Isolation and Characterization of Mutations
That Alter the Quorum Response in *Bacillus subtilis*

by

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ABSTRACT

Many microbes use cell-cell signaling to modulate gene expression. In various organisms cell-cell signaling regulates bioluminescence, aggregation, sporulation, nodulation, competence, plasmid transfer, conjugation, and virulence, in response to cell density. The ability to sense and respond to population density is called the quorum response.

This thesis describes the isolation and characterization of mutations that affect the production of two peptide pheromones involved in the quorum response in *Bacillus subtilis*. The activity of the key transcription factor (ComA) required for the reprogramming of gene expression in response to increasing cell density is controlled by its phosphorylation state. The kinase that activates it is activated by one of the pheromones, ComX pheromone. The phosphatase which inactivates it is inactivated by the other peptide pheromone, Competence and Sporulation Factor (CSF). Both pheromones accumulate extracellularly as culture density increases. ComX pheromone is a cell density factor and CSF, as I demonstrate in this thesis, modulates the quorum response by integrating information on cell density and growth conditions.

I isolated 27 mutations (at least 18 of which are independent) that decrease the expression of a quorum-responsive gene. Each of these mutations, which cause the elimination of ComX pheromone production, is in comQ or comX. ComX is the precursor of ComX pheromone and ComQ is similar to isoprenoid-binding proteins. ComQ may be involved in modification of ComX pheromone. No other genes were identified in this search for genes involved in ComX pheromone production. The others are likely to encode proteins that are essential for viability, redundant, and/or extracellular.

I isolated 51 mutations that increase the expression of a quorum-responsive gene and characterized 5 of them. These ups (up for srfA expression) mutations affect the production of CSF. The mutations, which result in the activation of the quorum response at low cell density, affect the phosphoenolpyruvate:sugar phosphotransferase system (PTS) and the pyridine nucleotide cycle (PNC) revealing a connection between carbon and energy physiology and cell-cell signaling. The mutations cause inefficient carbon utilization (resulting slow growth) and increased activity of the alternate sigma factor sigma-H. Since the production of CSF is sigma-H-dependent, conditions that support poor growth cause increased CSF production. Increased CSF production sensitizes the cell to ComX pheromone allowing the quorum response to be activated when there is little extracellular ComX pheromone. I found that by using CSF to integrate information on cell density and physiology cells can change the cell density at which the quorum response is activated in response to nutritional conditions.
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Finally, I’d like to thank my husband Brad Palmer. Thank you for dealing so well with the expansions and contractions of ‘lab time’, the peaks and valleys of day-to-day life with a grad student, and long hours caring for our son while I finished my thesis.

This thesis is dedicated to my son Kenneth Bradley Joseph Palmer. You are the most interesting and delightful experiment I have ever begun.
# Table of Contents

Abstract 2
Acknowledgements 3
Table of Contents 4
List of Tables 5
List of Figures 6
Thesis Plan 8

Chapter 1: Introduction to Quorum Sensing 10

Chapter 2: Isolation and Characterization of Mutations that Eliminate ComX Pheromone Production 45

Chapter 2A: A Sequence-based Search for the ComX Pheromone Exporter 63

Chapter 3: Growth Conditions Modulate the Quorum Response by Affecting CSF Production 79

Chapter 4: Summary and Perspectives 113

Chapter 5: Materials and Methods 123

References 138
List of Tables

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>Table 1-1</td>
<td>Peptide permeases involved in signaling</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Table 1-2</td>
<td>Post-translational modifications that add 206-336 daltons</td>
<td>34</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Table 2-1</td>
<td>Each mutation was mapped to \textit{comQX}</td>
<td>58</td>
</tr>
<tr>
<td>Chapter 2A</td>
<td>Table 2A-1</td>
<td>Putative peptide exporters encoded in the \textit{B. subtilis} genome</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Table 2A-2</td>
<td>Plasmids</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Table 2A-3</td>
<td>Primers</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Table 2A-4</td>
<td>Strains</td>
<td>73</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Table 3-1</td>
<td>The \textit{ups} mutations map to genes involved in carbon and energy metabolism</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Table 3-2</td>
<td>The \textit{ups} mutations cause slow growth in defined minimal medium containing glucose as a carbon source</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Table 3-3</td>
<td>CSF accumulation is greater in \textit{ups} mutants than in wild type cells during early exponential growth</td>
<td>102</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Table 5-1</td>
<td>Strains</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Table 5-2</td>
<td>Plasmids</td>
<td>127</td>
</tr>
</tbody>
</table>
List of Figures

Chapter 1

Figure 1-1 Structure of N-acyl homoserine lactone and gamma-butyrolactone signals 17
Figure 1-2 Peptide signaling cassettes are found in six genera 24
Figure 1-3 *Bacillus subtilis* quorum response 30
Figure 1-4 Production of ComX pheromone 32
Figure 1-5 Production of CSF 36
Figure 1-6 A family of phosphatase/peptide regulator pairs in *B. subtilis* 37
Figure 1-7 The regulation of competence development 41
Figure 1-8 Multiple inputs into the initiation of sporulation 43

Chapter 2

Figure 2-1 Alignment of multiple known isoprenyl diphosphate synthases and ComQ 47
Figure 2-2 Extracellular ComX pheromone activity increases as cell density increases 51
Figure 2-3 The expression of *comQX-lacZ* varies less than two-fold during exponential growth 52
Figure 2-4 The number of copies of *comQX* affects the expression of *srfA-lacZ* 54

Chapter 2A

Figure 2A-1 Cells lacking one putative peptide exporter exhibit a wild type response to increasing cell density 75
Figure 2A-2 Cells lacking four putative peptide exporters exhibit a wild type response to increasing cell density 77

Chapter 3

Figure 3-1 The expression of *srfA-lacZ* is induced at lower cell density in *ups* mutants than in wild type cells 82
Figure 3-2 Null mutations in *ptsG*, *gicT*, and *gicR* cause an early induction of the quorum response 87
Figure 3-3 The phosphoenolpyruvate:sugar phosphotransferase system (PTS) 89
Figure 3-4 Regulation of *ptsG* expression in *B. subtilis* 91
Figure 3-5 Synthesis and recycling of NAD 94
Figure 3-6  Conditions that support an extended doubling time and the relief of catabolite repression cause an early induction of the quorum response

Figure 3-7  A ptsG150 mutant behaves like wild type in defined minimal medium containing glycerol as a carbon source

Figure 3-8  Production of CSF is elevated under conditions that support poor growth

Figure 3-9  The early induction of srfA-lacZ expression in an ups mutant depends upon the production of CSF

Figure 3-10  Model predicting a hyper-response to ComX pheromone resulting from an increase in CSF production

Figure 3-11  An ups mutation causes hyper-response to both ComX pheromone and CSF

Figure 3-12  The expression of the CSF precursor gene, phrC, is elevated in ups mutants

Figure 3-13  Sigma-H is required to mediate the effect of an ups mutation on the quorum response

Chapter 4  Figure 4-1  Growth conditions modulate the quorum response in B. subtilis

Figure 4-2  Regulation of sigma-H activity
Chapter 1 is an introduction to quorum sensing in bacteria. I begin with a discussion of the quorum response in bacteria that focuses on the identity of the cell-cell signaling molecules involved, production of the signaling molecules, signal transduction, and signal output. I then describe the quorum response of Bacillus subtilis in detail. The regulation of the development of genetic competence and the initiation of sporulation in B. subtilis are presented as examples of developmental processes that are regulated by multiple inputs, including cell-cell signaling and the quorum response.

In Chapter 2 I describe the isolation and characterization of mutations that affect the expression of srfA (comS), a quorum-responsive gene. I isolated 27 mutants that made negligible amounts of ComX pheromone. Each of these mutants contained a mutation in comQ or comX, two genes previously known to be involved in ComX pheromone production. The implications of this result are presented.

In Chapter 2A (an appendix to Chapter 2) I describe a sequence-based search for the ComX pheromone exporter. The competed B. subtilis genome was searched for ATP-binding cassette (ABC) transporters that share sequence similarity with ABC transporters known to export peptide signaling molecules in others systems. Seven such exporters were identified. One encodes the sublancin exporter and was not considered further. Two encode essential ABC exporters and were not considered further at this point. Four encode dispensible ABC exporters. None of these four appear to play a role (alone or in combination) in ComX pheromone export.

Chapter 3 is a presentation of work demonstrating that growth conditions modulate the quorum response in B. subtilis by affecting the production of CSF. Five mutants with increased expression of srfA-lacZ were characterized. Each contains a mutation in a gene involved in carbon and energy metabolism. These mutations cause slow growth, which leads to induction of the quorum response at lower cell density. The early induction of the quorum response depends upon the alternate sigma factor sigma-H. Increased sigma-H activity in these mutants results in
increased transcription of \textit{phrC}, the gene encoding the CSF precursor, and increased production of CSF. These findings indicate that multiple signals can be integrated to affect the production of a signaling molecule allowing both cell density and physiological inputs to contribute to the quorum response.

In Chapter 4 I discuss how the work presented in this thesis provides another example of signal integration. I go on to discuss the regulation of sigma-H and to propose possible mechanisms by which growth conditions may affect sigma-H activity. I conclude with the suggestion that growth conditions may modulate the quorum response in multiple systems.

Chapter 5 contains a description of the materials and methods used in the work described in Chapters 2 and 3.
Chapter 1

Introduction to Quorum Sensing
Despite the predominant perception of single-celled organisms as solitary beings acting independently of their neighbors, unicellular creatures actually communicate quite elegantly with each other. In dozens of characterized examples, individual cells have been found to produce signaling molecules that are perceived by other cells of the same species [reviewed in (Dunny and Winans, 1999)]. Such signaling systems are likely to exist in nearly all bacteria. These signals induce biologically relevant responses by altering gene expression, development and/or behavior. This only makes sense. Why carry out an action that requires a large population if there is but a single being? Only a foolhardy individual would try to build the Great Pyramids of Egypt alone.

Many bacterial processes require large numbers of cells in order to have an effect. An antibiotic produced by one cell has no effect on other bacteria since it is diluted out into the environment. Get billions of cells producing that same antibiotic in a small space and competing bacteria now have something to worry about. (The same can be said for the production of extracellular enzymes such as proteases or pectate lyases.) A similar argument can be made for the use of cell-cell signaling by pathogenic organisms. A single bacterium making virulence factors deep within the lung of a man or woman is just wasting its time and energy. After colonization, that bacterium and its progeny doing the same thing cause pneumonia.

Recognition of successful colonization in order to have strength of numbers is not the only function of cell-cell signaling. Intra-specific genetic exchange requires the presence of a DNA donor as well as a DNA recipient. It’s a sad individual who prims for the prom only to sit in an empty gymnasium. Enterococcus faecalis uses cell-cell signaling to delay the mating response until the presence of a plasmid recipient is sensed [reviewed in (Clewell, 1999)]. Streptococcus pneumoniae and Bacillus subtilis delay the development of genetic competence (the ability to bind to and take up DNA from the environment) until the population of con-specific organisms is dense enough to make the uptake of useful DNA likely [reviewed in (Grossman, 1995; Havarstein and Morrison, 1999; Lazazzera et al., 1999)].

Finally, some developmental processes require great energy expenditure and result in a cell type that is not actively reproducing. Formation of such a cell type is great for the population as a
whole if resources are scarce and required by a large number of individuals since a dormant cell can be dispersed and persist until resources are bountiful again. It might be a strategic mistake for a cell to become dormant if it has few competitors, even if nutrient levels are very low. If nutrient levels increase suddenly, cells that have become dormant would be at a competitive disadvantage to cells that are actively metabolizing and poised for cell division. Thus several organisms, including *B. subtilis, Myxococcus xanthus*, and *Streptomyces griseus*, sporulate only when cells are both starving and crowded by members of their own species [reviewed in (Grossman, 1995; Stragier and Losick, 1996; Horinouchi, 1999; Plamann and Kaplan, 1999)].

The mechanism by which individual cells sense that they are amongst friends, lovers, or competitors is called the quorum response and is the subject of this chapter.

THE QUORUM RESPONSE

Definition of the quorum response Antibiotic production, pathogenesis, conjugation, genetic competence, sporulation – all are processes that occur at high cell density in particular organisms. In each case, a reprogramming of gene expression leads to the production of a new set of proteins. This occurs in response to the perception of a signaling molecule that is at the appropriate concentration to signal a change in transcription patterns. This critical concentration is reached only when the cells are at the appropriate cell density for carrying out the cell density-regulated process. Thus, the quorum response is a change in gene expression in response to the sensing of a signaling molecule that accumulates extracellularly at high cell density (Fuqua et al., 1994).

The first hint of inter-cellular communication in a bacterial system came many decades ago, but it has only been a few years since a 'complete' understanding of a quorum response was developed. In the 1930s Dawson noted that Streptococci become competent only transiently [reviewed in (Havarstein and Morrison, 1999)]. It was presumed that there must be a means by which the cells recognize that they are at the proper cell density for competence development, thus some sort of inter-cellular signaling was hypothesized. It wasn’t until the mid-1960s that Tomasz
and Mosser demonstrated that the cells themselves produce a factor that potentiates competence development (Tomasz and Mosser, 1966). By 1972, Joenje had demonstrated that one of these competence-inducing factors, in this case that of *B. subtilis*, is produced in a cell density-dependent manner (Joenje et al., 1972). A few years later and in yet another system, Eberhard et al. presented the first molecular characterization of a cell-cell signaling molecule. An N-acyl homoserine lactone (AHL), N-3- (oxohexanoyl) homoserine lactone, produced by *Vibrio (Photobacterium) fischeri* was found to be required for the induction of bioluminescence at high cell density (Eberhard et al., 1981). With the cloning of the *lux* genes from *V. fischeri* in 1983, a model for the first fully-characterized quorum response (including details on factor production, factor sensing, signal transduction, and alteration of gene expression) emerged (Engebrecht et al., 1983). Along the way many nay-sayers contended that bacteria could not exhibit ‘multicellular’ behavior (discussed in (Hellingwerf, 1988; Hastings and Greenberg, 1999; Nealson, 1999) but by 1999 inter-cellular signaling in bacteria was so widely-characterized and well-accepted that the American Society of Microbiology (ASM) published an entire volume the phenomenon (Dunny and Winans, 1999).

