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Title: QseC controls biofilm formation of non-typeable *Haemophilus influenzae* in addition to an AI-2 dependent mechanism

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Running title: QseC controls *Haemophilus influenzae* biofilm

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Abstract

Non-typeable *Haemophilus influenzae* (NTHi) is a common pathogen associated with diseases such as acute otitis media or exacerbations in patients with chronic obstructive pulmonary disease. The biofilm forming capability substantially contributes to the persistence of NTHi. However, the regulation of biofilm formation is not completely understood. Quorum sensing regulated by autoinducer-2 produced by *luxS* is until now the only described regulatory mechanism. In this study, we show that the two component signalling system QseB/C is involved in the biofilm formation of NTHi *in vitro*. An isogenic NTHi mutant of *qseC* (Hi3655KR2) showed a significant decrease in biofilm formation under static and semi-static conditions as assessed by crystal violet staining. In addition, under constant flow conditions the Δ qseC mutant Hi3655KR2 formed less biofilm after 48 h. The biofilm defects were irrespective of autoinducer-2 levels. Hence, here we suggest for the first time a regulatory circuit in NTHi, which controls biofilm formation by mechanisms other than or in addition to *luxS*-dependent factors.

(158 words)

Key words: biofilm, NTHi, quorum sensing, signalling

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Introduction

Haemophilus influenzae is a Gram-negative commensal that colonizes the upper respiratory tract of humans (Erwin and Smith, 2007). It can be differentiated due to the presence or absence of a polysaccharide capsule into typeable (types a-f) and non-typeable strains (NTHi), respectively. *H. influenzae* is a major respiratory pathogen in pre-school children and in adults with chronic obstructive pulmonary disease (COPD). Since the introduction of a conjugate polysaccharide vaccine against the dominant *H. influenzae* type b (Hib) in the 1990s, the incidence accounted to this serotype was successfully reduced. However, there is strong evidence for a trend towards NTHi replacing Hib in the context of invasive disease in humans (Resman *et al.*, 2010). NTHi is one of the main causes for recurrent otitis media (OM) and tonsillitis, and is often associated with COPD (Murphy, 2006; Leibovitz *et al.*, 2010). OM and COPD are major diseases, with acute OM being responsible for 80 % morbidity among children under the age of 3 years, and COPD causing 5 % of the annual deaths globally with enormous health costs (Nazir and Erbland, 2009). Clinical studies support the hypothesis that biofilm formation is important for severity and persistence of OM as well as COPD (Eldika and Sethi, 2006; Bakaletz, 2007).

Biofilms are complex three-dimensional structures which facilitate bacteria to survive in hostile environments during colonization. Bacteria in biofilms constitute functionally heterogeneous bacterial communities that can exhibit differences in the expression of surface molecules, antibiotic resistance and virulence factors all contributing to persistence in the human host (Hall-Stoodley and Stoodley, 2009). The role of biofilms in chronic diseases caused by NTHi is generally acknowledged (Bakaletz, 2007). There are microscopical observations of bacterial aggregates or micro-colonies of NTHi in patient biopsy samples (Hoa *et al.* 2009). In addition, many NTHi isolates recovered from patients are able to attach to abiotic surfaces as estimated from biofilm formation assays (Moriyama *et al.*, 2009).

Several genetic determinants are identified, which can affect the biofilm properties of NTHi. However, the number of existing studies dealing with regulatory mechanisms controlling the formation of biofilms by NTHi is limited. One such mechanism is quorum

sensing (QS) where small signalling molecules enable the bacteria to sense their population density (Freeman and Bassler, 1999; Piazza *et al.*, 1999). NTHi possesses a *luxS* gene, which is responsible for the production of the signalling molecule autoinducer-2 (AI-2). However, the role of *luxS* and AI-2 during biofilm formation and its contribution to virulence is controversial (Daines *et al.*, 2005; Armbruster *et al.*, 2009). In a more recent study, NTHi-derived AI-2 was also brought into connection with interspecies communication in polymicrobial biofilms in an *in vivo* OM animal model (Armbruster *et al.*, 2010). Another key regulatory mechanism consists of two component signalling systems, which are substantial for adaptation to changing environmental conditions and often are part of quorum sensing processes. The first component, the sensor kinase, senses environmental changes by detecting signalling molecules. This information is transmitted via a phosphorelay to the second component, the respective response regulator, which acts as a transcriptional regulator by up- or down-regulating the activity of its target genes (Eguchi and Utsumi, 2008; Casino *et al.*, 2010).

NTHi possesses several known and putative two-component signalling systems, which are explored only to a limited extent or like in the case of the QseB/C-system are yet not characterized. The ortholog of the QseB/C-system in NTHi, encoded by the *qseB* and *qseC* genes organized in an operon (Fig. 2A), was first described in enterohemorrhagic *E. coli* (EHEC) and was found to regulate the expression of virulence genes in connection with QS independent of AI-2 (Sperandio, Torres, and Kaper, 2002). More recent studies point at virulence associated functions of this novel phosphorelay system also in other pathogens (Moreira *et al.*, 2010; Novak *et al.*, 2010; Pullinger *et al.*, 2010).

In this study, we evaluated the influence of *qseC* on the *in vitro* biofilm characteristics of NTHi. A *qseC* deletion mutant was created and tested in crystal violet staining assays as well as in an experimental model using constant flow conditions. In addition, possible effects on AI-2 production were examined.

Methods

Bacterial strains and culture conditions. Hi3655wt and its isogenic mutants were grown overnight on chocolate agar plates at 37 °C with 5 % CO₂. Liquid cultures were prepared in BHI (BD Bacto™) supplemented with 10 µg/ml Hemin (Sigma-Aldrich) and 10 µg/ml NAD⁺ (Fluka) (sBHI). *Vibrio harveyi* MM32 (BAA-1121™) was purchased from ATCC® and grown in autoinducer bioassay (AB) medium with 10 µg/ml kanamycin and 2 µg/ml chloramphenicol at 30 °C. The autoinducer bioassay medium contained 0.3 M NaCl, 0.05 M MgSO₄, and 0.2 % (w/v) casamino acids adjusted to pH 7.5. After autoclaving, the medium was supplemented with sterile 10 mM K₂HPO₄/KH₂PO₄ (pH 7.0), 1 mM L-arginine and 1 % (v/v) glycerol (E. P. Greenberg *et al.*, 1979).

