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RESEARCH ARTICLE

A quantitative study of gene regulatory pathways in *Bacillus subtilis* for virulence and competence phenotype by quorum sensing

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Abstract Quorum sensing (QS) is a process which allows a population of bacteria to coordinately regulate gene expression of their entire community. *Bacillus subtilis* is a soil organism which uses QS to alternate between competence for DNA uptake and sporulation. We propose a model to describe the components involved in QS and to analyze reaction species involved in the regulation of QS machinery. We targeted only those QS phenotypes for which the genetic organization and molecular characterization of the components are fully elucidated. We have analyzed simulations for concentration of different species involved in competence as well as sporulation pathways at diverse time period using quantitative methods. It was observed that there is possibility of achieving different measurement from reactions taken place between species by applying irreversible Michaelis–Menten kinetic law. We obtain variation in measurement on changing parameters such as concentrations ranging from 0.3 to 50 μM in stepwise manner by setting end time in the range of 0.1–100 ms. Additionally we observe covariance between different reaction species involved in QS by fluctuating their quantities in real-time simulations. Our model mimics correctly the phenotype for competence and virulence. We concluded that time factor play major role to determine rate kinetics of diverse reaction species as compared to their concentrations and support the hypothesis of getting genetic stability while colonies are in synchronization.

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Introduction

Quorum sensing (QS) is a process found in nature that allows bacterial communication. QS enables to control the bacterial functions or processes in an effective way when undertaken by a group while it is ineffective when commenced by an individual. Quorum sensing is achieved through the coordinated production of special kind of particles called autoinducers. Different strains of bacteria uses different autoinducer for communication i.e. Gram negative bacteria mainly uses AHL (Acyl Homoserine Lactone) autoinducer while Gram positive bacteria uses oligopeptide as autoinducer respectively. The overall process could be regarded as a communication mechanism amongst bacteria and they proceeds to transmit it through secretion. The message encoded in the density of particles is decoded and treated as a gene regulation expression mechanism. There are various functions such as virulence, biofilm formation, bioluminescence, sporulation and mating which is being controlled by QS (Dunny and Leonard 1997; Davies et al. 1998; Lazazzera 2000; Krasnogor et al. 2005; Rumbaugh et al. 2007; Williams et al. 2007).

Bacillus subtilis has become the best studied gram-positive model organism for a number of reasons which includes its relative ease of handling in wet lab experiments, its growth rate is relatively fast (comparable to that of *Escherichia coli*), it possesses a natural competence system and the machinery for homologous recombination (Baca-DeLancey et al. 1999). In Gram positive bacteria signal molecule is a post-translationally processed secreted

peptide for the communication. This secreted peptide, called pheromone is recognized by typical sensor component of a two-component signal transduction systems. As a first step towards understanding the interplay between a pathogen and its host from a systems biology standpoint, we focused on the entities involved in virulence and competence pathways (Stephenson and Lewis 2005). The analysis predicts a regulatory network in which all regulators and their associated set of genes are being included. We performed simulations on our regulatory model by a subset of the regulators of known virulence and competence factors located within *B. subtilis*.

There are number of extracellular proteins which play important role in the pathogenesis of *B. subtilis* infection which is regulated by cell density (Hengge-Aronis 1999; Deep et al. 2011). The virulence phenotype is mediated by RNA-III, whose expression is regulated by accessory gene regulator (agr) locus. This agr locus contain two divergent transcription units. The larger of two transcripts include agr operon encoding sensor (agrC) and response regulator (agrA), and two open reading frames (ORFs), agrB and agrD. RNAIII has a key regulatory role in agr-response. Production of agrD derived peptide pheromone requires agrB gene and might be involved in post-translational modification of agrD gene product. agrC act as peptide pheromones sensor and via phosphorylation of agrA, stimulate RNAIII production. Some environmental factors such as glucose concentration and pH have strong influence on agr expression (Solomon and Grossman 1996; Kleerebezem et al. 1997; Fujiya et al. 2007).