Since the initial discovery of the quorum response, cell density-dependent signaling has been found to regulate a variety of adaptive processes in bacterial systems. As described below, the best characterized examples are the induction of bioluminescence in *V. fischeri*, which colonize the light organs of squid, and the induction of competence in the pathogen *S. pneumoniae* [reviewed in (Fuqua et al., 1996; Havarstein and Morrison, 1999)]. The induction of bioluminescence in *V. fischeri*, conjugation in *Agrobacterium tumefaciens*, and pathogenesis in several *Pseudomonas* species (and many other gram negative plant and animal pathogens) are each regulated by a quorum response activated by one or more AHL signaling molecules [reviewed in (Gray, 1997)]. The development of competence in *S. pneumoniae* and *B. subtilis*, sporulation in *B. subtilis*, virulence in *Staphylococcus aureus*, conjugation in *E. faecalis*, and the production of antibiotics in various lactic acid bacteria are induced by the accumulation of peptide signals [reviewed in (Dunny and Leonard, 1997)]. In each process there is an accumulation of a signaling
molecule, perception of the signaling molecule, activation of a transcription factor, and a reprogramming of gene expression.

Single-celled eukaryotes also utilize cell–cell signaling to affect developmental changes. The best characterized example is the regulation of mating in the yeast *Saccharomyces cerevisiae* by two peptide pheromones [reviewed in (Kurjan, 1993)]. During the yeast life cycle, cells go through a haploid state. In this haploid state, cells of one mating type, a, produce a-factor and cells of the other mating type, alpha, produce alpha-factor. a-factor is a farnesylated dodecapeptide (Anderegg et al., 1988) that activates a membrane-bound receptor on a nearby alpha cell (Hagen et al., 1986), while alpha-factor is an unmodified 13-amino-acid peptide (Nakano and Zuber, 1993) that activates a different membrane-bound receptor on a nearby a cell (Jenness et al., 1983). Since there is asymmetry in factor secretion (Kuchler et al., 1993), this sets up a mating pair consisting of one a cell and one alpha cell. Both pheromone receptors are coupled to the same signaling pathway (Bender and Sprague, 1986; Kurjan, 1993). This pathway leads to the activation of the Ste12 transcription factor (Fields and Herskowitz, 1985). Under the direction of Ste12, changes in gene expression result in a cell cycle arrest and fusion of the a and alpha cells. After the cells fuse, the a and alpha nuclei fuse to form a diploid nucleus and mating is complete.

**Bacterial Processes Regulated by Cell Density: 2 Examples**

**Bioluminescence in Vibrio fischeri** The bacterial quorum response was initially characterized in the gram negative organism *V. fischeri*, which uses two N-acyl homoserine lactones (AHLs) to regulate bioluminescence during the colonization of the light organ of the squid *Euprymna scolopes* [reviewed in (Ruby, 1996)]. Inside the light organs the AHLs cannot diffuse into the vast volume of the sea; this leads to an accumulation of AHLs both inside and outside the cells. As the bacteria grow to high cell density, the AHLs reach concentrations high enough to activate the expression of the genes encoding luciferase and those that encode the proteins that produce its aliphatic substrate. Luciferase converts this substrate and oxygen to light and water. At night, while luminescing, the bacteria are at cell densities exceeding $10^9$ cells per milliliter. Each
morning, when light is no longer needed, the squid ejects 90-95% of the bacteria from the light organ. This reduction in cell density inside the light organ allows a shut off of bioluminescence during the daylight hours when it is unneeded. The bacteria multiply throughout the day reaching high cell density in time to generate more light the following night. In this well-designed symbiosis the bacterial symbiont provides the squid with light that protects it from predators while the squid provides the bacterium with nutrients for growth and a means of dispersal.

**Competence development in *Streptococcus pneumoniae*** The most thoroughly characterized gram positive quorum response is that which governs competence development in *S. pneumoniae* [reviewed in (Havarstein and Morrison, 1999)]. During growth *S. pneumoniae* produces an unmodified 17-amino-acid signaling peptide. As described below, the sensing of this peptide, as well as the sensing of other uncharacterized peptide signaling molecules, results in the activation of the transcription factor ComE. ComE governs the expression of at least 6 (and perhaps 8 or more) genes, all of which are required for the binding, uptake, or recombination into the genome of DNA from the extracellular milieu (Campbell et al., 1998; Claverys and Martin, 1998). In this way the quorum response leads directly to the development of genetic competence in this organism.

The development of genetic competence only at high cell density in gram positive organisms is an energy-saving strategy. In general, gram positive organisms that become competent to take up exogenous DNA do so without regard to the sequence or source of that DNA [reviewed in (Solomon and Grossman, 1996)]. Since incoming DNA can only be recombined into the genome if it is reasonably complementary, it makes sense to regulate competence development in a cell density-dependent manner. An organism that becomes competent only when surrounded by many members of its own species is more likely to take up DNA highly similar to its own.

**A Rule Emerges** In general, gram negative organisms use N-acyl homoserine lactone signals in cell-density signaling, while gram positive organisms use peptide-based signals. AHLs are produced inside the cell and freely diffuse across the cell membrane, reaching higher concentrations both inside and outside the cell as cell density increases (Nealson, 1977; Eberhard et
AHLs can activate the quorum response in one of two ways. In most cases they bind to and activate an intracellular receptor/transcription factor (Showalter et al., 1990; Schaefer et al., 1996). However, in the case of *V. harveyi* two AHL autoinducers act via a branched phosphorelay to regulate the activity of a transcription factor; this demonstrates that AHLs can also act via (expanded) two-component systems (Bassler et al., 1993; Bassler et al., 1994a; Freeman and Bassler, 1999a; Freeman and Bassler, 1999b). Both mechanisms of signal transduction alter gene expression.

Peptide signals are produced inside the cell and actively exported to the extracellular milieu where they may undergo further processing [reviewed in (Dunny and Leonard, 1997; Lazazzera et al., 1999)]. Peptide signaling molecules accumulate extracellularly and either (1) activate membrane-bound histidine protein kinases leading to the activation of the cognate response regulator transcription factor [reviewed in (Kleerebezem et al., 1997)] or (2) are imported back into the cell via an oligopeptide permease where they interact with intracellular targets to affect gene expression [reviewed in (Lazazzera and Grossman, 1998)].

**Exceptions to the Rule** *Streptomyces* species are gram positive. While some of them use peptide signaling molecules, many of them use gamma-butyrolactones, signaling molecules that are structurally similar to the AHL signals used by gram negative organisms (Fig. 1-1) [reviewed in (Horinouchi and Beppu, 1994; Horinouchi, 1999)]. Antibiotic production, aerial mycelium formation, and sporulation in *S. griseus* depend on a gamma-butyrolactone called A-factor. Like the AHLs, A-factor is synthesized intracellularly from metabolic intermediates by a specific synthase and is hypothesized to diffuse freely across the cell membrane to reach equal concentration inside and outside the cell (Horinouchi et al., 1989; Ando et al., 1997). Like AHLs, A-factor interacts with an intracellular receptor/transcriptional regulator to affect gene expression (Miyake et al., 1989; Onaka et al., 1995). In contrast with the AHLs, A-factor activates gene expression by inhibiting the binding of a transcriptional repressor instead of stimulating the binding of a transcriptional activator (Onaka et al., 1995). Although gamma-butyrolactone signaling is
<table>
<thead>
<tr>
<th>Organism</th>
<th>Synthase</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio fischeri</em></td>
<td>LuxI</td>
<td><img src="image" alt="Structural formula for LuxI" /></td>
</tr>
<tr>
<td><em>Vibrio fischeri</em></td>
<td>AinS</td>
<td><img src="image" alt="Structural formula for AinS" /></td>
</tr>
<tr>
<td><em>Rhizobium leguminosarum</em></td>
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<td><img src="image" alt="Structural formula for unknown synthase" /></td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td>AfsA</td>
<td><img src="image" alt="Structural formula for AfsA" /></td>
</tr>
</tbody>
</table>

Figure 1-1. Structure of N-acyl homoserine lactone and gamma-butyrolactone signaling molecules

The structures of representative N-acyl homoserine lactone and gamma-butyrolactone signaling molecules are depicted. Each signaling molecule is produced by the indicated organism, and production is dependent upon the activity of the indicated synthase.
similar in many respects to AHL signaling, the two evolved separately illustrating the usefulness of this particular signaling mechanism.

The gram negative organism *Myxococcus xanthus* is also an exception to the rule. Fruiting body formation and sporulation in *M. xanthus* are governed by a complex developmental pathway regulated by multiple extracellular signals [reviewed in (Kim et al., 1992)]. The factor that indicates cell density, A-factor, has two components: extracellular proteases and the amino acids liberated by these proteases (Kuspa et al., 1992; Plamann et al., 1992). So, instead of using an N-acyl homoserine lactone to signal cell density, *M. xanthus* uses amino acids derived from peptides. Although A-factor was purified based on its ability to induce expression of a cell density-regulated gene, little is known about the molecular mechanisms that underlie A-signaling.

**AHL SIGNALING IN GRAM NEGATIVE ORGANISMS**

As discussed above, the best characterized example of AHL signaling is the use of N-3-(oxohexanoyl) N-acyl homoserine lactone to regulate the induction of bioluminescence in the marine symbiont *V. fischeri*. In this section I discuss the structure and production of AHL signaling molecules and then describe the signal transduction pathway that allows AHLs to alter gene expression. Most of the information is drawn from *V. fischeri*, but information from other organisms is used when necessary to highlight specific points or to present a more complete description.

**Nature of the Signals** AHL signaling molecules are comprised of a homoserine lactone moiety (derived from amino acid biosynthesis) linked via a peptide bond to an acyl moiety (derived from fatty acid biosynthesis). Some of the organisms that make AHLs produce more than one AHL and some AHLs are produced by more than one organism. Each different AHL activates the quorum response of the organism(s) that produces it. Specificity arises from variations in the acyl moiety. In different AHLs the acyl group varies in length, in the degree of saturation of the carbon backbone, and/or in modification or oxidation (Fuqua and Eberhard, 1999) (Fig. 1-1).
Synthesis of AHLs  AHLs are produced by specific synthases. There appear to be two unrelated families of synthases. The first is comprised of synthases that are similar in sequence to LuxI, the AHL synthase responsible for N-3-(oxohexanoyl) homoserine lactone synthesis in V. fischeri. Homologues are found in at least 17 organisms (Fuqua and Eberhard, 1999). The second family is composed of proteins that are similar to AinS, the N-octanoyl-L-homoserine lactone synthase of V. fischeri (Gilson et al., 1995). Although they synthesize AHLs, the AinS-like proteins share little sequence similarity with LuxI. To date AinS homologues have been found only in V. fischeri and V. harveyi. Little is known about the mechanism by which these proteins synthesize AHLs. The current model is that AHLs are synthesized from S-adenosylmethionine and an acyl carrier protein conjugated to the appropriate acyl group (Eberhard et al., 1981). This was confirmed by the in vitro synthesis of N-(3-oxo-octanoyl)-L-homoserine lactone from S-adenosylmethionine and 3-oxo-octanoyl-acyl carrier protein by TraI, the LuxI homologue of Agrobacterium tumefaciens (More et al., 1996).

The production of AHLs is autoinduced. At low cell density, the extracellular (and intracellular) concentration of AHLs is very low; at high cell density it is higher [reviewed in (Fuqua et al., 1994)]. The sensing of AHL leads to the production of more AHL; thus, the concentration of AHL increases at a higher rate than does cell density. Specifically, since LuxR, the transcriptional activator activated by N-3-(oxohexanoyl) homoserine lactone, activates transcription of luxI, perception of very low levels of this AHL soon leads to a rapid increase in AHL production and accumulation (Engebrecht and Silverman, 1984; Engebrecht and Silverman, 1987). A similar autoinduction loop is found in other AHL-based signaling systems [reviewed in (Fuqua et al., 1996)].

Analysis of the sequences of the various AHL synthases has revealed little about the generation of the various AHL structures. Although the LuxI homologues are only 28-34% identical there are no obvious region of divergence that could account for the generation of signal diversity seen in AHLs (Fuqua and Eberhard, 1999).
Signal Transduction in AHL Signaling  In general, AHLs interact with and activate a cognate member of the LuxR family. LuxR is the transcriptional activator responsible for reprogramming gene expression in V. fischeri in response to N-3-(oxohexanoyl) homoserine lactone (Engebrecht and Silverman, 1987; Shadel and Baldwin, 1991). Upon binding to AHL the LuxR homologues multimerize resulting in increased affinity for a DNA site exhibiting dyad symmetry, so-called ‘lux boxes’ (Devine et al., 1989). Binding of LuxR to a lux box facilitates binding of RNA polymerase resulting in activation of transcription of the downstream gene(s) (Stevens et al., 1994). In V. fischeri this results in activation of luxICDABEG (Engebrecht and Silverman, 1984). Since these genes encode the autoinducer synthase, luciferase, and the enzymes that synthesize the luciferase substrate, the sensing of the AHL signal results in further signal production as well as the induction of bioluminescence.

The two AHLs (one produced by a LuxI family member and one produced by an AinS family member) produced by V. harveyi act via a branched phosphorelay composed of two histidine protein kinase –response regulator (HPK-RR) proteins, a phosphotransfer protein, and a response regulator/ transcription factor (Freeman and Bassler, 1999b) [reviewed in (Bassler, 1999)]. The response regulator negatively regulates the quorum response in V. harveyi (Bassler et al., 1994b; Bassler et al., 1994a). The current model is that at low cell density the kinases are active, allowing phosphate to flow from ATP through the phosphorelay to the response regulator activating it as a repressor. At high cell density, the interaction of the AHLs with their HPK-RR receptors results in activation of the receptor’s phosphatase activity. This leads to backwards flow of phosphate through the phosphorelay, dephosphorylation and deactivation of the response regulator/ repressor, and, finally, to activation of the quorum response (Bassler, 1999).

Output of AHL Signaling  As described above, the response to an AHL signal results in increased expression of genes encoding the AHL synthase and genes involved in the particular process that is subject to cell-density control. While the quorum response in B. subtilis leads to activation of a variety of genes affecting several processes (see below), to date the quorum...
response in gram negative organisms appears to be limited to one specific adaptation to high cell density (i.e. bioluminescence, virulence, conjugation, etc.).