Construction of knock-out mutants. An isogenic *qseC* and *luxS* knock-out mutants of Hi3655wt were generated by homologous recombination. The up- and down-stream flanking regions of the target gene were amplified with overhangs complementary to the 5' and 3' regions of the ORF of the respective resistance gene and merged together in a joining PCR (Wach, 1996) (Table 1). Phusion® polymerase (Finnzymes) was used as proof reading polymerase for all PCR reactions. Approximately 1 µg purified linear knock-out construct was introduced to the bacteria by using natural transformation according to Poje and Redfield (2003). Transformants were selected on chocolate agar containing 1 µg/ml chloramphenicol, 4 µg/ml zeocin or 20 µg/ml kanamycin, respectively. Resulting clones were checked by PCR (Fig. 1A and B).

Complementation of qseC. Complementation of *qseC* was achieved by stable insertion into a previously reported intergenic region on the chromosome with following modifications (Jones *et al.*, 2002). The corresponding intergenic region in NTHi3655 (CGSHi3655_06559) was amplified with the primers KS36 and KS37 (Table 1) and cloned into pBluescript II SK (Stratagene) using the terminal restriction sites BamHI and XhoI yielding the plasmid pKR1 (Table 1). Proteomic studies in our laboratory showed that the protein encoded by the gene with the locus number CGSHi3655_02309 is highly expressed (unpublished data). Hence, the promoter region of this gene (named *hpf* from now on) was amplified using the primers

PFprom_For and PFprom_Rev and cloned into the intergenic region in pKR1 with EcoRV and SalI yielding pKR2. In the following step, the zeocin[®] resistance gene (*bleo*) was amplified with the primers Zeo^R_F and Zeo^R_R and cloned into pKR2 using the restriction sites SphI and SalI resulting in pKR9.1. In a final step the *qseC* gene was amplified with the help of QseC_F2 and QseC_R2 and cloned into pKR9.1, which was linearized with NheI and BglIII. This produced the plasmid pKR9.1*qseC* carrying the complementation construct consisting of the *qseC* ORF driven by the promoter of *hpf* coupled to zeocin[®] resistance gene (*bleo*) which was cloned downstream and in reverse direction of *Phpf-qseC* (Fig. 1C). The constructs for complementation with or without *Phpf-qseC* were amplified from pKR9.1*qseC* and pKR9.1, respectively, with KS36 and KS37 using Phusion[®] Polymerase (Finnzymes). Purified PCR products were used to transform Hi3655wt and Hi3655KR2 by the MIV method as described above. Resulting clones were selected on chocolate agar plates containing 4 µg/ml zeocin[®] (Thermo Lifesciences), and the gene complementation was verified by PCR (Fig. 1D).

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Biofilm formation assay. Hi3655wt and its isogenic mutants (Table 2) were grown overnight on chocolate agar plates, and resuspended in BHI containing 10 µg/ml NAD⁺ and 10 µg/ml hemin (sBHI) to a final OD₆₀₀ 0.1. The suspensions (200 µl) were inoculated in triplicates for each tested strain in 96-well microtiter plates with flat or round bottom (NUNC, MicroWell™). While bacteria in round bottom plates were shaken at 37 °C and 200 rpm, plates with flat bottom were statically kept at 37 °C. In each plate wells with only sBHI were included as a control. After 24 h bacteria in suspension were removed and washed once with PBS. The remaining material was dried at 70 °C for 10 min and stained with 200 µl/well of 0.2 % (w/v) crystal violet staining solution in PBS. After 30 min, the dye solution was removed and the wells were excessively washed with H₂O_{dd}. Bound dye was solubilized in 33 % (v/v) acetic acid in H₂O_{dd}. The suspension (100 µl) was transferred into a fresh plate and the absorption at an OD 600 nm was measured.

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Biofilm formation in flow cells. NTHi biofilms under flow conditions were grown in channel slides (µ-Slide VI, ibidi[®]). The surface was coated with poly-L-lysine (Sigma-Aldrich, P4707) to

achieve proper attachment. Coating was done by applying 50 μ l of the solution into each flow cell and incubating either 30 min at RT or overnight at 4 °C. Hi3655wt and Hi3655KR2 were grown overnight on chocolate agar and resuspended in BHI diluted with PBS (1:3) supplemented with 2 μ g/ml hemin and 10 μ g/ml NAD⁺ to a final OD₆₀₀ of 0.05. Fifty microliters of each suspension were inoculated into the flow cells. Before the slide was connected to the pump, bacteria were allowed to settle for 30 min at 37 °C. The flow cells were flooded with fresh BHI:PBS at a pumping rate of 72 ml/h, and thereafter the run was continued at 3.6 ml/h for 24 or 48 h. Before disassembling, loose bacteria were removed by increasing the flow to 18 ml/h for 5 min. After disassembling, medium was removed by three washes with PBS and bacteria were visualized by using the LiveDead[®] BacLight[™] staining (Invitrogen) according to the manufacturer's instructions. Biofilm formation was analysed with a confocal microscope (LSM 710, Zeiss) using a 40x oil immersion objective and a pin hole allowing an optical slice of ~1 μ m. For z-stacks, 1 μ m steps were used between each optical slice. Images were analysed with the software *bioImage_L* (Chávez de Paz, 2009).