Natural genetic competence is the ability of cell to take up exogenous DNA. A subpopulation from *B. subtilis* culture are differentiate to become competent. These competent cells are metabolically less active and produce number of proteins that can bind and take up DNA independently of its nucleotide sequence. *B. subtilis* takes up DNA at the transition from exponential phase to stationary phase. This DNA is used as a repository of genetic material for use in repairing damaged chromosome. This makes it easy to manipulate genetically (Stragier and Losick 1996). The precursor of peptide pheromone are encoded by ComX gene. The cellular response to ComX derived peptide pheromone is mediated by sensor (ComA) and response regulator (ComP) protein encoded by ComA and ComP respectively. Data suggest that ComP act as sensor for ComX-derived peptide pheromone and activate the ComS transcription via signal transduction to ComA, which result in development of genetic competence (Fuqua et al. 1996; Ishihama 1997; Kleerebezem et al. 1997).

Systems biology studies the way in which interaction of different types of molecules results in complex behavior at the cellular, organism, disease and higher levels (Krasnogor et al. 2005; Melke et al. 2010; Panigrahi and Singh 2012; Ulas et al. 2012). It approaches these questions using a combination of high-throughput experiments, mathematical and computational models, and information storage in specialized databases. In this study we performed simulations for concentrations of different species (biological entities in our model) involved in competence as well as sporulation pathways. We targeted only those QS phenotypes for which the genetic organization and molecular characterization of the components are fully elucidated (Schauder and Bassler 2012; Mader et al. 2012). We proposed a simple model to represent competence and virulence phenotypes and to determine pathway steps to limit the flux of the molecular processes. We performed a quantitative analysis of our proposed model to simulate competence and virulence phenotypes in *B. subtilis* to enhance coordination amongst involved molecular entities. This analysis would provide new insights in understanding the mechanisms of competence and sporulation in *B. subtilis* and may possibly work as a future model for other bacterial species.

Materials and methods

To understand the competence and virulence mechanisms in *B. subtilis*, we fabricated a computational model to study temporal dynamics of genes and proteins. We applied CellDesigner (Funahashi et al. 2003) for modeling and simulation purpose. CellDesigner is a modeling tool for biochemical and gene-regulatory network. CellDesigner was selected for simulation purposes against other contemporary methods as it supports parameter search, estimation, and simulations through sophisticated graphical user interface (Funahashi et al. 2003). For quantitative methods and analysis CellDesigner is comparable to other contemporary tools. Based on a fundamental hypothesis, existing global biological knowledge is collected and processed to build up an integrative conceptual model of a biological system. This conceptual model is then extended with more information to get the corresponding analytical model. For the analytical model, constraints have to be considered in the buildup model while, the parameters that are missing have to be guessed and diverse assumptions have to be made to get a usable model. We have applied the same approach where we started our model as phenomenological to a comprehensive one while progressing and refining it step-by-step. Computer simulations were performed for the developed model. The results obtained from the simulation were compared to the data from the biological knowledge of explicit experiments. The once constructed model has to be revised continuously in an iterative process and adapted dynamically to a validated model. This validated model can then be used for diagnostic purposes.

A combined model has been developed for both virulence and competence phenotype. Our model comprise of 9 genes ComA, ComB, ComC, ComD, ComE, agrA, agrB, agrC, agrD, 2 proteins Protein1 and peptide pheromone precursor protein, response regulators, and receptors. Simulations have been performed in combinations as well as individually for both conditions at various time intervals. Concentrations of species in the model have also been altered during simulations to evaluate their consequences on final outcome. Parameter values for our model were determined from available experimental data. CellDesigner being an integrated modeling and simulation environments provides the flexibility of using number of simulation algorithms. We have applied deterministic algorithm for our model. In studying biological model, we require solution for a given set of parameter values along with their examination for dependency towards other species in the model. SBML based ODE Solver (Funahashi et al. 2003; Machné et al. 2006) was used through CellDesigner, which enables us to run ODE-based simulations. ODE based simulations are preferred method for quantitative analysis as they surpass logical methods in term of computational efficiency, while considering species concentration and time as major parameters. The SBML ODE Solver Library (SOSLib) is a C++ programming library for symbolic and numerical analysis of chemical reaction network models encoded in SBML (Machné et al. 2006). The simulation engine itself is executed by the native library, and the results are shown in a GUI window written in Java. This tool enables the constructed model equation to directly generate machine code for all the equations of the model.