PEPTIDE SIGNALING IN GRAM POSITIVE ORGANISMS

The best characterized peptide-based quorum responses are those that govern the development of genetic competence in *S. pneumoniae* and *B. subtilis*. *S. pneumoniae* uses competence stimulating peptide (CSP) and other uncharacterized peptides [reviewed in (Havarstein and Morrison, 1999)], while *B. subtilis* uses ComX pheromone and competence and sporulation factor (CSF) [reviewed in (Grossman, 1995; Lazazzera et al., 1999)]. In each case the major signaling peptide (CSP and ComX pheromone, respectively) is a so-called peptide signaling cassette (PSC) peptide (defined below) that is sensed by a two-component system. The other peptides (uncharacterized in the case of *S. pneumoniae* and CSF in the case of *B. subtilis*) are imported into the cell and interact with intracellular targets. Below, I discuss each type of signaling. I have attempted to use *S. pneumoniae* to illustrate peptide signaling, but information from other organisms is presented when necessary to illustrate a point or to present a more complete description.

Peptide Signaling via Two-component Systems

Nature of the Signals In general peptide signaling molecules used for quorum sensing are derived via proteolytic cleavage of larger ribosomally-synthesized precursor peptides. Therefore the backbone of each of the peptide-based signaling molecules is a series of amino acids linked by peptide bonds. Some of the signaling peptides are also post-translationally modified. These include simple modifications, such as the introduction of a internal anhydride bond in the autoinducing peptide (AIP) of *S. aureus* (Ji et al., 1995), more complex modifications, such as the uncharacterized hydrophobic modification of a tryptophan found in ComX pheromone of *B. subtilis* (Magnuson et al., 1994), and multiple unusual modifications, such as those that create the poly-cyclic structure of the lantibiotics nisin (produced by *Lactococcus lactis*) and subtilin
(produced by \textit{B. subtilis}), which serve as autoinducers of their own synthesis (de Ruyter et al., 1996a; de Ruyter et al., 1996b; Kleerebezem et al., 1999).

\textbf{Synthesis of Peptide Signals} The unmodified PSC peptides, such as CSP of \textit{S. pneumoniae}, are each cleaved and exported in a similar manner, while those that are modified (ComX pheromone of \textit{B. subtilis} and AIP of \textit{S. aureus}) appear to have more divergent means of production. Unmodified PSC peptides are liberated upon the concomitant cleavage of the precursor at a gly-gly cleavage site and export of the active peptide from the cell. The mechanisms by which modified PSC peptides are cleaved and exported remains unknown (see Chapter 2), although there has been some insight into how ComX pheromone and AIP are modified.

The precursors of the unmodified PSC peptides share three characteristics: (1) a conserved leader peptide, (2) a gly-gly cleavage site, (3) similar amphipathicity profiles (Havarstein et al., 1995b). The conserved leader peptide is believed to be important for recognition by the conserved proteolytic domain of the PSC exporter, while the gly-gly cleavage site is necessary for cleavage. The amphipathic nature of the signaling peptide may be responsible for increasing the local concentration of the peptide at the cell surface and facilitating interaction with the histidine protein kinase receptor.

The PSC peptides that contain a gly-gly cleavage site are cleaved during export by a dedicated ATP-binding cassette (ABC) exporter (Havarstein et al., 1995b). Each exporter is composed of three domains: a conserved N-terminal domain, a transmembrane domain typical of ABC exporters, and a C-terminal ATP-binding domain. The transmembrane and ATP-binding domains share similarity with multiple ABC exporters that export peptides or small proteins. The lactococcin G exporter, LagD, has been shown to cleave the lactococcin G precursor LagA at the gly-gly cleavage site, demonstrating that the proteolytic activity resides in LagD itself (Havarstein et al., 1995b). Mutation of cysteines conserved in the N-terminal domains of ABC exporters that act on peptides with gly-gly cleavage sites destroyed the proteolytic activity of LagD suggesting that the N-terminal domain is the proteolytic domain (Havarstein et al., 1995b). Although the other
dedicated exporters are presumed to act similarly, the proteolytic activity of the N-terminal extension has been characterized only in LagD.

Not all PSC peptides are unmodified. Both ComX pheromone of *B. subtilis* and AIP of *S. aureus* are modified. ComX pheromone is hydrophobically-modified on a tryptophan residue (Magnuson et al., 1994) while AIP contains an internal anhydride bond that cyclizes its last 5 amino acids (Ji et al., 1995). In both cases the modification is absolutely required for activity. The precursors of these peptides lack both typical sec-dependent signal sequences and the leader peptides and gly-gly cleavage sites of the unmodified PSC peptides. The peptides also lack the amphipathic character of the unmodified PSC peptide precursors. It is tempting to speculate that these differences necessitate the modifications found in these peptides. Perhaps the modifications are needed to facilitate interaction with the protease or exporter. More intriguing, given the essential nature of the modifications, is the possibility that they somehow compensate for the lack of amphipathicity of the peptide backbones.

**Genetics of Peptide Signal Production** Similarly arranged sets of genes required for cell density-dependent gene expression, which I call ‘peptide signaling cassettes’ (PSCs), have been characterized in multiple gram positive organisms (Fig. 1-2). Each peptide signaling cassette consists of at least three genes. The first encodes a precursor peptide that is cleaved to generate the peptide (moiety of the) signal. The next two genes encode the two-component histidine protein kinase/ response regulator pair required for response to the peptide signal. When the signaling peptide is modified, an additional gene upstream of these three is required for signal production and is believed to be involved in modification of the peptide. Although they can be found elsewhere, genes encoding a sec-independent processing and secretion system required for peptide signal export via an ATP-binding cassette (ABC) exporter are often found adjacent to the PSC. This paradigm for cell-cell signaling has been characterized in six species to date (Fig. 1-2) (Magnuson et al., 1994; Diep et al., 1995; Ji et al., 1995; Pestova et al., 1996; Brurberg et al., 1997; Quadri et al., 1997). The existence of PSCs in a variety of organisms suggests that they may have spread by horizontal transfer.
Figure 1-2. Peptide signaling cassettes

Peptide signaling cassettes (PSCs) are found in at least six genera and regulate the indicated process. The structures of the six characterized PSCs are as indicated. Each PSC is comprised of a pheromone precursor gene, a histidine protein kinase gene and one (or more) response regulator genes. Response to each peptide is dependent upon the two-component system encoded downstream of its precursor gene. In the case of *B. subtilis* and *S. aureus* a gene just upstream of the precursor gene is required for production of the active signaling peptide.
Given the high level of conservation, both in terms of sequence similarity and gene arrangements, one should be able to look for PSCs in any of the completed genome sequence. This maybe a straightforward way of identifying possible targets for drugs aimed at pathogens that depend upon a quorum-response for colonization. It may also lead to the discovery of new bacteriocins, since many type II bacteriocins are encoded in PSCs.

**Signal Transduction in PSC Peptide Signaling**  PSC peptides affect gene transcription by activating classical two-component systems [reviewed in (Kleerebezem et al., 1997)]. In each case it is hypothesized that the extracellular signaling factor interacts with the transmembrane domain of the histidine protein kinase to change its activity. It is not clear how this happens. The ligand may induce kinase activity or inhibit phosphatase activity by enhancing dimerization of the histidine protein kinase or altering the conformation of an already existing dimer. Phosphate is then transferred from the kinase to the cognate response regulator, resulting in its activation as a transcription factor.

The specificity in these signaling systems lies in the peptide:histidine protein kinase pair. The work of Havarstein et al. with two pherotypes of *Streptococcus gordonii* illustrates this quite elegantly (Havarstein et al., 1996). In *S. gordonii*, competence is induced by a peptide derived from the protein encoded by the comC gene. This competence factor (CF) activates a two-component system composed of the kinase ComD and the response regulator ComE. Two strains of *S. gordonii*, Challis and NCTC 7865, produce strain-specific CF in that the CF of Challis normally does not induce competence in NCTC 7865 (and vice versa). After introducing the *comD* gene of Challis into NCTC 7865 (and simultaneously mutating the native *comD* gene) Havarstein et al. were able to make NCTC 7865 competent by exposing it to Challis CF (Havarstein et al., 1996). Since only the Challis kinase gene was moved into NCTC 7865 this shows quite succinctly that the specificity for CF signaling lies in the interaction between CF and the membrane-bound histidine protein kinase.
Output of PSC Peptide Signaling  Like quorum responses in general, the perception of PSC peptide signals leads to a change in gene expression. In *S. pneumoniae* each of the genes induced by the quorum response has a conserved DNA sequence upstream of the promoter (a *cin* box) which results in activation of transcription upon activation of the quorum response (Campbell et al., 1998). Analysis of the *S. pneumoniae* genome revealed the presence of 7 operons preceded by *cin* boxes (Campbell et al., 1998; Claverys and Martin, 1998). All members of this putative competence regulon share sequence similarity with proteins required for competence and transformation in other systems. Five of them have been shown to be induced during competence development (the other two have not been tested) (Campbell et al., 1998). This is in contrast to the ComA regulon of *B. subtilis* (discussed below) which consists of genes involved in multiple processes, only one of which is competence development.

Opp-Mediated Peptide Signaling

Oligopeptide permeases (Opps) are ABC (ATP binding cassette) transporters that are responsible for the (relatively) non-specific transport of peptides ranging in length from approximately 2 to 5 amino acids (Sleigh et al., 1997). Opps are composed of 5 polypeptides: 2 ATPases, 2 transmembrane proteins, and a ligand binding protein (Higgins, 1992). Ligand binding proteins have been implicated in the regulation of multiple developmental processes in bacteria. For several years there was debate as to whether Opp acts as a receptor for a (peptide) signal or a transporter. Recent work in *B. subtilis* suggests that Opp serves primarily a transport role in cell-cell signaling (Lazazzera et al., 1997).

There are multiple forms of peptide signaling that depend upon oligopeptide permeases to import the signaling molecules. The most widespread is the use of Opp-transported peptides to modulate the response to a primary cell density factor. Signaling like this is well characterized in *B. subtilis* and is discussed in the section on *B. subtilis* below. It is also likely to occur in *S. pneumoniae* and other organisms (Table 1-1) (Rudner et al., 1991; Ruhfel et al., 1993; Tanimoto et
### Table 1-1. Oligopeptide permeases involved in cell-cell signaling

<table>
<thead>
<tr>
<th>organism</th>
<th>process(es) regulated</th>
<th>ligand-binding protein</th>
<th>oligopeptide permease</th>
<th>peptide signal</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>competence</td>
<td>Spo0KA (OppA)</td>
<td>Spo0K (Opp)</td>
<td>CSF</td>
</tr>
<tr>
<td></td>
<td>sporulation</td>
<td>Spo0KA (OppA)</td>
<td>Spo0K (Opp)</td>
<td>CSF, PhrA</td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td>competence</td>
<td>HppA</td>
<td>Hpp</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>competence</td>
<td>AmiA, AliA, AliB</td>
<td>Ami</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>conjugation</td>
<td>PrgZ, TraC</td>
<td>Opp</td>
<td>sex pheromones</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em></td>
<td>sporulation</td>
<td>BldKA</td>
<td>BldK</td>
<td>modified peptide?</td>
</tr>
</tbody>
</table>
In Streptococci several ligand binding proteins are proposed to interact with the oligopeptide permease and at least one plays a role in competence development. In *S. gordonii*, there are three identified ligand binding proteins: HppA, HppG, and HppH. One of these, HppA (SarA), is required for normal competence development, while the others appear to play no role in competence development (Jenkinson et al., 1995; Jenkinson et al., 1996). Delayed competence development in an *hppA* mutant suggests that the Hpp oligopeptide permease has a positive influence on competence development. However, the Ami oligopeptide permease appears to have a negative effect on competence development in *S. pneumoniae*. A triple (null) mutant lacking all three Ami ligand binding proteins (AmiA, AliA, AliB) has no competence defect while a strain containing a truncated form of one of them (AliB) is not competent (Alloing et al., 1994). In both *S. gordonii* and *S. pneumoniae* Opp-imported signals are hypothesized to modulate the quorum response in response to nutritional or growth conditions (Jenkinson et al., 1995; Alloing et al., 1998). The mechanism for this is uncharacterized and it is intriguing to speculate that it may be similar to the means by which CSF and Opp (Spo0K) modulate the quorum response in *B. subtilis* in response to carbon and energy metabolism as described in this thesis.

Opp-transported peptides also play roles in developmental processes where cell-cell signaling, but not necessarily a quorum response, is important. For example, in *Enterococcus faecalis* Opp-transported peptides are the primary cell-to-cell signal for conjugation and plasmid transfer [reviewed in (Clewell, 1999)]. In this system a few molecules are hypothesized to be enough to set up a donor-recipient pair (Mori et al., 1988). An Opp-imported peptide (or peptides) is also proposed to be used in the signaling pathway that leads to sporulation in *Streptomyces coelicolor* (Nodwell et al., 1996; Nodwell and Losick, 1998).
The Quorum Response in *Bacillus subtilis*

**Historical Aspects** Cell density has long been known to play a role in competence development in *B. subtilis*. A role for cell density (as opposed to nutritional conditions) became even more evident with the discovery that a key regulatory gene in the competence development pathway, *srfA* (*comS*), is induced during exponential growth (Hahn and Dubnau, 1991; Magnuson et al., 1994). Medium in which cells had been grown to high cell density and then removed was shown to induce the expression of *srfA* (*comS*) when added to cells at low cell density suggesting a role for cell-cell signaling (Magnuson et al., 1994). Proof that cell-cell signaling regulates competence development came with the isolation and purification of two extracellular peptides, ComX pheromone and competence and sporulation factor (CSF), from the spent medium of high density cultures that when added to *B. subtilis* cells at low cell density stimulate the expression of *srfA* (*comS*) (Magnuson et al., 1994; Solomon et al., 1996).

Sporulation has long been known to occur after the transition to stationary phase, a time when the cells are both starving and at high cell density. A role for cell density separate from starvation was demonstrated in 1988, when it was reported that *B. subtilis* cells sporulate at a much lower frequency at low cell density than at high cell density even when other conditions are identical (Grossman and Losick, 1988). Grossman also reported that medium from which cells had been grown to high cell density and then removed was capable of inducing sporulation in cells at low cell density, demonstrating that there was indeed an extracellular signal involved (Grossman and Losick, 1988). Both CSF and ComX pheromone can act as sporulation factors (Magnuson et al., 1994; Solomon et al., 1996). There are at least two more uncharacterized extracellular factors that regulate sporulation (Waldburger et al., 1993; Solomon et al., 1996).