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Autoinducer-2 (AI-2) bioassay. In order to assess the AI-2 production of NTHi 3655 and its isogenic mutants, bacteria were grown in sBHI and at different time points aliquots were saved for further analysis. Bacteria were removed by centrifugation (12,000 g, 3 min, room temperature) and sterile filtration (0.2 μ m syringe top filters, Sartorius) of the supernatant. Samples were stored at -20 °C if not used immediately. *V. harveyi* MM32 was cultured overnight in AB medium and diluted 1:5,000 in fresh AB medium containing antibiotics. 90 μ l of this suspension were transferred into black 96-well plates (NUNC, #237108) and mixed with 10 μ l of NTHi supernatant diluted 1:3 with fresh BHI. Bacteria with supernatant were kept for 5 h at 30 °C and the luminescence was thereafter measured with a β -counter (MicroBeta[®] Trilux, PerkinElmer[®]) set to measure luminescence signals with 1 sec integration per well. *V. harveyi* incubated with BHI only was included as a background control.

Statistics. Student's *t*-test for paired data was used for statistical calculations. $p \leq 0.05$ was considered as statistically significant.

Results

NTHi QseC is homologous to E. coli and Aggregatibacter actinomycetemcomitans known sensor kinases. Recently QseC has been evaluated for regulation of metabolic genes and virulence capacity in enterohemorrhagic *E. coli* (Hughes *et al.*, 2009). In addition, QseC of *A. actinomycetemcomitans* that also belongs to the family of *Pastareullaceae* has been shown to control biofilm formation and is part of the AI-2 regulon (Novak *et al.*, 2010). To determine the similarity between these known QseC, we aligned the protein sequences of QseC from *H. influenzae* (Q4QJN5) with *E. coli* (F1YCP8) and *A. actinomycetemcomitans* (C9R3Y8) and also predicted different functional domains. *H. influenzae* exhibited an overall 61.4% identity and 75.8% similarity with *A. actinomycetemcomitans*, and 43.9% identity and 65.1% similarity with *E. coli* QseC. QseB/C is a simple two-component system consisting of a sensor kinase that contains a signal sensing domain, a histidine kinase domain with a conserved histidine residue for phosphorylation and an ATPase domain (Fig 2A and B). The cytoplasmic cognate response regulator QseB has a receiver domain with a conserved aspartate residue that receives PO_4^{3-} from QseC (Fig. 2B). The histidine kinase domains and regulatory domains of QseC are conserved, while the sensing domains, which account for perceiving various signals, are diverse (Fig. 2C).

QseC deletion affects biofilm formation under static and semi-static conditions. In order to evaluate the impact of the two-component signalling system on biofilm formation, an isogenic $\Delta qseC$ mutant (Hi3655KR2) was constructed and compared to the corresponding Hi3655wt. NTHi strains adjusted to a starting OD_{600} of 0.1 in sBHI were grown either in standing F-bottom (static condition) or in shaking U-bottom (semi-static) microtiter plates for 24 h at 37 °C. After incubation, bacteria in suspension were removed and the remaining material attached to the plastic surface was stained with a 0.2 % (w/v) crystal violet solution. The $\Delta qseC$ mutant Hi3655KR2 showed a significant decrease in biofilm formation under static (Fig. 3A) as well as under semi-static conditions (Fig. 3B) as measured by the quantity of bound crystal violet ($p \leq 0.01$). In order to confirm the influence of *qseC* on the observed difference in biofilm formation an isogenic complementation mutant was generated by

inserting *qseC* under the control of the promoter of the *hpf* gene into the intergenic locus CGSHi3655_06559 (Hi3655KR4). In parallel, a control WT strain and a control knock-out strain were generated by inserting an empty construct into the same locus (Hi3655KR1 and Hi3655KR3, respectively). The same biofilm difference was observed between Hi3655KR1 and Hi3655KR3 indicating that the insertion did not have a detrimental effect on the phenotype. Further on, complementation of *qseC* caused an increase in biofilm forming capacity of Hi3655KR4 compared to Hi3655KR3. Although the complementation did not allow a fully restored phenotype, the increase was statistically significant ($p \leq 0.01$) (Fig. 3A and B).

QseC is involved in biofilm under continuous flow. We further went on by comparing the biofilm formation of Hi3655wt and the Hi3655KR2 under continuous flow conditions in chamber slides coated with poly-L-lysine. In this experimental set-up, biofilm development of both strains showed large differences up to 48 h. Already at 24 h, the surface coverage was significantly reduced ($p \leq 0.001$) in the case of the $\Delta qseC$ mutant Hi3655KR2 (20.4 ± 6.8 %) compared to the WT (58.0 ± 16.4 %) (Fig. 3C), although the total biomass in the chambers was comparable for both strains ($49.8 \cdot 10^3 \pm 17.3 \cdot 10^3 \mu\text{m}^3$ and $52.5 \cdot 10^3 \pm 14.5 \cdot 10^3 \mu\text{m}^3$ for the WT and $\Delta qseC$ Hi3655wt and Hi3655KR2, respectively) (Fig. 3D). In biofilms grown for 48 h, however, the $\Delta qseC$ mutant Hi3655KR2 had a total biomass of $60.7 \cdot 10^3 \pm 24.4 \cdot 10^3 \mu\text{m}^3$, whereas the total biomass of the WT strain was significantly higher, *i.e.*, $142.8 \cdot 10^3 \pm 36.1 \cdot 10^3 \mu\text{m}^3$, significantly higher ($p \leq 0.05$) (Fig. 2D). The viability of both bacterial strains at 48 h was 79.2 ± 5.3 % live cells for NTHi 3655 WTwt as compared to 84.3 ± 4.8 % for the $\Delta qseC$ mutant Hi3655KR2, and thus not significantly differed as determined by live/dead staining (Fig. 3E and F). Growth and viability were additionally checked by measuring OD₆₀₀ in liquid shaking culture over 8 h and counting CFU after 24 h of culturing, respectively. In both cases no significant difference was observed between Hi3655wt and Hi3655KR2 (data not shown see supplementary Fig. 1).