Results and discussion

We illustrated a simple model of QS using autoinduction which is based on the known biochemistry of *B. subtilis* (Fig. 1). Considering the fact that all the regulators in this study have biological functions during QS, suggests that they could be part of a coordinated network. Applying simulations to this model we demonstrate that quorum sensing works because of the rate of elimination of autoinducer depends on the colony size and density. The report table provides detailed information for setup, model, and output (Table 1). It is an easy way to change initial concentrations, simulation setup and multiple running in background. All the simulation operations were performed on hardware configuration comprises of Intel dual core processor of 2.30 GHz and 2 GB RAM on windows platform.

We performed two tier simulations where concentrations of species in reactions were fixed in first tier and flexible in the other tier. By doing this we describe Quantitative description of dynamics of the model was performed according to Li et al. (2008). It has been observed that when we kept initial amount of ComA and ComB equal and observe the behavior of ComC at simulation end time 30 ms. In this simulation ComA regarded as principal axis of the graph and ComC attain peak somewhere close to 33 μM at time 9 ms. Further we tried to simulate the same species reaction at end time 10 ms and the graph is somewhat different from previous one in which line of ComC is more taper and attain stationary position at time 7.5 ms. Here, one point to be noted that all simulation operation are performed with default error tolerance of −6, which signifies how much our defined model is close to real system. In another case we increase the amount of ComA to just double the amount of ComB but the effect on graph is not very significant. But when we increase the amount of ComA and ComB to same extent and observe the effect on gene ComC, we find that ComC attain its activation energy in much less time. Similarly, we observe the change in behavior of species involved in another phenotype (sporulation/virulence) at different time period of 5, 10 and 30 ms respectively. Simulation studies produced a cascade of appearance for few species involved in regulatory reactions (Fig. 2a–d; Table 1).

We also investigated whether any kind of relation persist between competence and virulence. We performed simulation at a condition where all species of competence are there in reaction while in other condition we dropped ComA and kept all other species of competence and virulence, and then observed the behavior of all species involved in virulence (sporulation). Here we found that ComB, C and D are less effective to draw any impact on species related to virulence (Figs. 3, 4; Table 1). From this study we found that concentration and time-factor are important key player to decide the behavior of any species related to any biological phenomenon. Our model predicts species mutual interchange for competence and virulence and mimics correctly the phenotype phenomenon for sporulation.

On analyzing the graph at 0.4 ms, we noticed the behavior of ComC, Protein1 and Peptide pheromone at initial amount 20 μM, ComC shows an exponential growth at this point (Fig. 5). The protein1 added to the system at 17.6 μM, rises up to the level of 22.6 μM concentration. It is to be predicted that behavior of ComC and Protein1 is antagonist to cell density dependent phenotype. On analyzing the same graph at simulation time 0.6 ms the behavior of these species are more contrasted, in this ComC rises from 20 μM concentration to 34.5 μM after they got stabilized. On the other hand cell density dependent phenotype grow exponentially in negative direction (Fig. 5). After examining the graph at end time 0.9 ms, the ComC and phenotype show parabolic growth. Here, one point to be noticed was that on increasing run time further,