Two extracellular peptides accumulate at high cell density and stimulate the quorum response in *B. subtilis* (Fig. 1-3). ComX pheromone activates a two-component system composed of the histidine protein kinase ComP and the response regulator ComA. After import into the cell via Opp (Spo0K), CSF modulates the activity of ComA by inhibiting the activity of the putative ComA-P phosphatase RapC. Together the opposing kinase (ComP) and phosphatase
Figure 1-3. *B. subtilis* quorum response Extracellular ComX pheromone and CSF activate the quorum response. ComX pheromone activates the membrane-bound kinase ComP. ComP donates phosphate to the response regulator ComA resulting in its activation as a transcription factor. Extracellular CSF is imported into the cell by the oligopeptide permease Opp (Spo0K). Once inside the cell, CSF inhibits the activity of the phosphatase RapC to stimulate further accumulation of ComA-P. ComA-P is required for the expression of genes which constitute the quorum regulon of *B. subtilis.*
(RapC) activities control the amount of active ComA–P and the timing of the ComA-dependent changes in transcription which constitute the quorum response.

**Identification of Two Extracellular Signaling Molecules** ComX pheromone and CSF were originally isolated from conditioned medium on the basis of their ability to stimulate the expression of *srfA-lacZ* when added to cells at low cell density (Magnuson et al., 1994). The ability to activate the quorum response when added to cultures at low cell density makes both peptides cell density factors. ComX pheromone is the major cell density signal, while CSF modulates the quorum response.

Both ComX pheromone and CSF are small peptides derived from larger precursor peptides. ComX pheromone is a modified 10 amino acid peptide derived from the carboxyl terminus of the 55 amino acid ComX precursor (Magnuson et al., 1994). The peptide moiety of ComX pheromone is ADPITRQ(W)GD (Magnuson et al., 1994). The modification of ComX pheromone is on the tryptophan three residues from the carboxyl terminus (Magnuson et al., 1994). CSF is an unmodified pentapeptide, ERGMT, derived from a 40 amino acid precursor, PhrC (Solomon et al., 1996).

**Production of ComX Pheromone** Little is known about ComX pheromone production. It is likely to involve multiple proteins since the precursor must be produced, the leader peptide removed, the modification produced, the tryptophan modified, and some form of the peptide secreted from the cell (Fig. 1-4). If production of ComX pheromone is analogous to that of other PSC peptides one would expect to find a regulator of expression, a modifying enzyme, and a dedicated export and processing system. However, it is formally possible that *comX* expression is unregulated and that processing, modification, and export are carried out by one protein.

The genes required for the ComX pheromone-dependent element of the quorum response (*comQ, comX, comP*, and *comA*) are adjacent to each other on the *B. subtilis* genome in a peptide signaling cassette (Fig. 1-2, Fig. 1-4). As described above, the core of a PSC consists of the genes encoding the precursor peptide, the histidine protein kinase, and the response regulator. In
ComX Pheromone

**Figure 1-4. Production of ComX pheromone** ComX pheromone is derived from the 55 amino acid ComX precursor encoded by *comX*. ComX must be processed to yield a 10 amino acid peptide, modified at the tryptophan residue 3 amino acids from the C-terminal end, and exported from the cell in order to generate active extracellular ComX pheromone. The peptide signaling cassette encoding proteins required for ComX pheromone production and response is drawn to scale with the known promoter indicated by the arrowhead and putative promoters indicated parenthetically.
B. subtilis those genes are \textit{comX}, \textit{comP}, and \textit{comA}, respectively. The additional gene, \textit{comQ}, is believed to be involved in the modification of ComX pheromone (Chapter 2). Except for \textit{comQ} and \textit{comX}, other genes involved in ComX pheromone production have not been identified.

The ComX pheromone precursor, ComX, is a 55 amino acid protein that shares little sequence similarity with the precursors of other PSC peptides. As discussed above, PSC precursors have similar (1) leader peptides, (2) cleavage sites, and (3) amphipathicity profiles. ComX lacks both a typical \textit{sec}-dependent leader sequence and the leader peptide characteristic of the precursors of typical PSC-encoded peptide signals that are secreted by an ABC transporter containing a protease domain. This suggests that ComX pheromone may be exported by a unique means or, at the very least, by an ABC transporter lacking a protease domain. Not only does ComX lack the gly-gly cleavage site seen in other PSC peptides, its cleavage site is not typical of any well-characterized protease. This makes it hard to know which proteases, if any, are good candidates for a processing enzyme. The structure of the peptide backbone also differs from canonical PSC peptides. Most PSC peptides form amphipathic alpha-helices. The backbone of ComX pheromone does not. As mentioned above, this may account for the necessity of the tryptophan modification.

The exact nature of the modification of ComX pheromone remains unknown, although it is known to be hydrophobic since the active pheromone is eluted from a C-18 reverse phase column only under relatively hydrophobic conditions (Magnuson et al., 1994). Based on available mass spectrometry data, the mass of the modification is between 206 and 336 daltons (Magnuson et al., 1994). The modification would be 206 daltons if the tryptophan side chain is intact, while it would be 336 daltons if the side chain is completely removed. Post-translational modifications of this mass are listed in Table 1-2 (Krishna and Wold, 1993). Although naturally-occurring isoprenylation of tryptophan has not been reported, the most likely candidates are the isoprenoids farnesyl or geranylgeranyl (Chapter 2). Identification of the modifying enzymes as well as further structural analysis of purified ComX pheromone should shed light on the exact nature of the modification.
Table 1-2. Post-translational modifications that add 206-330 daltons

<table>
<thead>
<tr>
<th>Mass Added (Daltons)</th>
<th>Modification</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>206</td>
<td>S-Farnesyl</td>
<td>Cys</td>
</tr>
<tr>
<td>210</td>
<td>N-Myristoyl</td>
<td>(amino terminus)</td>
</tr>
<tr>
<td>226</td>
<td>N-Biotionyl</td>
<td>Lys</td>
</tr>
<tr>
<td>229</td>
<td>N-Pyridoxyl</td>
<td>Lys</td>
</tr>
<tr>
<td>238</td>
<td>S-Palmitoyl</td>
<td>Cys</td>
</tr>
<tr>
<td>252</td>
<td>3,5-Diiodo</td>
<td>Tyr</td>
</tr>
<tr>
<td>259</td>
<td>N-alpha-(gamma-Glutamyl)-Glu$_2$</td>
<td>Gln</td>
</tr>
<tr>
<td>266</td>
<td>O-(GlcNAc-1-phosphoryl)</td>
<td>Ser</td>
</tr>
<tr>
<td>276</td>
<td>S-Geranylgeranyl</td>
<td>Cys</td>
</tr>
<tr>
<td>306</td>
<td>O-Uridylyl</td>
<td>Tyr</td>
</tr>
<tr>
<td>324</td>
<td>O-Pantetheinephosphoryl</td>
<td>Ser</td>
</tr>
<tr>
<td>324</td>
<td>S-(Hexosyl)$_2$</td>
<td>Cys</td>
</tr>
<tr>
<td>329</td>
<td>O-Adenylyl</td>
<td>Tyr</td>
</tr>
</tbody>
</table>
Since ComX does not contain a gly-gly cleavage site, it is possible that processing and export are not linked for this peptide. Whatever the means of ComX pheromone proteolysis and secretion, the enzymes responsible for these activities must be essential for cell viability and/or redundant in their function (Chapter 2).

**Production of CSF**  The production of CSF is fairly well-understood. CSF consists of the C-terminal 5 amino acids of the 40 amino acid precursor PhrC (Solomon et al., 1996). It is liberated following secretion by the sec-dependent secretion pathway, cleavage by a signal peptidase yielding a peptide of 11-25 amino acids, and further cleavage(s) by an unknown protease(s) to yield the 5 amino acid peptide that accumulates extracellularly (Fig. 1-5).

The CSF precursor gene, *phrC*, is under the control of two promoters (Fig. 1-5) (Solomon et al., 1996; Lazazzera et al., 1999). The first is ComA-dependent and is the primary promoter responsible for *phrC* expression during exponential growth since that is when the quorum response is activated. The second is dependent upon the alternative sigma factor sigma-H. Sigma-H is usually activated during the transition to stationary phase. This results in a more rapid accumulation of CSF as the culture enters stationary phase (Lazazzera et al., 1999).

*B. subtilis* contains a family of 7 genes encoding peptides related to PhrC (Perego et al., 1996; Lazazzera and Grossman, 1998) (Fig. 1-6). It is likely that each of these is processed to generate an extracellular signaling peptide that regulates the aspartyl-phosphate phosphatase encoded upstream of its precursor gene or one of the four aspartyl-phosphate phosphatases not encoded in an operon with a *phr* gene. In fact, PhrA is the precursor for a 5 amino acid peptide that accumulates extracellularly and regulates the activity of its cognate aspartyl-phosphate phosphatase RapA to affect the initiation of sporulation (see below) (Perego and Hoch, 1996; Perego, 1997), while CSF also regulates RapB, which is not encoded in an operon with a *phr* gene (Perego et al., 1996; Lazazzera et al., 1997). It is likely that one or more of the sigma-H-dependent *phr* genes encodes a peptide that plays a very minor role in the quorum response.
Figure 1-5. Production of CSF  CSF is derived from the 40 amino acid PhrC precursor encoded by *phrC*. PhrC is exported by the *sec*-dependent secretion apparatus. Putative peptidase cleavage sites are indicated by arrows above the primary sequence of PhrC. The final 5 amino acid active form of CSF is generated upon cleavage by an unknown protease. The structure of the operon encoding PhrC is as indicated. The two promoters driving *phrC* expression are indicated by arrows. The ComA box upstream of the operon is indicated by a small black box. The transcription terminator is indicated by a stem-loop.
Figure 1-6. A family of phosphatase/peptide regulator pairs in *B. subtilis* There are 11 aspartyl-phosphate phosphatases and 7 Phr signaling peptides encoded in the *B. subtilis* genome. Each Phr peptide is hypothesized to regulate the activity of its cognate phosphatase. The genes encoding the phosphatases are indicated by open boxes, while those encoding the Phr peptides are indicated by hatched boxes. ComA boxes are indicated by small black boxes. The known sigma-H-dependent promoter upstream of *phrC* is indicated by an arrow, while putative sigma-H-dependent promoters upstream of 5 of the other *phr* genes are indicated parenthetically.
It is also probable that one (or more) of the sigma-H-dependent *phr* genes encodes another sporulation factor (Lazazzera and Grossman, 1998).

**Signal Transduction in the *B. subtilis* Quorum Response** Both ComX pheromone and CSF stimulate the activity of the transcription factor ComA (Weinrauch et al., 1989; Weinrauch et al., 1990; Magnuson et al., 1994; Solomon et al., 1995). They do so by controlling opposing kinase and phosphatase activities. ComX pheromone activates the ComA kinase ComP (Solomon et al., 1995). CSF inhibits the ComA–P phosphatase RapC (Solomon et al., 1996; Lazazzera et al., 1997). Since the responses are integrated at the level of ComA activation, the quorum response pathway is branched in *B. subtilis* (Solomon et al., 1995) (Fig. 1-3).

ComX pheromone activates the ComP/ComA two-component system in which ComP is a membrane-bound histidine protein kinase and ComA is its cognate response regulator transcription factor (Weinrauch et al., 1989; Weinrauch et al., 1990; Solomon et al., 1995). It is not known whether ComX pheromone interacts directly with the extracellular loops of ComP or if it must first interact somehow with the membrane. Since the backbone of ComX pheromone does not appear to form an amphipathic alpha-helix, if ComX pheromone does interact with the membrane it does not do so in the same way as other PSC peptides.

After being imported into the cell CSF activates the quorum response by inhibiting the activity of the aspartyl-phosphate phosphatase RapC (Solomon et al., 1996; Lazazzera et al., 1997). CSF is likely to interact directly with RapC to inhibit its phosphatase activity, in a manner similar to the inhibition of aspartyl-phosphate phosphatase RapA by the 5 amino acid PhrA peptide (Perego et al., 1996; Perego and Hoch, 1996; Perego, 1997). RapC negatively controls the expression of ComA-dependent genes, including *srfA (comS)* (Solomon et al., 1996; Lazazzera et al., 1999). Most likely RapC directly dephosphorylates ComA–P, since two other Rap phosphatases (RapA and RapB) have been shown to dephosphorylate a response regulator (Perego et al., 1994; Perego and Hoch, 1996). By inhibiting an inhibitor of ComA activation CSF stimulates the expression of quorum-responsive genes.
At the high extracellular concentrations (50 – 100 nM) that accumulate as cells enter stationary phase CSF inhibits the quorum response (and, as discussed below, stimulates sporulation) (Lazazzera et al., 1997). The intracellular target for the inhibition of the quorum response is unknown, although it is known to be distinct from the targets which activate the quorum response and the initiation of sporulation (Lazazzera et al., 1997). This and other undefined signals lead to the shutoff of the quorum response as cells enter stationary phase.

**Output of the B. subtilis Quorum Response** The output of the quorum response is the transcription of a set of cell density-controlled genes that are dependent upon the quorum-activated transcription factor ComA for their expression. To date, there are 5 known members of the ComA regulon: srfA(comS), rapCphrC, rapAphrA, degQ, and pelA (Msadek et al., 1991; Nakano and Zuber, 1991; Mueller et al., 1992; Roggiani and Dubnau, 1993; Lazazzera et al., 1999) (RSM and ADG, unpublished results). ComA binds to a site (or sites) upstream of the promoter to activate transcription (Roggiani and Dubnau, 1993). An analysis of the B. subtilis genome revealed 17 other operons encoding 21 other genes which are likely to be regulated by ComA via the binding of ComA~P to a ComA box upstream of their promoters (Lazazzera et al., 1999). Together these genes constitute a putative ComA regulon.