QseC is linked to a system not related to AI-2 production. NTHi possesses an intact *luxS* homologous gene and produces the quorum sensing molecule autoinducer-2 (AI-2). A

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luxS deletion mutant was shown to be impaired in its biofilm forming ability (Armbruster *et al.*, 2009). Because of the possible interconnection between AI-2 and NTHi-biofilms we analysed whether Δ qseCHi3655KR2 produced less AI-2 compared to the non-mutated WT. AI-2 production was monitored by adding bacteria free NTHi culture supernatants collected at different time points (0, 3 and 8 h) to a diluted culture of the bioluminescent reporter strain *Vibrio harveyi* MM32. After incubation for 5 h at 30 °C, the light production was measured. The *luxS*-deletion mutant Hi3655KR5 was used as a negative control. In this assay no difference could be observed in the AI-2 production capacity of Hi3655wt and Hi3655KR2, whereas the supernatant of Hi3655KR5 did not induce any bioluminescence (Fig. 4). Thus, we could exclude that the observed biofilm defects were due to an impaired AI-2 production.

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Discussion

NTHi occasionally causes persistent infections, and its ability to form biofilms is a major virulence determinant in this respect as shown with biofilm deficient mutants in animal models (Jurcisek *et al.* 2005; Hong *et al.* 2007). Most of the mutations leading to defects in biofilm formation are found in sialic acid and polysaccharide biosynthesis gene clusters (Swords *et al.* 2004; L. L. Greiner *et al.* 2004; Jurcisek *et al.* 2005). In contrast, the regulatory mechanisms controlling the development of biofilm and host colonisation of NTHi populations largely remain unexplored.

It is clear from our results that QseC controls biofilm formation under static and flow conditions. In addition, further work is needed to completely evaluate the role of QseC during biofilm formation. One possibility might be that QseC regulates the expression of adhesin or LOS-biosynthesis genes influencing the adherence capabilities due to changes in the surface characteristics. An indication in this direction is the difference in surface coverage between Hi3655WTwt and its isogenic *qseC* deletion mutant Hi3655KR2 (Fig. 3C, see also suppl. Fig. 2). Nevertheless, both strains developed the same amount of biomass during the first 24 h of growth. Apparently, the deletion affected the attachment to the poly-L-lysine coated surface but had no detrimental effect on growth and interbacterial attachment. In accordance with this, no difference in aggregation could be observed between Hi3655wt and Hi3655KR2. Similarly the *qseC* complementation mutant, Hi3655KR4, showed no significant difference in an aggregationthe same assay compared to the isogenic deletion mutant Hi3655KR3 and the corresponding wild type control strain Hi3655KR1 (data not shownsee supplementary Fig. 3). However, a significant decrease in biomass could be observed in case of the $\Delta qseC$ mutantHi3655KR2 when grown for 48 h (Fig. 3D). Since deletion of *qseC* did not affect bacterial growth and viability as measured in liquid growth culture, the observed biofilm differences indicate a possible quorum sensing defect, which prevents the bacteria from adjusting their physiology to increasing population densities in order to maintain an intact biofilm structure.

Until now AI-2 is the only described quorum sensing signal in NTHi with an RbsB

homolog as a recently identified receptor (Armbruster *et al.*, 2011). We could show that deletion of *qseC* had no effect on AI-2 production as assessed by an autoinducer bioassay with *V. harveyi* MM32 as a reporter strain (Fig. 4). Thus, the observed biofilm defect is not due to AI-2-deficiency. However, QseC can be a possible additional receptor for AI-2 or may be acting downstream of the primary AI-2 receptor RbsB as it was speculated for *A. actinomycetemcomitans* (Novak *et al.* 2010). Therefore, reporter assays should be developed in order to identify possible AI-2 dependent gene regulatory mechanisms and used for the comparison of the WT strain and its isogenic *qseC* deletion mutant. Further on, the identification of an alternative signalling molecule is of great interest. In EHEC, the sensor kinase QseC was shown to sense human epinephrine (Epi) or norepinephrine (NE), probably receiving signals when entering its host (Clarke *et al.* 2006). However, there is also a postulated bacterial QS-molecule, named AI-3, which is able to replace Epi or NE (Hughes *et al.* 2009). Preliminary tests with the known agonist, epinephrine, and an antagonist, propranolol, like it was shown for QseC of EHEC (Clarke *et al.* 2006), did not result in any increase or decrease in biofilm formation in crystal violet assays, respectively (unpublished data). The alignment of protein sequences of QseC from NTHi, *E. coli* and *A. actinomycetemcomitans* showed higher sequence diversity in the sensing domains compared to the rest of the protein (Fig. 2C). Thus the experimental evidences that did not show any involvement of AI-2 and Epi/NE signals would be supporting for a unique and novel signal that might be sensed by QseC of *H. influenzae*.

Our results show that QseC influences NTHi biofilm formation, whereas the exact regulatory network downstream of the QseB/C phosphorelay system as well as a possible cross-talk with other NTHi phosphorelays like ArcA/B (Manukhov *et al.*, 2000) has to be evaluated. Until now there are two reports which link *qseB/C* directly to biofilm in other bacterial species. Interestingly deletion of *qseC* in *Aggregatibacter actinomycetemcomitans* resulted in decreased biofilm formation similar to our observations (Novak *et al.*, 2011), while in *Aeromonas hydrophila* in the absence of *qseB* a stronger biofilm phenotype was observed (Khajanchi *et al.*, 2011). In the light of these and our observations it can be speculated that the sensor kinase QseC of this two-component system is responsible for the switch from a non-

biofilm to a biofilm phenotype once the yet to be identified signal is perceived.