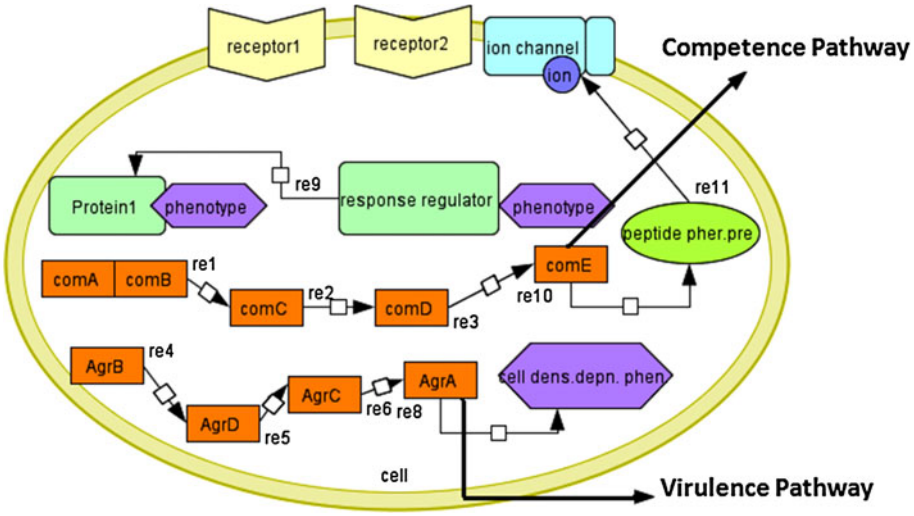

Fig. 1 Representation of simple model for *Bacillus subtilis* for virulence and competence. All involved genes, proteins, and other regulators are shown in a connected and coordinated manner. agrA is connected with cell density dependent phenotype in virulence pathway while ComE is connected with peptide pheromone precursor in competence pathway

Table 1 Simulation configuration and conditions for the proposed model in deterministic simulation environment

Configuration			Time span			Model	
Simulation environment (pathways)	Solver	End time	No. of points	Tolerance	Simulation time (stabilization)	Species	Reactions
Deterministic (common)	SOSLib	50	100	−6	13.0	14	11
Deterministic (competence)	SOSLib	20	100	−6	10.0	6	4
Deterministic (competence)	SOSLib	40	100	−6	8.0	6	4
Deterministic (competence)	SOSLib	100	100	−6	10.0	6	4
Deterministic (competence)	SOSLib	10	100	−6	8.0	4	2
Deterministic (common)	SOSLib	30	100	−6	7.5	8	2
Deterministic (sporulation)	SOSLib	5	100	−6	4.5	5	4
Deterministic (sporulation)	SOSLib	10	100	−6	5.5	5	4
Deterministic (sporulation)	SOSLib	0.4	100	−6	3.0	5	4
Deterministic (sporulation)	SOSLib	0.4	100	−6	3.0	4	4

SOSLib was used as simulation solver. To obtain realistic simulations tolerance (error) rate was kept −6. Selected and significant entries have been included in the table for modeling and simulation configurations

the axis of ComC and protein1 intersect at 27.8 μM concentration, this might be due to competitive inhibition between these entities whereas cell dependent phenotype get stabilized at 0.9 ms (Fig. 5; Table 1).

There are evidences where work on survey of QS protein domain essential for communication using HMM (Wuster and Madan Babu 2008) and mathematical modeling of QS process for biofilm formation had been done (Davies et al. 1998). Qualitative modeling using Petri nets have also been performed for sporulation in *B. subtilis* (Steggles et al. 2007). Major drawback of logical models (qualitative) is incapability in capturing the real dynamic behavior of networks while continuous (quantitative) models could better represent and capture the dynamicity of biological networks. Currently, QS is considered as a potential target for antimicrobial therapy to control multiple drug resistant infection. Thus, the autoinducer production switches to its high state when the elimination of autoinducer from the extracellular space is decreased. This biochemical switch is hysteretic so that autoinducer production switches on at different (higher) levels than it switches off. This hysteresis is possibly important in the regulation of the production of pheromones, which tends to decrease bacterial density. One limitation of our study is that the hysteresis predicted by this model investigation has not been verified experimentally. Experimental verification could provide more insight into the process of competence, and sporulation regulation.