Members of the ComA regulon are involved in a diverse array of processes. The genes which are known to be regulated by ComA are involved in surfactin production (srfA), competence development [comS (embedded within srfA) and rapC], sporulation (rapA), and extracellular degradative enzyme production (degQ and pelA) (Yang et al., 1986; Nakano et al., 1991; D'Souza et al., 1994; Perego et al., 1994; Hamoen et al., 1995; Perego et al., 1996; Solomon et al., 1996; Kunst et al., 1997). The significance of quorum-control of the members of the putative ComA regulon is not known, although one could hypothesize that changes in translation, lipid composition, and nucleotide metabolism could be part of a general adaptation to life at high cell density.
Adaptations to High Cell Density in *Bacillus subtilis*

The effect of the quorum response in *B. subtilis* is best understood in terms of the development of genetic competence and the formation of dormant endospores, two processes that occur at high cell density. The uptake of DNA by competent cells is believed to make the population more competitive under adverse conditions by increasing genetic diversity. The formation of dormant endospores creates a cell type able to withstand the most severe conditions and germinate when conditions are once again favorable for growth. Although high cell density is required for both competence development and sporulation, it is certainly not the only regulatory input into either process.

**The Control of Competence Development**

The machinery required for the binding and uptake of DNA from the environment is encoded by the so-called late competence genes, genes which depend upon the competence transcription factor ComK for their expression (van Sinderen et al., 1994; van Sinderen et al., 1995). The activity and expression of ComK, and therefore the development of competence, is controlled by multiple inputs (Fig. 1-7), only one of which is cell density. As described above, the quorum response leads to the expression of *comS* (a small open reading frame embedded in *srfA*) which encodes a small peptide (D'Souza et al., 1994; Hamoen et al., 1995). ComS inhibits the activity of MecA which together with ClpC (and ClpP) inhibits ComK activity (Turgay et al., 1997; Turgay et al., 1998). The binding of ComS to MecA leads to the release of ComK from a MecA-ClpC-ComK complex at high cell density (Turgay et al., 1997). Once released from the complex ComK activates its own expression, as well as the expression of the late competence genes (van Sinderen and Venema, 1994; van Sinderen et al., 1995).

The CodY, AbrB, SinR, and DegU proteins also regulate the expression of ComK and the development of competence (Hahn et al., 1994; van Sinderen and Venema, 1994; Hahn et al., 1996; Serror and Sonenshein, 1996). CodY represses the transcription of target genes, including ComK, during exponential growth in media containing mixtures of complex amino acids (Serror and Sonenshein, 1996). In this way it mediates one aspect of the nutritional control of competence development. SinR, DegU, and AbrB (at low concentration) are believed to be co-activators of
Figure 1-7. The regulation of competence development  The development of genetic competence in *B. subtilis* is dependent upon activation of the competence transcription factor ComK. The expression of *comK* is regulated by multiple proteins. DegU, SinR, and low levels of AbrB stimulate *comK* transcription, while CodY and high levels of AbrB repress *comK* transcription. ComK activity is also regulated post-transcriptionally. At low cell density ComK is complexed with ClpC and MecA which targets ComK for proteolysis by ClpP. Under conditions of high cell density, when ComS accumulates, MecA switches partners from ComK to ComS freeing active ComK. ComK then activates transcription of the late competence genes.
ComK transcription, enhancing the activity of ComK at its own promoter (Hahn et al., 1996). Since AbrB expression is repressed as cells enter stationary phase, AbrB may integrate information on growth phase into the competence development pathway (Perego et al., 1988). Although the signals conveyed by SinR and DegU are uncharacterized, SinR is involved in the switch between competence development and sporulation (Bai et al., 1993).

The initiation of sporulation Sporulation is a profound morphological differentiation under complex temporal and spatial control [reviewed in (Grossman, 1995; Stragier and Losick, 1996)]. Activation of the key transcription factor for the initiation of sporulation, Spo0A, is controlled by a phosphorelay that integrates the multiple signals which affect sporulation (Burbulys et al., 1991) (Fig. 1-8). The phosphorelay is composed of two phosphotransfer proteins that transfer phosphate from ATP via three kinases to the response regulator Spo0A (Burbulys et al., 1991). The flow of phosphate through the phosphorelay is modulated by at least three kinases which act on the phosphotransfer protein Spo0F or Spo0A (Burbulys et al., 1991; Ohlsen et al., 1994; LeDeaux and Grossman, 1995; Perego et al., 1996). Each member of the phosphorelay is a possible target for receiving information on the many factors which affect the initiation of sporulation: cell density, starvation, activity of the TCA cycle, DNA replication, and DNA damage [reviewed in (Grossman, 1995)]. The two aspartyl-phosphate phosphatases, RapA and RapB, which dephosphorylate the phosphotransfer protein Spo0F, are regulated by cell-cell signaling and cell density (Perego et al., 1994; Perego et al., 1996; Perego and Hoch, 1996; Solomon et al., 1996; Lazazzera et al., 1997). Each phosphatase is inhibited by a Phr-derived peptide that accumulates extracellularly and is transported into the cell by the oligopeptide permease Opp (Spo0K) [reviewed in (Lazazzera et al., 1999; Perego, 1999)]. RapB is inhibited by CSF (Solomon et al., 1996; Lazazzera et al., 1997). Since CSF is a cell density factor, the action of RapB conveys information on cell density. RapA is inhibited by PhrA (Perego, 1997). The signal conveyed by RapA is less well understood. Since it accumulates at high cell density, it may be a sort of timer that fine-tunes the phosphorelay under the conditions of high cell density required for the expression of the rapAphrA operon (Perego, 1997).
Figure 1-8. Multiple inputs into the initiation of sporulation  The initiation of sporulation in *B. subtilis* is dependent upon the activation of Spo0A by the phosphorelay. Phosphate is transferred from ATP via three kinases (KinA, KinB, and KinC) to the phosphotransfer protein Spo0F, from Spo0F to a second phosphotransfer protein Spo0B, and finally to the transcription factor Spo0A. Spo0A-P then activates sporulation gene expression. A variety of inputs modulate the flow of phosphate through the phosphorelay. Cell density signals are mediated by the extracellular peptides PhrA and CSF, which after import via the oligopeptide permease Opp (Spo0K) inhibit the Spo0F-P phosphatases RapA and RapB, respectively. It is hypothesized that Obg senses GTP. The signal, if any, regulating Spo0E activity is unknown. The molecular mechanisms for the perception of TCA cycle activity, DNA damage/synthesis, and nutrient deprivation signals are also unknown.
The Use of Multiple Cell Density Factors Allows Signal Integration

It would appear that there are quite enough signals being integrated into each of these pathways as it is. However, as presented in this thesis, the production of the Phr peptides, particularly CSF, is yet another point at which signal integration can occur. The production of CSF is dependent upon the quorum-activated transcription factor ComA, the alternate sigma factor sigma-H, and the factors that affect the activity of these two proteins. As presented in Chapter 3, the activity of sigma-H is regulated by growth conditions, allowing growth conditions to modulate the quorum response in *B. subtilis.*
Chapter 2

Isolation and Characterization of Mutations
that Eliminate ComX Pheromone Production
ComX pheromone is the major peptide pheromone utilized in the quorum response of *B. subtilis*. It is a modified peptide derived from a larger precursor peptide. During the initial characterization of ComX pheromone, two genes were identified that are essential for its production (Magnuson et al., 1994). One of these, *comX*, encodes the peptide precursor of ComX pheromone. At the time of its identification, the second, *comQ*, had no homologs in the available databases (Weinrauch et al., 1991; Magnuson et al., 1994). ComQ shows no similarity to proteases or exporters, so I hypothesized that additional gene products must be required for the production of ComX pheromone. In this chapter I describe sequence analysis of ComQ, characterization of the production of ComX pheromone and the expression of *comQX*, and the outcome of a hunt for additional genes involved in ComX pheromone production.

**ComQ, a Putative Isoprenoid-Binding Protein** ComQ is essential for ComX pheromone production (Magnuson et al., 1994). Comparison of ComQ to proteins of known function, described below, suggests that ComQ is likely to be involved in the isoprenylation of ComX pheromone. Although the exact nature of the ComX pheromone modification is unknown, the mass and hydrophobicity of the modification (Chapter 1) are consistent with isoprenylation. Specifically, the additional mass attributable to the modification is 206 daltons, assuming that the trp side chain is intact (Magnuson et al., 1994). This mass, 206 daltons, is the exact mass added to proteins upon farnesylation (Krishna and Wold, 1993).

ComQ is most similar to the bifunctional short chain diphosphate synthase, IdsA, of *Methanobacterium thermoautotrophicum* (21% identical, 51% similar). Isoprenyl diphosphate synthases (IPPases) such as IdsA catalyze the polymerization of polyisoprenoids from dimethylallyl pyrophosphate (DMAPP) and isopentyl pyrophosphate (IPP). An alignment of ComQ with multiple bacterial IPPases (Fig. 2-1) shows that ComQ contains multiple motifs that are conserved in IPPases (Koyama et al., 1993; Chen et al., 1994). Especially noteworthy is the conservation in regions II and V, particularly the aspartate-rich motif in region II. These two regions are believed to interact with diphosphate groups of DMAPP and the growing isoprene.
<table>
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<tr>
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**Conservation Region:**

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Figure 2.1. Alignment of multiple known isoprenyl diphosphate synthases and ComQ. ClustalW was used to align the sequences of ComQ and multiple known isoprenyl diphosphate synthases. 1. Geranylgeranyl pyrophosphate synthase (CrtE) of *Erwinia herbicola*; 2. Geranylgeranyl pyrophosphate synthase (CrtE) of *Erwinia uredovora*; 3. Farnesyl-diphosphate synthase (IspA) of *Bacillus stearothermophilus*; 4. Farnesyl-diphosphate synthase (IspA) of *Escherichia coli*; 5. Bifunctional short chain isoprenyl diphosphate synthase (IdsA) of *Methanobacterium thermoautotrophicum*; 6. Geranylgeranyl pyrophosphate synthase of *Sulfolobus acidocaldarius*; 7. Probable heptaprenyl diphosphate synthase (GerC3) of *Bacillus subtilis*; 8. Geranylgeranyl pyrophosphate synthase (CrtE) of *Rhodobacter capsulatus*; 9. ComQ of *Bacillus subtilis*. Conserved regions are labeled I-V and consensus sequences of each are indicated below the alignment [Chen, 1994, Protein Science]. Residues conserved in known bacterial IPPases are indicated by a *. Residues conserved in known bacterial IPPases and ComQ are indicated by a *. Similar/identical residues are indicated in bold type.
chain to position the two substrates of IPPases in the proper orientation for catalysis (Ashby et al., 1990). In a likely model, the growing isoprene chain (IPP or geranyl pyrophosphate) interacts with region II, while the 5-carbon building block (DMAPP) is bound by region V (Chen et al., 1994). I hypothesize that ComQ binds to isoprenoid groups, most likely via the well-conserved aspartate-rich motif in region II. Given that region V is less well-conserved, it seems likely that ComQ is involved in attaching an isoprenyl modification to ComX rather than in synthesizing the modification.

Work by Kate Bacon (KTB and ADG, unpublished results) suggests that region II is indeed required for ComQ activity. Upon mutation of either of two of the conserved aspartates in region II (D67, D71) to glutamate, ComQ accumulates in the cell, as evidenced by Western blot, but is not able to function in ComX pheromone production (KTB and ADG, unpublished results). Multiple mutational analyses of isoprenyl diphosphate synthases (IPPases) indicate that the conserved aspartates are required for activity. In rat farnesyl diphosphate synthase the conserved aspartates in regions II and V are required for in vitro activity (Marrero et al., 1992; Joly and Edwards, 1993). Similar experiments were done to show that aspartates in regions II and V are required for activity of yeast farnesyl diphosphate synthase (Song and Poulter, 1994).

Further structural analyses of ComX pheromone and in vitro work with ComQ may go hand-in-hand in the characterization of ComX pheromone modification. Identification of the modification would identify the probable substrate of the modifying enzyme. That may allow in vitro characterization of the modification reaction. If one makes an educated guess as to the nature of the substrate and can demonstrate isoprenylation of ComX by ComQ in vitro, then the structural analysis may become more straightforward.

The Production of ComX Pheromone Early characterization of ComX pheromone production during cell growth revealed simply that there was more ComX pheromone at high cell density than at low cell density (Magnuson et al., 1994). In order to get a more complete understanding of the production of ComX pheromone, I measured the amount of extracellular ComX pheromone as a function of increasing cell density. Cells unable to make CSF (phrC
mutants) were grown in defined minimal medium and conditioned medium was harvested at time points throughout growth. The amount of extracellular factor activity, all attributable to ComX pheromone in the absence of CSF production, was assayed. The amount of extracellular ComX pheromone increases as cell density increases (Fig. 2-2). In contrast with the signals in acyl homoserine lactone-based signaling systems and many of the peptide signaling cassette-based signaling systems where perception of the signal leads to further signal production, ComX pheromone production is not autoinduced. There is very little ComX pheromone present extracellularly at low cell density. The concentration of ComX pheromone increases in proportion to cell density throughout exponential growth and continues to increase as cells enter stationary phase.

To monitor the transcription of comQ and comX, I constructed transcriptional fusions of comQ and comQX to lacZ and placed them at the non-essential amyE locus. The expression of comQX varies less than two-fold under the conditions used to monitor comQX expression (Fig. 2-3). The relatively constant expression of comX at these cell densities is consistent with its role as an indicator of cell density. (Neither expression of comQX nor ComX pheromone production were monitored at lower cell densities; this leaves open the possibility that they are regulated at very low cell densities.) The decrease in ß-galactosidase activity after transition to stationary phase may reflect the instability of ß-galactosidase in stationary phase cells and decrease in transcription. The regulatory factors that control the expression of comQX, if they exist, are unknown (Weinrauch et al., 1991).