Biofilms are one of the most important common features of many nosocomial and chronic infections, allowing bacterial pathogens to colonize diverse niches and to resist anti-infective therapy due to increased antibiotic resistance or mechanical resilience. Biofilm formation depends on many different factors including the signals bacteria perceive from their environment. Quorum sensing and two-component signalling mechanisms play a pivotal role during these processes (Senadheera and Cvitkovitch, 2008; Dickschat, 2010). Both are considered as promising alternative drug targets, since gene deletions in both systems are generally not lethal under laboratory conditions but affect the fitness of bacteria in certain niches at restricted time points as it is the case during host colonization (Gotoh *et al.*, 2010; Sintim *et al.*, 2010). Therefore, the analysis of mechanisms underlying biofilm dynamics of NTHi is of uttermost interest, and will facilitate new treatment strategies against this with disease commonly associated pathogen.

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FIGURE LEGENDS

Fig. 1: Knock out and complementation of *qseC* by homologous recombination in the genome. **A.** The ORF of *qseC* organized in the *qseB/C* operon was one to one replaced by the ORF of *nptI* (kanamycin resistance), which was fused to the up- and downstream flanking regions of *qseC* in a joining PCR and yielding the isogenic k.o.-strain Hi3655KR2. **B.** Transformants were verified by colony-PCR using the primer combination KmR_F/QseC_DFR. Shown is a representative 1 % agarose gel with WT-control (lane 1) and three clones, where the expected PCR product of ca. 1400 bp is generated (lanes 2-4). **C.** Diagram depicting the complementation strategy employed to insert the *qseC* gene, as well as the necessary controls, in Hi3655wt and Hi3655KR2. Genomic insertion occurs in the locus CGSHi3655_06559 annotated in the draft genome of NTHi3655 and either yields the strains Hi3655KR1 and Hi3655KR3 (insertion of the empty construct) or the strain Hi3655KR4 (insertion of the complementation construct bearing *qseC*). **D.** Transformants were checked by colony-PCR using the primer combination Rec3655_F/PFprom_R (lanes 1, 3, 4 and 6) or PFpProm_F/QseC_R2 (lanes 2, 5 and 7). Shown are representative 1 % agarose gels. In lanes 3, 4 and 6 the expected bands for a correct integration of the complementation construct are observed while only in lane 7 the expected band indicating the presence of *qseC* in the construct is present. Depicted are also the negative PCR-result of Hi3655wt with the primer combination Rec3655_F/PFprom_R (lane 1) and a non-specific PCR product of the primer combination PFpProm_F/QseC_R2 (lane 2).

Fig. 2: *QseC* of NTHi, *E. coli* and *A. actinomycetemcomitans* are homologous. **A.** Schematic representation of the genetic organization of the *qseB/C* locus and the protein products with their respective protein domains. The open reading frames of *qseB* and *qseC* are organized in an operon. **B.** The gene NTHi2015 encodes for sensor kinase anchored to bacterial inner membrane by transmembrane 1 (TM1) and TM2 domains. The sensing domain is situated between TM1 and TM2 exposed in periplasmic space, followed by HAMP (Histidine kinases, Adenylyl cyclases, Methyl-binding proteins, Phosphatases) linker domain. The cytoplasmic part of QseC consists of Histidine kinase domain that has a conserved

histidine for phosphorylation and a regulatory ATPase domain. QseB has a receiver domain that receives high energy phosphoryl group from QseC, followed by a DNA binding helix-turn-helix (HTH) motif. Under non-phosphorylated conditions, it does not bind to DNA, but phosphorylation induces conformational changes that lead to binding to DNA and altering gene expression. **C.** ClustalW alignment of QseC of NTHi, *E. coli* and *A. actinomycetemcomitans* along with functional domains predicted by SMART server (<http://smart.embl.de/>) and Pfam (<http://pfam.sanger.ac.uk/>) server.

Fig. 3: Comparison of biofilm development by crystal violet staining and in flow cells.

A and B. The *qseC* deletion mutant Hi3655KR2 was tested for its biofilm forming capacity in 96-well culture plates and compared to the isogenic wild type Hi3655wt. Bacteria were grown for 24 h either in shaking (A) or standing culture (B) at 37 °C and after removing bacteria in suspension, bound bacteria and material were stained with a 0.2 % (w/v) crystal violet solution. A significant decrease of biofilm formation was observed under both culture conditions in case of Hi3655KR2. The effect of *qseC* was confirmed by comparing the isogenic *qseC* complementation mutant Hi3655KR4 with the deletion mutant Hi3655KR3 carrying only the empty complementation construct. Hi3655KR4 formed under both conditions significantly more biofilm mass. **C.-F.** The biofilms of Hi3655wt and its Hi3655KR2 were compared microscopically after 24 and 48 h of growth under constant flow conditions. Bacteria were stained with Live/Dead staining kit for visualization. At 24 h the surface coverage was significantly lower in case of the $\Delta qseC$ -mutant (C), while the total biomass was comparable for both strains at this time point (D). The biofilm differed significantly as measured by biomass after 48 h of growth (D). Representative pictures of 48 h biofilms are depicted for Hi3655wt (E) and Hi3655KR2 (F). The graphs depict mean and SD of three independent experiments. Significance was calculated by two-sided paired Student's *t*-test; * $p \leq 0.05$, *** $p \leq 0.001$.

Fig. 4: *Deletion of qseC does not affect AI-2 production.* Production of AI-2 in Hi3655wt and Hi3655KR2 was measured over 8 h of growth by using the reporter strain *V. harveyi* MM32. Sterile culture supernatants were prepared at the indicated time points and were added to a fresh culture of *V. harveyi* MM32. After 5 h of incubation at 30 °C the bioluminescence was measured. The results are given in relative light units (RLU). The isogenic *luxS* deletion mutant of Hi3655wt was used as a negative control. The graph represents the mean and standard deviation of three independent experiments with triplicates for each strain.