We interpret the behavior of species by implying different condition of time and concentration and these simulations validated the regulatory model and showed that the response regulator ComE and agrA are the terminal

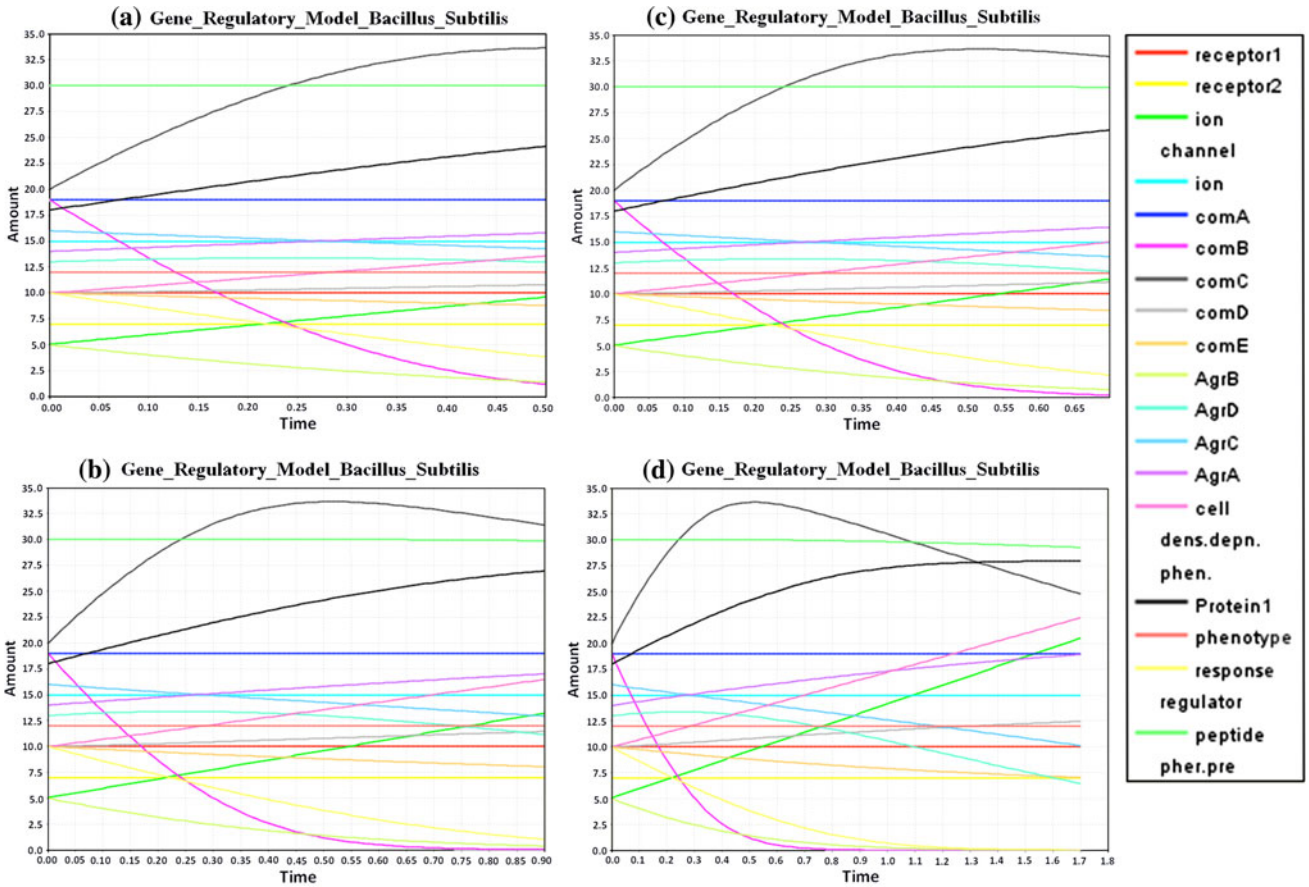

Fig. 2 Cascade of simulations at fixed concentrations (max. limit 35 μM) but at different end time ranging from 0.4 to 1.9 ms. **a** Simulation performed at end time 0.5 ms. **b** Simulation performed at end time 0.7 ms. **c** Simulation performed at end time 0.9 ms. **d** Simulation performed at end time 1.7 ms. Simulations were performed in real time environment using interactive options to validate dynamicity of reaction equations for all the reaction species. Important reaction species identified after this cascades were ComB, ComC, agrA, cell density dependent phenotype and the protein produced (Protein 1)