**Rationale for Mutant Hunt.** With much still to be learned about the quorum response, including a complete picture of ComX pheromone production and the identity of factors involved in the expression of comQX-lacZ, two genetic approaches were considered: (1) a broad hunt for mutations affecting the quorum response and (2) a broad hunt for mutations affecting the production of ComX pheromone. I chose to pursue a broad hunt for mutations affecting the quorum response since this was fairly certain to yield new information and did not preclude insights into any specific aspect of the quorum response.
Figure 2-2. Extracellular ComX pheromone activity increases as cell density increases. Cells containing a phrC::erm mutation (JMS751) were grown in defined minimal medium and conditioned medium was harvested at the indicated cell densities. The amount of extracellular srfA-inducing activity (in this case, ComX pheromone) was assayed by mixing conditioned medium with cells containing the srfA-lacZ Δ374 fusion (TMH144) at low cell density and assaying for increased β-galactosidase activity. Relative ComX pheromone expressed as a percentage of the maximum ComX pheromone activity, closed circles; growth, open squares. Data has been corrected for background β-galactosidase activity.
Figure 2-3. The expression of \textit{comQX-lacZ} varies less than two-fold during exponential growth TMH500, containing the \textit{comQX-lacZ} \textit{O}500 fusion, was grown in defined minimal medium and samples taken at the indicated cell densities for determination of \(\beta\)-galactosidase activity. \(\beta\)-galactosidase specific activity, open squares; growth, closed circles.
Mutants with altered expression of *srfA-lacZ* might reasonably be expected to have altered production of cell-cell signaling molecules. Strains containing a multi-copy plasmid (5-10 copies (Haima et al., 1987)] carrying *comQX* have significantly elevated production of ComX pheromone compared to wild type cells (data not shown), whereas *comQ* mutants produce no ComX pheromone (Magnuson et al., 1994). Strains with altered production of ComX pheromone have altered induction of the quorum response as assayed by *srfA-lacZ* expression (Figure 2-4). Cells which make excess ComX pheromone (multicopy *comQX* cells) exhibit induction of *srfA-lacZ* at a significantly lower cell density than do wild type cells, while cells that do not make ComX pheromone have negligible expression of *srfA-lacZ*.

Although much is known about the response to cellular crowding, little is known about how the quorum response is modulated by other factors. Several proteins, including sigma-H (Spo0H), Opp (Spo0K), and CodY, affect the expression of *srfA-lacZ* independently of the quorum response. A null mutation in either *spo0H* or *spo0K* causes a much greater decrease in the expression of *srfA-lacZ* than does a mutation that eliminates CSF production (*phrC* null) (data not shown; JMS and ADG, unpublished data). This suggests that both the alternate sigma factor sigma-H and the oligopeptide permease Opp (Spo0K) have uncharacterized, CSF-independent roles in the expression of *srfA-lacZ*. The transcriptional repressor CodY represses expression of several genes in the presence of complex mixtures of amino acids or short peptides (Serror and Sonenshein, 1996). Little is known about how CodY senses amino acids and peptides. It is possible that the study of mutants with altered expression of *srfA-lacZ* might give further insight into the CSF-independent roles of Spo0H and Opp (Spo0K), the mechanism of CodY repression, or the identity of additional stresses that modulate the quorum response.

**The Mutant Hunt** A very general hunt for mutants was devised. Mutations that increase or decrease the expression of the ComA-dependent, quorum-activated *srfA-lacZ* reporter fusion were isolated under conditions where the quorum response is active.

EMS (ethyl methanesulfonate)-mutagenized cells were screened for mutants with altered expression of *srfA-lacZ*. Mutagenized cells were plated onto solid competence (SpII) medium
Figure 2-4. The number of copies of \textit{comQX} affects the expression of \textit{srfA-lacZ}.

Strains containing the \textit{srfA-lacZ} \textit{Ω}374 fusion were grown in defined minimal medium and samples taken at the indicated cell densities for determination of β-galactosidase activity. Wild type, TMH144, open circles; pTH2 (multicopy \textit{comQX}), TMH145, closed squares; pH13 (multicopy vector), TMH146, closed circles; \textit{comQ}, TMH149, open squares.
containing Xgal under conditions where activation of the quorum response is easily observed using the \textit{srfA-lacZ} reporter fusion. All screening was done at low colony density (<150 colonies per plate) to prevent 'cross-talk' between colonies where an adjacent colony might provide ComX pheromone or CSF to a neighboring colony unable to make its own. Conditions (incubation temperature, length of incubation, colony density, Xgal concentration) where the greatest effect on Xgal color development was seen were tested and optimized by looking for \textit{comQ::spc} mutants among a large excess of wild type cells. Isolation of \textit{comQ} mutants during screening served as an internal control indicating that the screen was working.

The hunt for mutants was extensive and thorough. Approximately $2.6 \times 10^5$ colonies from 18 independent mutagenesis experiments were screened. The average \textit{B. subtilis} gene is ~800 bp and the genome is 4.2 MB (Kunst et al., 1997). Assuming random mutagenesis and a perfect screen, the probability of identifying a non-essential gene of 809 bp in a genome of 4.2MB is >99.999% in a screen of this size. The screen was essentially saturating down to a target size of 100 bp since the chance of missing a mutation in a target of this size is 0.2%. In other words, even if the desired target was 100 bp, one is 99.8% certain of hitting it at least once in a screen of 263,000 candidates, assuming an unbiased distribution of mutations.

Mutations that affect the expression of \textit{srfA-lacZ} were isolated. Approximately 1000 mutants with decreased expression of \textit{srfA-lacZ} were further characterized. These are discussed below. Fifty-one mutants with increased expression of \textit{srfA-lacZ} were isolated. The characterization of these mutants is discussed in Chapter 3.

\textbf{Isolation of Mutants Making Negligible Amounts of ComX Pheromone} Of the ~1000 mutants with decreased expression of \textit{srfA-lacZ}, 27 were found to make little or no ComX pheromone. Each of the ~1000 mutants was screened for the ability to make ComX pheromone using a simple plate assay based on the ability of cells that make ComX pheromone to donate it to cells that cannot. Since ComX pheromone is secreted to the extracellular milieu, ComX pheromone made by wild type cells is able to stimulate the expression of \textit{srfA-lacZ} in cells that are unable to make ComX pheromone (Magnuson et al., 1994). Mutants with decreased
expression of srfA-lacZ were grown on top of a lawn of cells that are unable to make ComX pheromone (comQ mutants) and that contain a srfA-lacZ reporter fusion. On solid medium containing Xgal those mutants that make ComX pheromone are able to stimulate srfA-lacZ expression in the lawn of comQ indicator cells; this results in a blue halo around the zone of mutant growth. However, mutants that are unable to make ComX pheromone are unable to stimulate the expression of srfA-lacZ in the comQ indicator cells and no blue halo appears. This secondary assay indicated that 27 of the mutants make negligible ComX pheromone.

Characterization of Mutations That Eliminate ComX Pheromone Production

Each of the mutations was mapped by plasmid rescue. Two approaches were utilized. In the first pNG15 (Magnuson et al., 1994), an integrative plasmid containing DNA spanning the 298 bp upstream of comQ through the middle of comX, was used to introduce a wild type copy of comQ and most of comX at their native loci in the mutants. If the mutation is in comQ or comX, then the wild type expression of srfA-lacZ would be restored in some percentage of cells transformed with pNG15.

Mutations in comQ would be rescued by complementation or gene conversion. In the case of a recessive mutation in comQ nearly all transformants should be wild type. (Those not rescued by complementation would be those in which the incoming copy of comQ was converted to the mutant form.) In the case of a dominant mutation in comQ nearly all transformants should retain the mutant phenotype. (Those exhibiting rescue would be those in which the mutant form of comQ had been gene converted to the wild type.)

The wild type copy of comX on pNG15 is truncated, so mutations in comX can not be rescued by complementation. In the case of mutations in comX rescue could occur in either of two ways: (1) a crossover downstream of the mutation would result in an intact wild type copy of comX and a truncated mutant copy (which would yield a wild type phenotype in the case of a recessive mutation or, after gene conversion, in the case of a dominant mutation) or (2) crossovers on either side of the mutation would yield a wild type phenotype in any case. The percentage of transformants exhibiting rescue would be very low if the mutation is in 5' end of comX contained
in the fragment cloned in pNG15 and no rescue would be evident if the mutation is in the 3’ end of comX (beyond the end of the cloned fragment of comX).

The defect in srfA-lacZ expression in 19 mutants was rescued by introduction of pNG15 (Table 2-1) indicating that the mutation(s) in each strain was located in the region carried on the plasmid.

A second approach was used to map the remaining mutations. In this complementation test pKB1, a plasmid containing the entire comQX locus cloned into the thrC gene, was used to insert an intact copy of comQX at the thrC locus in each of the mutants. In this situation if the mutation is anywhere in comQ or comX, then rescue of the srfA-lacZ phenotype would be seen nearly 100% of the time assuming that the mutation is recessive. If the mutation is not in comQ or comX, then no rescue would be evident. All mutants transformed with pKB1 exhibited ~100% rescue (Table 2-1). Taken together, these two experiments indicated that all 27 mutations are in comQ and/or comX.

It is unlikely that more extensive screening would have identified additional genes involved in ComX pheromone production. As described above, the 27 mutants that did not make ComX pheromone were isolated from 18 independent mutagenesis experiments. Since the same loci were mutated over and over again in separate experiments, the screen appears to be saturating in terms of mutations in non-essential, non-redundant genes required for ComX pheromone production. The remaining proteins are most likely essential for cell viability and/or redundant with other proteins, at least for their role in ComX pheromone production.

This screen was not optimized for the isolation of mutants that make slightly reduced amounts of ComX pheromone. The screen was devised using a known pheromone-deficient mutant (a comQ mutant) to develop screening conditions, so conditions were optimal for isolating other mutants that make no pheromone. These conditions are likely to be too stringent to allow isolation of mutations that decrease, but do not eliminate, ComX pheromone production. A screen for mutants with intermediate expression of srfA-lacZ may be more likely to identify proteins with partially-redundant roles in ComX pheromone production. It may be necessary to do such a screen.
Table 2.1. Each mutation was mapped to \textit{comQX}.

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<td>82%</td>
<td>n/a</td>
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</tr>
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<td>47%</td>
<td>n/a</td>
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<tr>
<td>132</td>
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<td>133</td>
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<td>yes#</td>
<td>n/a</td>
<td>\textit{comQX}</td>
</tr>
<tr>
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<td>287</td>
<td>yes#</td>
<td>n/a</td>
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</tr>
<tr>
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</tr>
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<td>290</td>
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<td>285</td>
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</tr>
<tr>
<td>197</td>
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</tr>
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<td>285</td>
<td>n/a</td>
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<td>\textit{comQX}</td>
</tr>
<tr>
<td>199</td>
<td>293</td>
<td>n/a</td>
<td>yes</td>
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<tr>
<td>217</td>
<td>301</td>
<td>n/a</td>
<td>yes</td>
<td>\textit{comQX}</td>
</tr>
</tbody>
</table>

\textit{n/a} not assayed

* large number of transformants, too crowded to count accurately but rescue evident

\* small number of transformants, true rescue likely to be <100\%
in a strain containing two (or more) copies of \textit{comQX} to avoid isolating more \textit{comQX} mutations.

It is possible that a step(s) in ComX pheromone biogenesis occurs extracellularly. Mutants defective in an extracellular activity required for ComX pheromone synthesis would not have been identified in this mutant hunt since such deficiencies would be complemented when the mutants were mixed with \textit{comQ} mutant cells in the test for ComX pheromone production. Currently the only method for measuring the production of active ComX pheromone involves mixing the sample to be tested with live cells. Therefore, for the time being, there is no way to circumvent this limitation.

**Alternative Genetic Approaches to Identifying Components of the ComX Pheromone Production Pathway**

It is unlikely that ComQ and ComX are the only proteins involved in the production of ComX pheromone. Since the unidentified components of the ComX pheromone biogenesis pathway appear to be essential for cell viability and/or redundant for their function in ComX pheromone production, there are several more elaborate approaches to identify additional genes involved in the production of ComX pheromone. To identify genes involved in ComX pheromone production that are essential for cell viability one could isolate conditional mutants (i.e. temperature sensitive mutants), and assess them at an intermediate condition (i.e. temperature) for an effect on ComX pheromone production. This is risky, as it assumes that an effect on ComX pheromone production would be evident at the intermediate condition. To identify redundant genes involved in ComX pheromone production, one could screen an even larger number of cells. This is difficult since the screen would be very large.

There are two approaches to identify genes involved in ComX pheromone production using increased ComX pheromone as a readout. These are less risky since (1) they would not require the partial ablation of an activity essential for cell viability and (2) they would not require simultaneously mutating multiple targets. One could identify genes that negatively regulate ComX pheromone production by screening a collection of mutated cells for increased ComX pheromone production. Alternatively, if one used a mutagenesis method that allowed for the isolation of point mutations, one could get increased production or activity of a rate limiting component in the
production pathway. Given that most mutations are loss of function mutations, the major drawback to this approach is that it assumes that there are negative regulators of ComX pheromone production (see next paragraph). Alternatively, one could identify genes that encode the components of the biosynthesis pathway (or positive regulators of it) by screening a library of cells containing multicopy plasmids carrying *B. subtilis* DNA. This approach may be quite fruitful.

In a way, the isolation of mutations with increased expression of *srfA-lacZ* (Chapter 3) was a screen for negative regulators of ComX pheromone production. Any of the 51 EMS-mutagenized strains with increased expression of *srfA-lacZ* could contain a mutation in a gene that negatively regulates ComX pheromone production. A null mutation in such a gene would allow increased production of ComX pheromone, leading to increased activation of the quorum response and increased expression of *srfA-lacZ*. As presented in Chapter 3, five of these mutations were characterized and none affected ComX pheromone production. It is possible that one or more of the remaining 46 mutants contains a mutation affecting ComX pheromone production.

**Genomics-based Approaches to Studying ComX Pheromone Production.**

The entire *B. subtilis* genome has been sequenced, opening up several interesting approaches to the study of ComX pheromone biosynthesis. The minimum set of activities required for ComX pheromone production include: a protease, a modifying enzyme, and an exporter. One could easily search the genome for candidate proteases, modifying enzymes, or transporters. See the Chapter 2A (an appendix to this chapter) details on a sequence-based search for the ComX pheromone exporter.

**Approaches to Studying ComX Pheromone Production Utilizing the *comQ* and *comX* Mutants** Further characterization of the nature of the mutations in *comQX* and their effects on the production of ComX pheromone may yield useful information. Specifically, it may allow insight into (1) the identity of other proteins involved in ComX pheromone production, (2) the steps in ComX pheromone production, or (3) the activity of ComQ.

There are both genetic and biochemical approaches that are feasible in each case. Before further work is done, one would need to identify mutations that result in the production of an
altered protein (as opposed to no protein at all). This could be accomplished by a combination of sequencing to identify missense mutations in comQ or comX and Western blotting to determine whether mutant forms of ComQ or ComX accumulate in the cell.