Figure 1

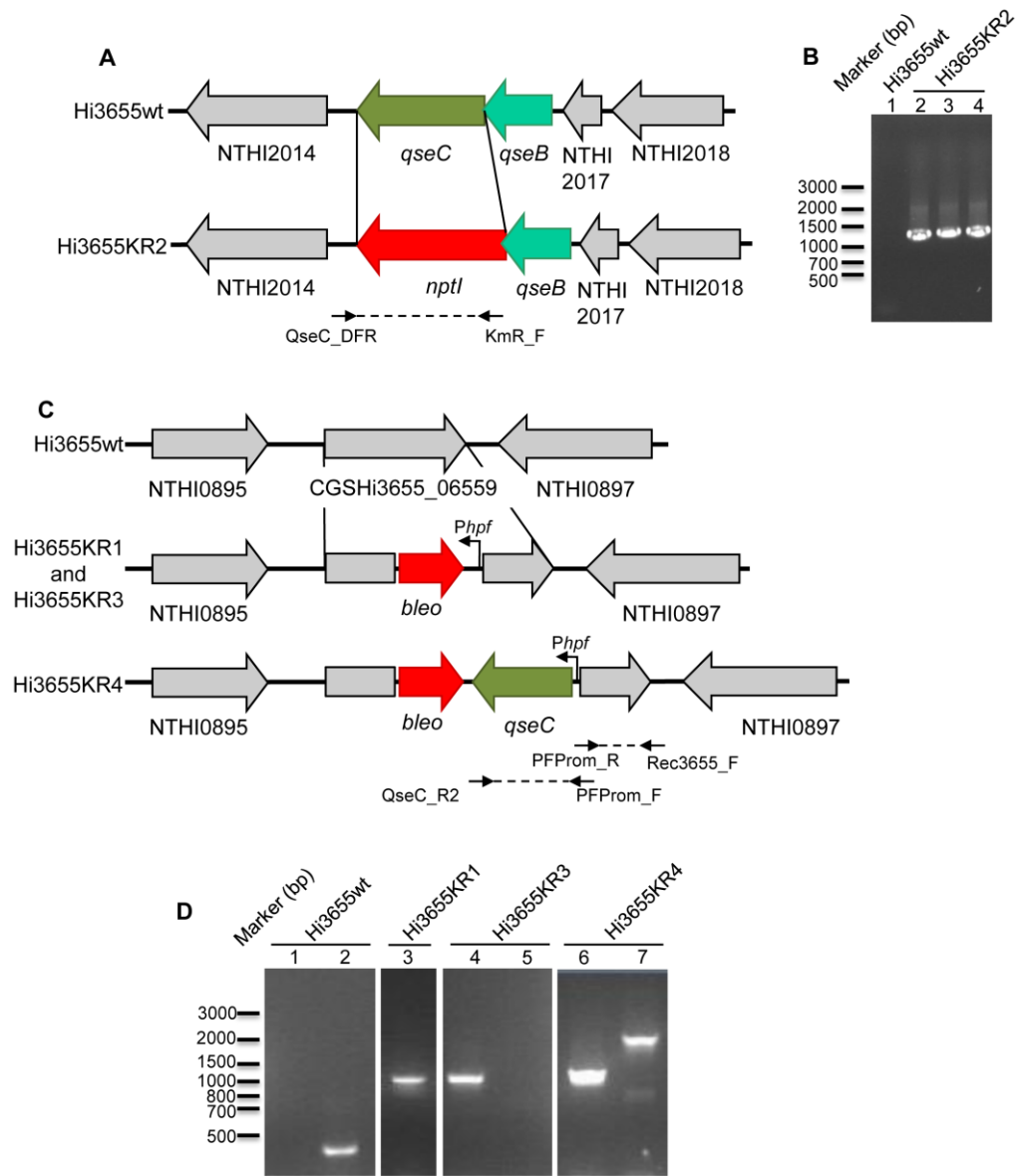


Figure 2

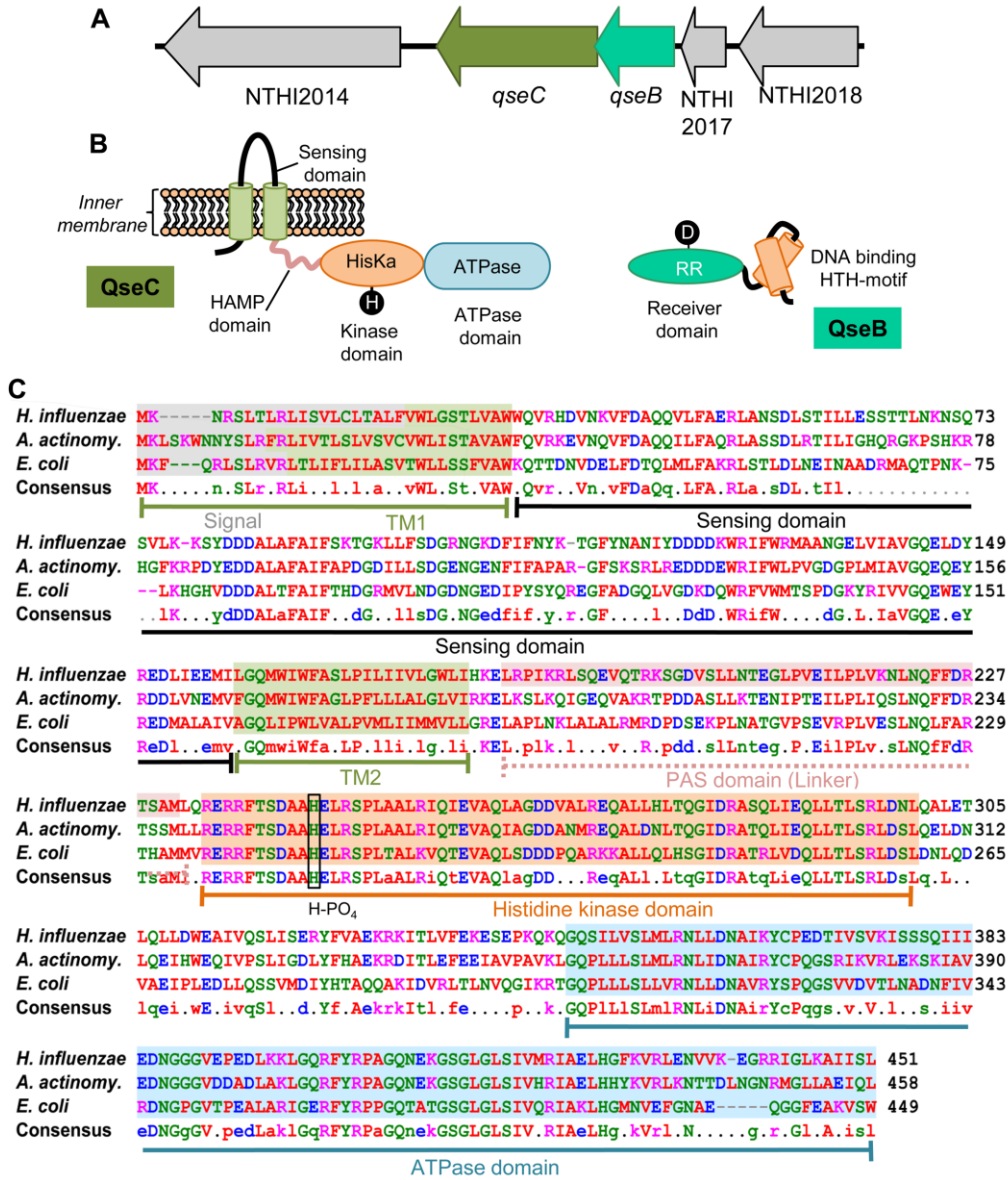


Figure 3

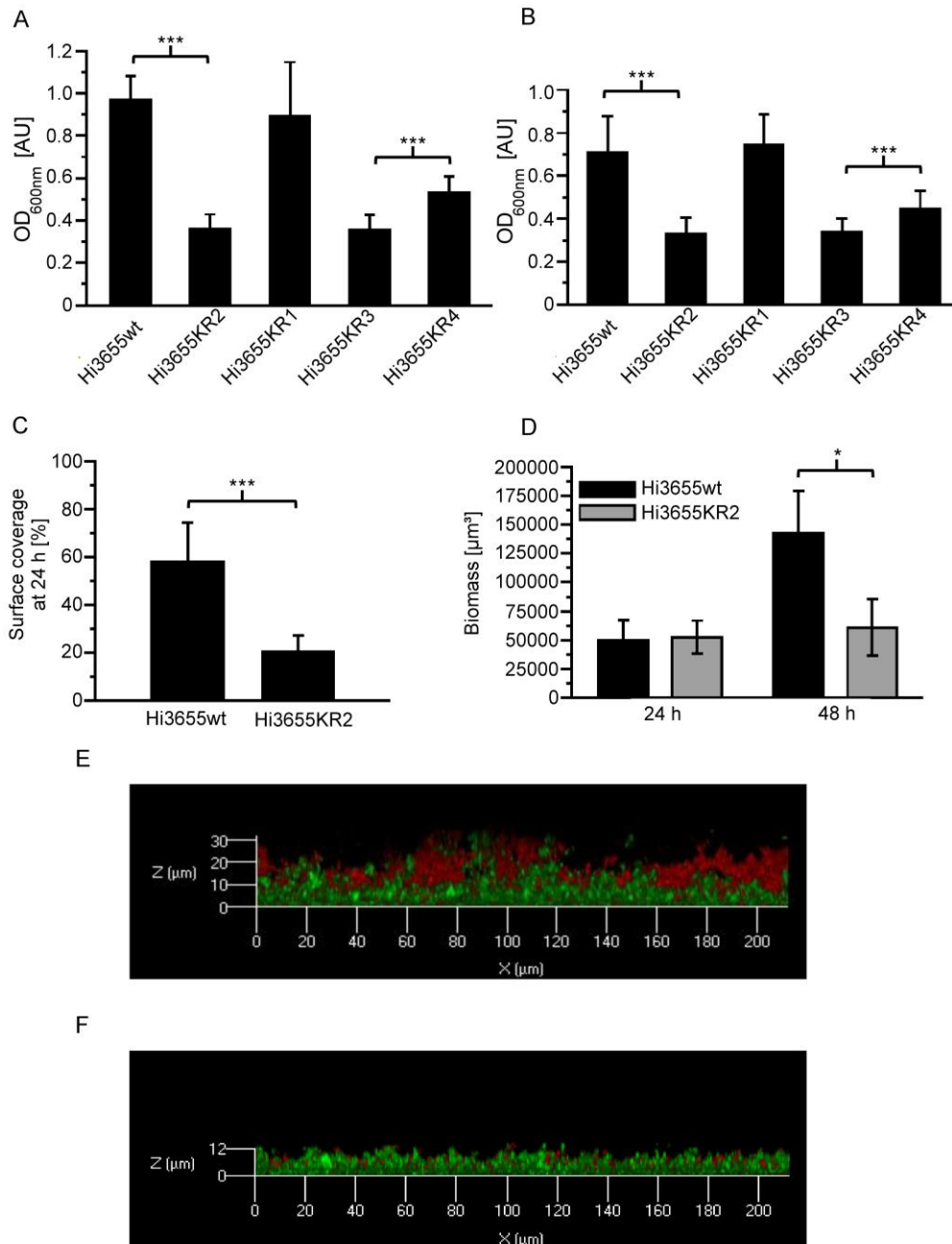