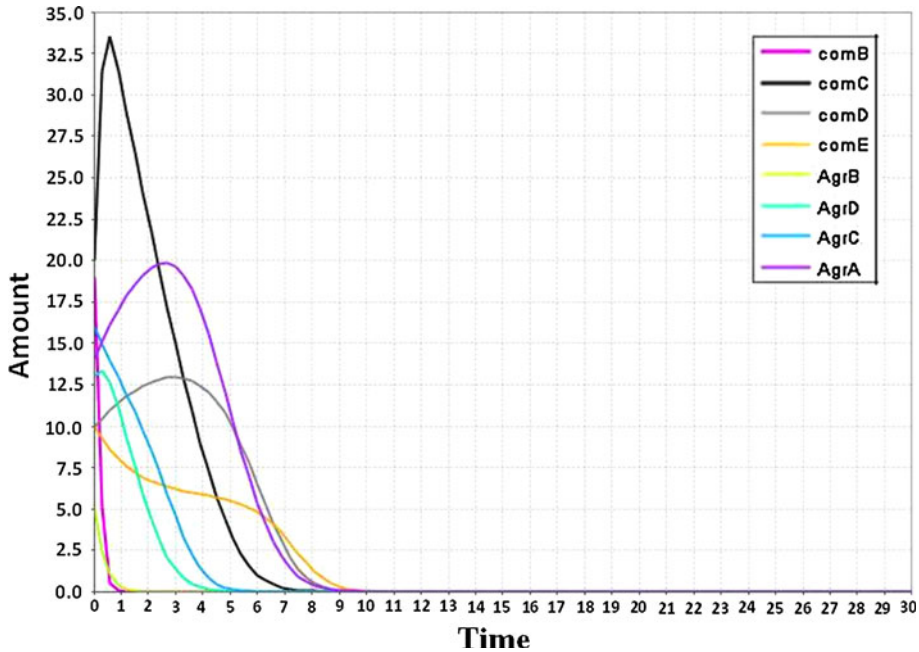

Fig. 3 Simulation results for reaction species involved in two pathways (all 4 Com and Agr in both competence and virulence pathways respectively), behaved in a harmony while excluding other interactive factors

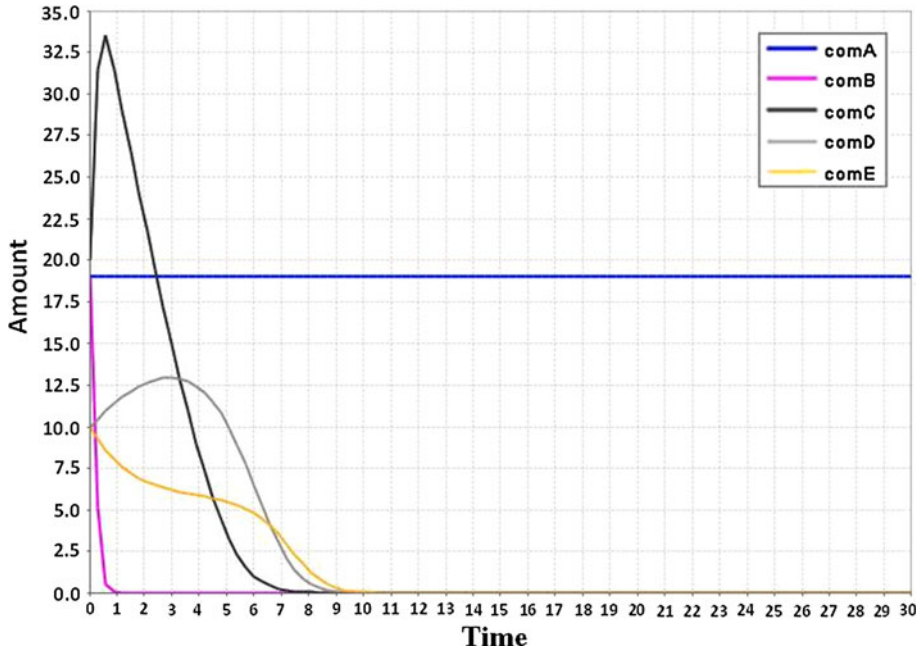

Fig. 4 Simulation results for reaction species involved in competence pathway. ComA behaved non dynamic (reason for exclusion from other analyses) while rest others found interactive with other reaction species