Mutant forms of ComQ and ComX may be useful in the identification of other proteins involved in ComX pheromone production. Hunts for extragenic suppressors of comQ or comX missense mutations may identify proteins that interact with the mutated protein. Extragenic suppressors of comX mutations may affect the gene encoding the protease, modifying enzyme, or exporter. Extragenic suppressors of comQ mutations may lie in genes encoding the modifying enzyme (if ComQ synthesizes the modification) or in the modification-synthesizing enzyme (if ComQ attaches the modification). One could also take complementary biochemical approaches involving immunoprecipitation of mutant forms of ComX or ComQ to identify interacting proteins. Mutant forms of ComX may be ‘stuck’ in a complex with the protease, modifying enzyme, or exporter. If production occurs in a multi-component complex, then immunoprecipitation of proteins that interact with wild type ComQ may identify new players. If such a complex is unstable, it may be more fruitful to use mutant forms of ComQ or ComX, which may muck-up the system and prevent dissociation, to precipitate the complex.

Missense mutations in comX that prevent production of active ComX pheromone could be used to isolate intermediates in the ComX pheromone biosynthetic pathway and perhaps even to order steps in the pathway. Following isolation of ComX from comX mutants that do not make ComX pheromone (a technical feat not currently achievable), one could do mass spectrometry to determine which form of ComX accumulates in each mutant. One could easily tell if ComX had been cleaved and/or modified. Such characterization may allow the ordering of modification relative to cleavage and allow the identification of ComX residues important for each process. If ComX isolation techniques become especially efficient, one could look for ComX intermediates in the conditioned medium from pheromone-deficient mutants. Analyses of these may allow the ordering of cleavage and/or modification relative to export.
The identification of extragenic suppressors of \textit{comX} mutations (as discussed above) may also help to order the pathway. Combining structural analysis to determine which step was blocked by the original mutation with sequence analysis to determine the function of the gene identified in a hunt for extragenic suppressors would allow the step involving the newly-identified gene to be placed at or after the step blocked by the \textit{comX} mutation.

Analysis of \textit{comQ} mutations may give further insight into the function of ComQ. As discussed above, ComQ shares several stretches of similarity with regions conserved in isoprenyl diphosphate synthases (IPPases) suggesting that ComQ may bind to isoprenoid groups. The identification of \textit{comQ} mutations that map to regions conserved in IPPases (Fig. 2-1) would be consistent with a role for ComQ in isoprenoid metabolism and would lend further support to the hypothesis that the modification may be an isoprenoid group synthesized or attached to ComX by ComQ.

One could also use \textit{comQ} mutations to map the interface between ComQ and ComX. To do this extragenic suppressors of \textit{comQ} mutations that lie in \textit{comX} (and vice versa) would be sequenced to determine the nature of the mutation. The amino acid affected by a mutation in \textit{comX} is likely to interact with the amino acid affected by the mutation in \textit{comQ}.

Although this mutant hunt did not identify any new components of the ComX pheromone production pathway, it did provide tools that may be useful in alternative approaches to the study of ComX pheromone biogenesis. It also led to the isolation of 51 mutants with increased expression of \textit{srfA-lacZ}. Characterization of five of these mutations revealed that growth conditions modulate the quorum response by affecting the production of CSF. This work is presented in the Chapter 3.
Chapter 2A  
(Appendix to Chapter 2)

A Sequence-based Search  
for the  
ComX Pheromone Exporter
Summary Seven putative ABC exporters that are similar to the PSC family of ABC exporters were identified. One of these (SunT) encodes the sublancin exporter and was not considered further. Two of the remaining 6 appear to be essential for viability and were not further investigated at this time. The remaining 4 were mutated alone and in combination. In no case was there an effect on the expression of srfA-lacZ, a quorum-activated gene dependent upon ComX pheromone for full expression. This suggests that none of these four exporters play a major role in ComX pheromone biogenesis. The two essential ABC exporters may be involved in ComX pheromone production and further work needs to be done to determine what, if any, role they play.

Background ComX pheromone is a modified decapeptide that serves as the major cell density signal in the quorum response in *B. subtilis* (Magnuson et al., 1994; Solomon et al., 1995). Little is known about how ComX pheromone is processed, modified, and secreted from the cell. Since the search for mutants that fail to make ComX pheromone failed to identify the ComX pheromone exporter (Chapter 2), I took a sequence-based approach to identify possible ComX pheromone exporters. As described in Chapter 1, the ComX pheromone precursor ComX is encoded in a peptide signaling cassette (reviewed in (Kleerebezem et al., 1997)). As depicted in Figure 1-2, peptide signaling cassettes are found in at least 6 genera. In each case the PSC-encoded peptide functions as a cell-cell signaling molecule. Several PSC-encoded signaling molecules and antibiotics are known to be exported by ATP-binding cassette (ABC) transporters (Havarstein et al., 1995b) [reviewed in (Kleerebezem et al., 1997)]. I hypothesized that a similar transporter may export ComX pheromone.

Identification of putative peptide exporters encoded in the *B. subtilis* genome Six peptide signaling cassettes are known to encode the precursors of signaling molecules (See Figure 1-2) (Brurberg et al., 1997; Diep et al., 1995; Ji et al., 1995; Magnuson et al., 1994; Pestova et al., 1996; Quadri et al., 1997). In four of these cases an ABC exporter and an accessory transport factor (of unknown function) are required for signal production. The four ABC exporters that have been shown to have a role in the production of a PSC-encoded cell-cell signaling molecule are ComA (*S. pneumoniae*), CbnT (*C. piscicola*), SppT (*L. sake*), and PlnG.
(L. plantarum) (Diep et al., 1996; Havarstein et al., 1995a; Huhne et al., 1996; Pestova et al., 1996; Quadri et al., 1997). As described in Chapter 1, a similar exporter (LagG) has been shown to cleave the lactococcin G precursor while exporting it (Havarstein et al., 1995b). It is hypothesized that each of these exporters processes the peptide that it secretes. In each case another protein, termed an accessory transport factor (ATF), is also required for signal production.

The completed B. subtilis genome was searched for ABC transporters that are similar to ComA, CbnT, SppT, and PlnG using the BLAST search engine at the Subtilist web server (http://genomeweb.pasteur.fr/GenoList/SubtiList/). Each of the known exporters was similar (approximately 25% similar on the protein level) to the same 7 putative B. subtilis ABC exporters, but not notably similar to any other B. subtilis proteins. One of these 7 ABC exporters, SunT, is the sublancin exporter (Kunst et al., 1997). The known PSC peptide exporters appear to be dedicated to the production of the PSC peptide. In no case has one been shown to export multiple peptides and/or antibiotics. Since SunT is the sublancin exporter it was not considered further. The other 6 putative peptide exporters, listed in Table 2A-1, were considered reasonable candidates for the ComX pheromone exporter.

The putative peptide exporters of B. subtilis are substantially different from the known PSC peptide exporters in two respects. First, the B. subtilis ABC exporters are significantly shorter than the known PSC peptide exporters because they lack the N-terminal protease domains (discussed in Chapter 1). This is not altogether unexpected, since ComX lacks the typical leader sequence and gly-gly cleavage sites found in most other PSC-encoded peptide precursors. It is reasonable to assume that ComX pheromone may be processed by some other means (see discussion below). Second, they appear to act without requiring accessory transport factors, since there are no recognizable ATFs encoded in the B. subtilis genome. Apparently SunT acts without an ATF. Presumably any other peptide exporter may also act without an ATF.

**Construction of mutations in putative ABC exporters** I constructed mutations designed to eliminate the activity of ygaD, yvcC, ywjA, yfiBC, yheiH, and yknUV. In each case
<table>
<thead>
<tr>
<th>exporter</th>
<th>size(s)</th>
<th>allele</th>
<th>deletion</th>
<th>insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>YgaD</td>
<td>589 aa</td>
<td>ygaD:cat</td>
<td>none</td>
<td>leaves 233 N-terminal amino acids pGEMcat integrated 699 bp downstream of 5' end of ygaD</td>
</tr>
<tr>
<td>YvcC</td>
<td>589 aa</td>
<td>yvcC::tetr</td>
<td>none</td>
<td>leaves 156 N-terminal amino acids tetrR cassette at bp 319 (PacI site) of yvcC</td>
</tr>
<tr>
<td>YwjA</td>
<td>575 aa</td>
<td>ΔywjA::spc</td>
<td>leaves 187 N-terminal amino acids (244 bp from bp 562 (EcoRV site) to bp 805 (BsiWI site) of ywjA)</td>
<td>spcR cassette</td>
</tr>
<tr>
<td>YfiBC</td>
<td>YfiB: 573 aa</td>
<td>YfiC: 604 aa</td>
<td>ΔyfiBC::kan</td>
<td>leaves 183 N-terminal amino acids of YfiB (1936 bp from bp 550 (BsgI site) of yfiB to bp 769 (BsgI site) of yfiC)</td>
</tr>
<tr>
<td>YheIH</td>
<td>YheI: 585 aa</td>
<td>YheH: 673 aa</td>
<td>ΔyheIH::phleo</td>
<td>leaves 335 N-terminal amino acids of YheI (1362 bp from bp 1006 (EcoRV site) of yheI to bp 205 (EagI site) of yheH)</td>
</tr>
<tr>
<td>YknUV</td>
<td>YknU: 585 aa</td>
<td>YknV: 604 aa</td>
<td>ΔyknUV::erm</td>
<td>Leaves 433 N-terminal amino acids of YknU 964 bp from bp 1300 (EagI site) of yknU to bp 494 (HincII site) of yknV</td>
</tr>
</tbody>
</table>
an internal fragment of the gene(s) was amplified by PCR and cloned into an integrative vector (see Tables 2A-2 and 2A-3). In one case this plasmid was integrated into the chromosome to disrupt the corresponding gene. In one case a drug-resistance gene was cloned into the cloned piece. In the other four cases a portion of the cloned gene fragment was replaced with a drug resistance gene. The construction of each of the mutations is outlined in Table 2A-1 and described briefly below.

The \textit{ygaD:cat} allele truncates \textit{YgaD} after 233 amino acids. To construct the \textit{ygaD} mutation, a plasmid containing an internal fragment of \textit{ygaD} (pTH86) was integrated into the chromosome of \textit{B. subtilis} by transformation and chloramphenicol-resistant transformants were selected.

The \textit{yvcC::tet} mutation truncates \textit{YvcC} after 156 amino acids. To construct the \textit{yvcC} mutation, a tetracycline-resistance cassette was cloned at the PacI site 319 bp downstream of the 5' end of the 1767 bp \textit{yvcC} open reading frame. The \textit{yvcC::tet} allele on pTH90 was introduced into \textit{B. subtilis} by transformation followed by selection for tetracycline-resistant transformants. Tetracycline-resistant transformants were screened for chloramphenicol sensitivity to identify transformants in which \textit{yvcC::tet} had integrated by double crossover.

The \textit{AywjA::spc} allele truncates \textit{YwjA} after 187 amino acids. To construct the \textit{ywjA} mutation, 244 bp from bp 562 to bp 805 of the 1725 bp \textit{ywjA} open reading frame were deleted and replaced with a spectinomycin-resistance cassette. The \textit{AywjA::spc} allele on pTH97 was introduced into \textit{B. subtilis} by transformation followed by selection for spectinomycin-resistant transformants. Spectinomycin-resistant transformants were screened for chloramphenicol sensitivity to identify transformants in which \textit{AywjA::spc} had integrated by double crossover.

The \textit{AyknUV::erm} allele truncates \textit{YknU} after 433 amino acids (and separates the remaining fragment of the \textit{yknV} open reading frame from its promoter). To construct the \textit{yknUV} mutation, 964 bp from bp 1300 of \textit{yknU} to bp 494 of \textit{yknV} were deleted and replaced with an erythromycin-resistance cassette. The \textit{AyknUV::erm} mutation on pTH101 was introduced into \textit{B. subtilis} by
<table>
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<th>insert</th>
<th>cloning</th>
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<td>PCR product was blunted with Klenow and cloned into cut vector</td>
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<td>pTH81</td>
<td>pGEMcat</td>
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<td>pGEMcat</td>
<td>internal fragment of <em>yfiBC</em> THP11-2 - THP21 Taq PCR product</td>
<td>PCR product was blunted with Klenow and cloned into cut vector</td>
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<td>pGEMcat</td>
<td>internal fragment of <em>ygaD</em> THP8 - THP9 Taq PCR product</td>
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<td>starting plasmid</td>
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<td>cloning</td>
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<td>pTH90</td>
<td>pTH82</td>
<td>tet&lt;sup&gt;R&lt;/sup&gt; cassette amplified from pDG1513 Vent PCR product gift from Janet Lindow</td>
<td>PCR product was cloned into Klenow-blunted cut pTH82</td>
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<td>pTH92</td>
<td>pTH83</td>
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<td>kan&lt;sup&gt;R&lt;/sup&gt; cassette was cloned into Klenow-blunted cut pTH83</td>
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<td>BamHI-NdeI fragment of pUS19 containing the spc&lt;sup&gt;R&lt;/sup&gt; cassette</td>
<td>spc&lt;sup&gt;R&lt;/sup&gt; cassette was blunted with Klenow and cloned into Klenow-blunted cut pTH84</td>
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<td>pTH97</td>
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<td>pTH81</td>
<td>XbaI-HindIII fragment of pUC18-ble1 containing the phleo&lt;sup&gt;R&lt;/sup&gt; cassette</td>
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<td>pTH101</td>
<td>pTH80</td>
<td>EcoRI-HindIII fragment of pJPM8 containing the erm&lt;sup&gt;R&lt;/sup&gt; cassette</td>
<td>erm&lt;sup&gt;R&lt;/sup&gt; cassette was blunted with Klenow and cloned into Klenow-blunted cut pTH80</td>
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<td>ΔyknUV::erm</td>
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<td>Amplifies to</td>
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<td>Primer</td>
<td>Sequence (5’→3’)</td>
<td>Amplifies from</td>
<td>Amplifies to</td>
</tr>
<tr>
<td>--------</td>
<td>------------------</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>THP38</td>
<td>GGAATCTGTTCAGGC</td>
<td>bp1045403 of genome (bp 605 of <em>yhel</em>)</td>
<td>bp 1047282 of genome (bp 730 of <em>yheH</em>)</td>
</tr>
<tr>
<td>THP39</td>
<td>GCCGTGAATATCCGA</td>
<td>bp1047282 of genome (bp 730 of <em>yheH</em>)</td>
<td></td>
</tr>
<tr>
<td>THP40</td>
<td>CTTTGTTGCT</td>
<td>bp1500109 of genome (bp 815 of <em>yknU</em>)</td>
<td></td>
</tr>
<tr>
<td>THP41</td>
<td>CGTACGTTTGCCAGGA</td>
<td>bp1501709 of genome (bp 646 of <em>yknV</em>)</td>
<td></td>
</tr>
</tbody>
</table>
transformation followed by selection for macrolide-lincosamide-streptogramin B antibiotic (MLS)-
resistance. MLS-resistant transformants were screened for chloramphenicol sensitivity to identify
transformants in which ΔyknUV::erm had integrated by double crossover.