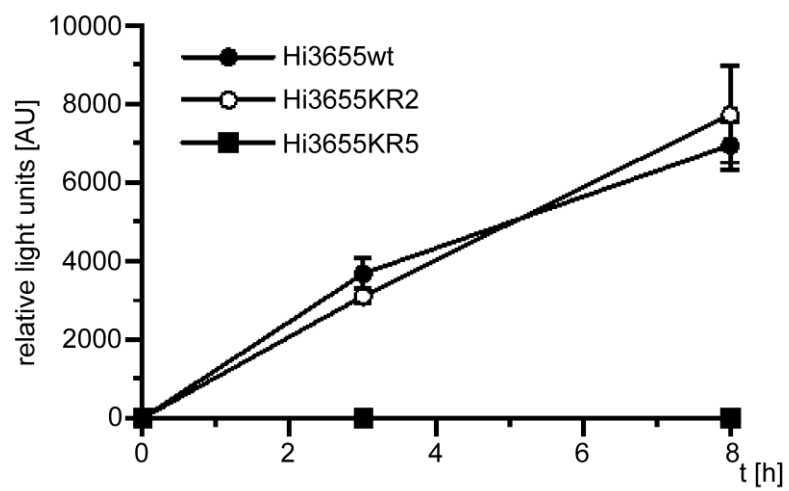


Figure 4



1 **Table 1:** List of primers and plasmids used in this study.