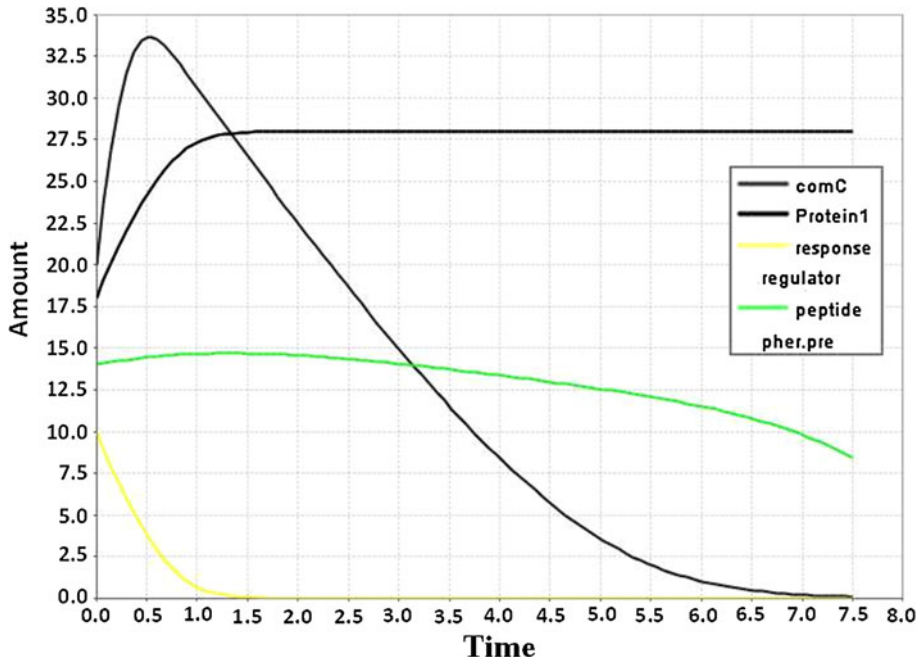

Fig. 5 Simulation of all final products and important entities for evaluation of their interactive behaviors. The axis of ComC and protein1 intersect at 27.8 μM concentration indicates competitive inhibition between these entities. At similar point of time peptide pheromone achieved its highest level and then started decreasing gradually

regulators in a cascade that integrates multiple signals. Furthermore, our approach addresses that in virulence relationship agrD can replace agrC and, in some cases, agrC can replace agrD for expression of cell dependent encoded virulence factors. Similarly, in some cases ComA and ComB can replace each other while demonstrating competence relationships. Since many model species (genes and proteins) are involved in regulating the competence and virulence of *B. subtilis* are conserved among other bacterial species, the proposed model may be applicable to other bacterial species which are important in agriculture, biotechnology, and medicine.

Conclusion

Bacteria adopt QS system to regulate a varieties of activities. QS allow population of individuals to coordinate global behavior and act as a multicellular unit. The processes controlled by QS are diverse and reflect the specific needs of

particular communities inhabiting unique niches. Competing bacteria and susceptible hosts have developed natural strategies for impeding QS bacteria by destroying the chemical signal molecules or producing autoinducer antagonists that interfere with recognition of the bona-fide signal molecule. We proposed a simple autoinduction and auto-regulation based model for *B. subtilis* where we anticipated some interchange and exchange amongst species involved in regulatory mechanisms for QS. Cell-density dependent phenotypes regulation could provide clues for cellular communications and structural–functional analyses of these sensor proteins should resolve the latent mystery. Continued study of bacterial QS systems promises to give biologists new insights into novel mechanisms of intra and intercellular signal transmission, intra and interspecies communication and the evolution of multicellular organisms. As the list of bacteria employ QS system to grow, so does the number of possibilities for exploiting regulatory mechanisms. In future, it will be interesting to see whether additional human pathogens utilize QS as a part of their pathogenic lifestyle and if so, whether production of signal molecule can be exploited to control infection. Proposed model may be applicable to other species of importance in agriculture, biotechnology, and medicine.

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