastline lahko najdemo seve z različnimi biokontrolnimi lastnostmi (tvorba biofilma, sinteza surfaktina), ki lahko pozitivno ali negativno vplivajo na rast rastlin

Bakterije običajno uporabljajo QS za modulacijo ekspresije genov, kar je metabolna naložba populacije v skupno dobro in bi naj povečalo preživetje. Te metabolne naložbe so zanimive tudi z antropogenega vidika, saj sistemi za zaznavanje kvoruma vplivajo na patogenost bakterij, na razvoj odpornosti na antibiotike ter so pomembna komponenta v biokontroli rastlin in njihovih škodljivcev. Evolucijska stabilnost QS je vroča tema in pogosto preučevana eksperimentalno. Do nedavnega je bil edini poznani fenotip okvare genov za sintezo QS signala nezmožnost sinteze tega signala, kar ima seveda lahko posledice na rast v okolju, kjer je potrebno izražanje genov, ki so uravnavani pozitivno s QS. V tej nalogi smo pokazali, da je koevolucija signala in receptorja pri *B. subtilis* pripeljala do sklopa signaliziranja in odziva znotraj ene celice. Slednji mehanizem je verjetno odgovoren za povečano občutljivost QSS-mutant na signal. Verjamemo, da posledično nastaja omejitev odmika funkcije sinteze ComX pri *B. subtilis,* ker v kokulturah divji tip vedno uspeva bolje kot mutant. Opazili smo, da imajo QS mutante povečano aktivnost primarnega metabolizma, vendar se ta dramatično zniža na račun sekundarnega metabolizma v prisotnosti ComX. To nakazuje na vlogo QS sistema v regulaciji ravnotežja med primarnim in sekundarnim metabolizmom celic. Znižan fitnes mutant je povezan tudi z drugim, od surfaktina odvisnim mehanizmom, ki deluje tudi takrat, ko ni metabolnih omejitev (ko ima mutant delecijo v genu *srfA* in je neodvisen od metabolnih stroškov sinteze surfaktina). Ta naloga predstavlja nov mehanizem regulacije QS pri po Gramu pozitivnih bakterijah in nakazuje, da je produkcija QS signalov lahko stabilna tekom evolucije, zato ker je neposredno koristno za celico. Razen sebične koristi, ComX lahko koristi drugim, ker pod selekcijskim pritiskom za izmenjavo DNA, omogoča razvoj kompetence in prevzem novim genov iz okolja. V tem primeru lahko mutante zelo uspešno goljufajo, ker postanejo v kokulturi še bolj kompetenčni kot divji tip. Vendar pa njihov uspeh ni dolgotrajen, saj plačujejo tudi stroške sinteze surfaktina. Rezultati tudi pokažejo, da v koloniji QS signal ni popolnoma skupen in je lahko njegova difuzija od producenta k mutantom, omejena.

Ugotovili smo, da osnovni dejavniki okolja vplivajo na QS, kar je odprlo zanimivo vprašanje o vlogi surfaktina pri povečani slanosti v biofilmih *B. subtilis*. Ugotovili smo tudi, da lahko različne jezikovne skupine (ferotipi) sobivajo v rizosferi ene rastline in da se izolati *B. subtilis* bistveno razlikujejo glede na biokontrolne lastnosti. Verjamemo, da ta študija odpira zanimivo vprašanje o vlogi te raznolikosti v oboroževalni tekmi med *B. subtilis* in rastlinskimi patogeni

ali o potencialni vlogi raznolikosti v interakcijah z rastlinskim gostiteljem. Verjamemo, da bi lahko uporaba različnih sevov ene vrste alternativa za načrtovanje biokontrolnih preparatov.

8 PODSUMOWANIE

Bakterie wykorzystują mechanizm komunikacji międzykomórkowej tzw. quorum sensing (QS) w celu regulacji ekspresji niektórych genów. W trakcie QS komórki bakteryjne ze stałą częstotliwością wydzielają cząstki sygnałowe, których koncentracja rośnie wraz z narastającą liczbą samych bakterii. Kiedy sygnały osiągną koncentrację krytyczną, aktywują specyficzne receptory na powierzchni komórek bakteryjnych, te z kolei indukują całą kaskadę sygnalizacji wewnątrzkomórkowej, która prowadzi do aktywacji czynników transkrypcyjnych i ekspresji określonych genów. W rezultacie bakteria aktywuje często kosztowne metabolicznie zachowania takie jak produkcja antybiotyków, ochronnego płaszcza polisacharydowego lub enzymów zewnątrzkomórkowych; ważne w walce o przetrwanie całej populacji, dopiero wtedy, kiedy znajdzie się w otoczeniu wysokiej koncentracji sygnałów, a więc najprawdopodobniej i wysokiej koncentracji identycznych sobie komórek-klonów. Właściwości bakterii będące pod kontrolą QS są bardzo często ważne z punktu widzenia człowieka, ponieważ odgrywają ważną rolę w patogenezie, transformacji bakteryjnej i ewolucji odporności na antybiotyki, proteolizie a także w biokontroli. Stabilność ewolucyjna samego procesu QS była kilkakrotnie stawiana pod znakiem zapytania. Jakie mechanizmy uniemożliwiają inwazję komórek-mutantów, które unikają aktywności QS tj. syntezy cząsteczek sygnałowych, czy też receptorów a więc i całej kosztownej metabolicznie kaskady odpowiedzi QS? Takie komórki, ponieważ same nie płacą metabolicznych kosztów produkcji sygnałów, lub też odpowiedzi na sygnały QS (w przypadku mutacji receptora), mogłyby dzielić się szybciej niż normalne QS-aktywne bakterie, i zdominować populację.