Primer Name	Sequence (5'→3')
QseC_UFF	CGCTATTTTTGTTTATAAAGT
QseC_UFR*	<u>CCGTTGAATATGGCTCATT</u> TTTTCAACTTGTCCTCAAAG
QseC_DFF*	GCTCGATGAGTTTTTCTAAAAATGGCTTTAAATAAATGATA
QseC_DFR	AGAAAGATAATGGGTAAATG
Km ^{RΔ}	ATGAGCCATATTCAACGG
Km ^R	TTAGAAAAACTCATCGAGC
LuxS_UFF	GAGCAAGAAAAAACCCAC
LuxS_UFR*	<u>TCCAGTGATTTTTTTCTCCATAAA</u> TTTTCTTATAAATCAGTTGG
LuxS_DFF*	<u>CAGGGCGGGGCGTAA</u> GGAAGACGCTTGATTAACCT
LuxS_DFR	GATACTGCCAACCTTGGT
Cm ^{RΔ}	ATGGAGAAAAAATCACTGGA
Cm ^R	TTACGCCCCGCCCTG
KS36 ^{†‡}	GGCGGATCCTG <u>ACCGCACT</u> TAGGGGGATAAAACAAAGG (BamHI)
KS37 ^{†‡}	GGCCTCGAG <u>AAGT</u> GCGGTCAGGCAAGTCCCTGTTCAAA (XhoI)
PFpProm_F [‡]	GCGGGGATATC <u>TTCATAAA</u> CATAAAAAACGCGAG (EcoRV)
PFprom_R [‡]	TAACGTCGACAGATCTGGCGCGCCTAG <u>GCTAGCG</u> GATAGATTGAATGCCT GAATAATCG (SalI, BglII, NheI)
QseC_F2 [‡]	ATTATCGCTAGCTTTTTAAATAAGGACATCTTATGAAAAATAGAA GCCTTACG (NheI)
QseC_R2 [‡]	TATACGTCTAC <u>AGATCT</u> CTACAAGGATATTATTGCTT (BglII)
Zeo ^R _F [‡]	ATTAAGCATGCGAGAGAATTTGATTACCAAC (SphI)
Zeo ^R _R [‡]	AATTAGT <u>CGACA</u> AGTGCGGTTTCAGTCTCTCCTCGGCCACG (SalI)
Rec3655_F	TAACGTAAGAAATAACGATAAGA

Plasmid name	Backbone	Characteristics
pKR1	pBluescript II SK (Stratagene)	Contains intergenic region (IR) from NTHi 3655 genomic DNA (CGSHi3655_06559) cloned with XhoI and BamHI; Amp ^{RA}
pKR2	pKR1	Contains promoter of <i>hpf</i> (<i>Phpf</i>) cloned into the IR with EcoRV and SalI; Amp ^R
pKR9.1	pKR2	Contains zeocin [®] cassette cloned downstream of <i>Phpf</i> in reverse direction with SphI and SalI; Amp ^R , Zeo ^{RA}
pKR9.1 <i>qseC</i>	pKR9.1	Contains ORF of <i>qseC</i> cloned under the control of <i>Phpf</i> with BglII and NheI; Amp ^R , Zeo ^R

2 *Underlined are the overhangs, which are complementary to the 5' and 3' regions of the corresponding
3 resistance gene.

4 †KS36 and KS37 contain additional uptake signal sequences (USS) highlighted in order to increase the
5 efficiency of natural transformation using the MIV method.

6 ‡Underlined are the relevant restriction sites. The corresponding restriction enzymes are listed in brackets in 5' to
7 3' direction.

8 ^ΔKm^R: kanamycin resistance (*nptI*), Cm^R: chloramphenicol resistance (*cat*), Amp^R: ampicillin resistance (*bla*),
9 Zeo^R: zeocin[®] resistance (*bleo*)

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12 **Table 2:** List of *NTHi* strains used in the present study.

Name	Description	Reference
Hi3655wt	NTHi 3655 wild type strain, clinical isolate kindly provided by Robert Munson (Ohio State University)	Ronander <i>et al.</i> , 2008
Hi3655KR1	Isogenic mutant of Hi3655wt in which the empty complementation construct amplified from pKR9.1 was inserted into the CGSHi3655_06559 locus, Zeo ^R	This study
Hi3655KR2	Isogenic <i>qseC</i> deletion mutant of Hi3655wt in which the <i>qseC</i> gene was replaced by <i>nptI</i> , Km ^R	This study
Hi3655KR3	Isogenic mutant of Hi3655KR2 in which the empty complementation construct amplified from pKR9.1 was inserted into the CGSHi3655_06559 locus; Km ^R , Zeo ^R	This study
Hi3655KR4	Isogenic mutant of Hi3655KR2 in which the <i>qseC</i> -bearing complementation construct amplified from pKR9.1 <i>qseC</i> was inserted into the CGSHi3655_06559 locus; Km ^R , Zeo ^R	This study
Hi3655KR5	Isogenic <i>luxS</i> deletion mutant of Hi3655wt in which the <i>luxS</i> gene was replaced by <i>cat</i> , Km ^R	This study

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