Dotychczas jedyną poznaną konsekwencją wynikającą z mutacji genu odpowiadającego za syntezę cząstek sygnałowych, była niezdolność syntezy tych cząsteczek. W tej pracy pokazaliśmy, że w wyniku ko-ewolucji cząstek sygnałowych i receptorów u bakterii *Bacillus subtilis*, doszło do wykształcenia się wewnątrz-cząsteczkowego sprzężenia funkcji sygnalizacji oraz odpowiedzi na sygnał. W konsekwencji, bakterie QSS- niezdolne do produkcji cząsteczek sygnałowych cechuje nadwrażliwość na sygnały wydzielane przez normalne, QS-aktywne komórki (QS+). Sprzężenie to w pewien sposób wymusza ewolucyjną stabilność produkcji cząsteczek sygnałowych, ponieważ mutanty QSS- przegrywają w rywalizacji z populacją QS+ w mieszanych kulturach bakteryjnych (QSS- vs QS+). Dodatkowo QSS- cechuje zwiększona wyjściowa aktywność na poziomie metabolizmu pierwszorzędowego oraz niska aktywność metabolizmu drugorzędowego, (ponieważ ten w znacznej mierze zależy od obecności sygnałów QS). W obecności cząstek sygnałowych z kolei, aktywność metabolizmu pierwszorzędowego QSS- jest obniżona, natomiast aktywność metabolizmu drugorzędowego dramatycznie wzrasta–, co podkreśla sprzężenie pomiędzy QS i regulacją równowagi metabolicznej komórki bakteryjnej.

Praca ta ujawnia, zatem nowy mechanizm regulacji komunikacji QS u bakterii gram dodatnich. Dodatkowo dowiadujemy się, że dzielenie cząstek sygnałowych z innymi komórkami w populacji pozostaje ewolucyjnie stabilne z powodu bezpośrednich korzyści samego procesu produkcji sygnałów.

Pokazaliśmy także, w jakich warunkach cząsteczka sygnałowa ComX może stanowić tzw. dobro publiczne, a więc przynosić bezpośrednią korzyść komórkom, które ją wykrywają. Sygnał ComX aktywuje proces rozwoju naturalnej kompetencji, która umożliwia przyjmowanie fragmentów DNA z otoczenia i wbudowywania ich w swój genom za pomocą mechanizmu rekombinacji homologicznej. W warunkach środowiska, które faworyzują nowe genotypy, populacja, które ma zdolność do rekombinacji, a więc do wymiany DNA miedzy istniejącymi genotypami, ma zwiększoną szansę na przeżycie. W takich warunkach sygnał ComX umożliwi wykształcenie się naturalnej kometencji, a wiec i rekombinację i przeżycie części populacji. Paradoksalnie nadwrażliwy mutant QSS- jest w stanie, pod wpływem ComX, mocniej aktywować geny odpowiedzialne za kompetencje i lepiej, w porównaniu z normalnym szczepem QS+, przejść przez ,,wąskie gardło'' faworyzujące nowe genotypy. Dominacja QSS- nie trwa jednak dugo, ponieważ w obecności ComX, nadprodukuje on także inne kosztowne metabolicznie dobra publiczne.

Wyniki tej pracy sugerują także, że w trakcie wzrostu kolonii bakteryjnej na podłożu stałym, cząstki sygnałowe nie dyfundują swobodnie od producenta do odbiorcy. Takie ograniczone dzielenie się produkowanymi dobrami publicznymi, może być kolejnym mechanizmem, który zapewnia stabilność ewolucyjną sygnalizacji.

Pokazaliśmy, że warunki środowiskowe takie jak temperatura, czy zasolenie, znacząco wpływają na QS. Nasze wyniki otwierają serię ciekawych pytań min. o rolę powiększonej odpowiedzi na cząstki sygnałowe w biofilmach w obecności wysokiej koncentracji soli.

Dowiedliśmy także, że szczepy *B. subtilis* zasiedlające korzenie roślin, mogą reprezentować różne ferotypy QS (grupy komunikacyjne, które efektywnie komunikują się tylko wewnątrz grupy, lecz nie mogą komunikować się pomiędzy grupami, analogicznie do języków u człowieka). Ponadto szczepy rezydujące na jednej roślinie mogą różnić się w potencjalnych właściwościach biokontrolnych a także różnie wpływać na wzrost korzeni i liści rośliny modelowej *A. thaliana*. Wierzymy, że wyniki te otwierają serie ciekawych pytań o rolę tej wewnątrzgatunkowej różnorodności w ewolucyjnym ,,wyścigu zbrojeń'' pomiędzy *B. subtilis* i patogenami roślinnymi, a także w samych oddziaływaniach pomiędzy *B. subtilis* a rośliną-gospodarzem. Wierzymy także, że zastosowanie kilku różnorodnych szczepów jednego gatunku mogłoby być atrakcyjną alternatywą dla istniejących preparatów biokontrolnych.

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