The ΔyfiBC::kan allele truncates YfiB after 183 amino acids (and separates the remaining
fragment of yfiC from its promoter). To construct the yfiBC mutation, 1936 bp from bp 550 of
yfiB to bp 769 of yfiC were deleted and replaced with a kanamycin-resistance cassette. I attempted
to introduce the ΔyfiBC::kan mutation on pTH92 into B. subtilis by transformation followed by
selection for kanamycin-resistance. These transformations were unsuccessful (see discussion of
yfiBC and yheIH below).

The ΔyheIH::phleo allele truncates YheI after 335 amino acids (and separates the remaining
fragment of yheH from its promoter). To construct the yheIH mutation, 1362 bp from bp 1006 of
yheI to bp 205 of yheH were deleted and replaced with a phleomycin-resistance cassette. I
attempted to introduce the ΔyheIH::phleo mutation on pTH98 into B. subtilis by transformation
followed by selection for phleomycin-resistance. These transformation were unsuccessful (see
discussion of yfiBC and yheIH below).

Strains used are listed in Table 2A-4. Strains containing multiple mutations and/or reporter
fusions were constructed by transformation using standard methods.

YfiBC and YheIH appear to be essential for viability. As discussed above, I was unable to introduce the ΔyfiBC::kan or the ΔyheIH::phleo mutations into B. subtilis. In the
case of ΔyfiBC::kan, after transformation of wild type B. subtilis with pTH92, kanamycin-
resistant transformants grew after several days' incubation at 37°C. The transformants grew very
poorly suggesting that the mutation impaired growth. I attempted to backcross the ΔyfiBC::kan
mutation from this strain into a clean background. I got very few transformants, but those that did
grow grew up overnight. Most likely these transformants arose from congression of a suppressor
mutation with the ΔyfiBC::kan mutation. The ΔyfiBC::kan mutation appears to be lethal on its
own.
Table 2A-4. Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMH280</td>
<td>amy::(srfA-lacZ (\Delta)682 neo) trpC2 pheA1</td>
</tr>
<tr>
<td>TMH575</td>
<td>(\Delta)ywjA::spc amy::(srfA-lacZ (\Delta)682 neo) trpC2 pheA1</td>
</tr>
<tr>
<td>TMH585</td>
<td>yvcC::tet amy::(srfA-lacZ (\Delta)682 neo) trpC2 pheA1</td>
</tr>
<tr>
<td>TMH597</td>
<td>(\Delta)yknUV::erm amy::(srfA-lacZ (\Delta)682 neo) trpC2 pheA1</td>
</tr>
<tr>
<td>TMH608</td>
<td>ygaD::cat amy::(srfA-lacZ (\Delta)682 neo) trpC2 pheA1</td>
</tr>
<tr>
<td>TMH612</td>
<td>ygaD::pTH86(cat) (\Delta)yknUV::erm (\Delta)ywjA::spc yvcC::tet amy::(srfA-lacZ (\Delta)682 neo) trpC2 pheA1</td>
</tr>
</tbody>
</table>
The introduction of ΔyleI::phleo gave similar results. After transformation of pTH98 into wild type *B. subtilis*, phleomycin-resistant transformants grew up after several days' incubation at 37°C. These were extremely sickly (slow-growing with a tendency to lyse on the transformation plates). I was not able to subculture them. After several more days (at room temperature), faster-growing mutants grew out of the lysed patches. I suspect that the ΔyleI::phleo mutation is also lethal.

Further work needs to be done to conclusively demonstrate the essential nature of *yfiBC* and *yleI*. In these experiments, I selected for growth on rich medium (LB) at 37°C. It is possible that conditions (i.e., lower temperature, minimal medium) can be found where strains lacking one or both of these putative ABC transporters can grow. If so, it may be relatively straightforward to assess their role in ComX pheromone production. If they are indeed essential, determining whether either (or both) has a role in ComX pheromone production will be trickier.

**YgaD, YvcC, YwjA, and YknUV appear to play no role in ComX pheromone production.** As shown in Chapter 2, changes in ComX pheromone production lead to changes in the timing of the quorum response (see Fig. 2-4). Two approaches were taken to look at whether the timing of the quorum response was altered in strains lacking one or more of the four dispensable putative peptide-exporting ABC transporters. In both approaches expression of a transcriptional fusion of *lacZ* to the *srfA* promoter was used as a measure of the activation of the quorum response. If ComX pheromone production was eliminated (or reduced), one would expect no (or later) induction of *srfA-lacZ* expression.

In the first approach, I simply looked at the expression of *srfA-lacZ* in strains lacking one or more of the ABC transporters. In strains lacking just one of the ABC transporters (*ygaD, yvcC, ywjA*, or *yknUV*) the expression of *srfA-lacZ* was indistinguishable from the expression of *srfA-lacZ* in a wild type strain (Fig. 2A-1). This indicates that quorum response is not perturbed by these mutations and suggests that ComX pheromone production is wild type in these strains. This is not surprising given that mutations in these genes were not isolated in the hunt for mutants unable to make ComX pheromone (Chapter 2).
Figure 2A-1. Cells lacking one putative peptide exporter exhibit a wild type response to increasing cell density. Strains containing the srfA-lacZ Ω682 fusion were grown in defined minimal medium and samples taken at the indicated cell densities for determination of β-galactosidase activity. In each panel expression in wild type (TMH280) is marked with open circles while expression in the indicated mutant is marked with closed circles: A. ygaD:cat, TMH608, B. ΔyknUV::erm, TMH597, C. yvcC::tet, TMH585, D. ΔywJ::spc, TMH575.
Since the results of the mutant hunt suggested that ComX pheromone may be exported by redundant exporters, I also looked at the expression of srfA-lacZ in a strain lacking all four of the dispensable candidates. The expression of srfA-lacZ in the ygaD ywjA yvcC yknUV quadruple mutant was indistinguishable from the expression of srfA-lacZ in wild type cells (Fig. 2A-2). As in the case of the single mutants, this leads to the conclusion that ComX pheromone production is wild type in this strain. If any of these transporters plays a role in ComX pheromone production, that role is completely redundant with that of another protein.

In the second approach I attempted to overwhelm the ComX pheromone exporter(s). To increase the amount of ComX going through the ComX pheromone biogenesis pathway I expressed comQX from a multicopy plasmid (pTH2). If ComX pheromone is exported by redundant exporters, it is possible that increasing the amount of ComX expressed in the cell could overwhelm the secretion apparatus in strains lacking one of the exporters. This should lead to a defect in ComX pheromone production relative to a wild type strain expressing elevated levels of ComX. However, the expression of srfA-lacZ in wild type strains carrying pTH2 is indistinguishable from the expression of srfA-lacZ in strains lacking one or more of the dispensable ABC transporters and carrying pTH2 (data not shown). This is further evidence that ygaD, yvcC, ywjA, and yknUV play little, if any, role in ComX pheromone production.

**Discussion** Seven putative peptide-exporting ABC transporters were identified in the *B. subtilis* genome. One of them is the sublancin exporter. Two others appear to be essential for cell viability. The remaining four were tested for a role in ComX pheromone production and appear to have no (or a completely redundant) role. At this time, export via an ABC transporter is still the most reasonable hypothesis to explain how ComX pheromone (produced from a precursor lacking a typical sec-dependent signal sequence) is secreted from the cell. Since the four dispensable putative peptide-exporting ABC transporters appear to play little role in ComX pheromone production, the other three candidates warrant closer inspection. Perhaps SunT exports both sublancin and ComX pheromone export. *sunT* should be mutated and the effect of the mutation on
Figure 2A-2. Cells lacking four putative peptide exporters exhibit a wild type response to increasing cell density. Strains containing the srfA-lacZ Ω682 fusion were grown in defined minimal medium and samples taken at the indicated cell densities for determination of β-galactosidase activity. wild type, TMH280, open circles; ygaD:cat ΔyknUV::erm ΔywJ::spc yvcC::tet, TMH612, closed circles.
ComX pheromone production assayed (alone and in combination with mutations affecting the other candidates). More reasonable (given the outcome of the hunt for ComX pheromone deficient mutants discussed in Chapter 2) is the possibility that one or both of the essential ABC exporters, YfiBC and YheIH, is involved in ComX pheromone production. The genes encoding these two transporters should be place under an inducible promoter ($P_{spac}$ or $P_{xyl}$) and the expression levels titrated to see if at some intermediate level of expression cells retain viability but produce reduced amounts of ComX pheromone.

If one of the putative peptide-exporting ABC transporters is involved in ComX pheromone production, sequence analysis suggests that it (they) may export an unprocessed or partially-processed form of ComX. A search of the non-redundant protein database using any of the putative ComX pheromone exporters returns an extensive list of similar proteins. Topping the list are other members of the $B. subtilis$ family, followed by the essential ABC exporter MsbA (of $E. coli$ and $Haemophilus influenzae$), and finally by a variety of toxin exporters. The toxin exporters export proteins (as opposed to peptides). If one then searches the $B. subtilis$ genome for proteins similar to the toxin exporters (for example HylB of $Pasteurella haemolytica$) the same $7$ ABC transporters top the list of similar proteins. This raises the possibility that one or more of the putative peptide exporters of $B. subtilis$ act on full-length (or at least not fully-processed) ComX. This would lead to the prediction that the processing of ComX to the final 10 amino acid form occurs extracellularly. It may be worthwhile to look for a protein in the conditioned medium of a ComX producing culture that is capable of processing ComX to the final 10 amino acid form.
Chapter 3

Changes in CSF Production
in Response to Growth Conditions
Modulate the Quorum Response
ups mutants have increased expression of srfA-lacZ. In a hunt for mutants with altered induction of the quorum response (Chapter 2), 51 mutants with increased expression of the quorum-activated, ComA-dependent gene srfA (comS) were isolated. These mutants were identified on the basis of increased β-galactosidase activity expressed from a transcriptional srfA-lacZ reporter fusion. With respect to the expression of srfA-lacZ, the ups mutants (which are 'up for srfA') behave similarly to a wild type strain carrying comQ and comX on a multicopy plasmid. Cells carrying multicopy comQ and comX produce elevated amounts of ComX pheromone, resulting in an early activation of the quorum response (Chapter 2). Given the intriguing similarity in the pattern of the expression of srfA-lacZ in the ups mutants and the multicopy comQ comX strain, the 51 ups mutants were further characterized to gain additional insight into how B. subtilis regulates its response to crowding.

To assay for effects on the quorum response, the expression of srfA-lacZ was examined in defined minimal medium. The expression of srfA-lacZ in ups mutants was compared to the expression of srfA-lacZ in wild type cells. In wild type cells the expression of srfA-lacZ is very low at low cell density, increases as cell density increases during exponential growth, peaks as cells near the transition to stationary phase, and drops as cells enter stationary phase (Magnuson et al., 1994). Various patterns of srfA-lacZ expression were observed amongst the 51 ups isolates (data from original isolates not shown). In some cases expression was induced at a lower cell density than in wild type cells. In other cases induction occurred at the same cell density as in wild type cells but reached a higher peak value. In most cases the pattern was a combination of these two. A large number of mutants were indistinguishable from wild type with respect to the pattern of the expression of srfA-lacZ in liquid minimal medium suggesting that in these mutants the increased expression on solid medium may be due to factors specific to growth on a solid surface and/or factors specific to the medium used in the mutant hunt.

The mutations that caused early and/or increased expression of srfA-lacZ were backcrossed and the resulting strains were assayed for effects on the expression of srfA-lacZ. Five
independently isolated mutants with the most markedly early induction of \textit{srfA-lacZ} expression were chosen for further study (Fig. 3-1).

\textbf{ups mutants allow an early induction of the quorum response.} The induction of the expression of all ComA-dependent, quorum-regulated genes tested occurs at lower cell density in \textit{ups} mutants than in wild type cells. The expression of \textit{srfA (comS)}, \textit{rapC}, and \textit{rapA}, three genes directly dependent upon ComA for expression (Mueller et al., 1992; Roggiani and Dubnau, 1993; Lazazzera et al., 1999), was examined during growth in defined minimal medium by assaying expression of \(\beta\)-galactosidase from transcriptional fusions to \textit{lacZ} as a function of growth. The effect of \textit{ups150 (ptsG150)} on the induction of \textit{rapC-lacZ} (Fig. 3-12B) and \textit{rapA-lacZ} (data not shown) expression was similar to the effect of these mutations on the induction of \textit{srfA-lacZ} (Fig. 3-1B) expression. These effects are generalizable to each of the \textit{ups} mutants. (Throughout this chapter \textit{ups150 (ptsG150)} will be used to exemplify the \textit{ups} mutants when the phenotype under discussion is generalizable to all the \textit{ups} mutants.) In the case of \textit{srfA-lacZ} and \textit{rapC-lacZ}, the induction of cell density-regulated gene expression occurs in the \textit{ups} mutants at approximately half the cell density at which it occurs in wild type cells and continues to increase throughout exponential growth. The induction of \textit{rapA-lacZ} expression also occurs at lower cell density in the \textit{ups} mutants than in wild type cells, however the pattern is more complex suggesting that there may be additional factors, which perhaps act independently of the quorum-response, that regulate the expression of \textit{rapA-lacZ}.

Similar effects on the expression of all three quorum-activated genes tested suggest a common mechanism for the effect of \textit{ups} mutations on the quorum response. The simplest model is that \textit{ups} mutations cause increased ComA activation at low cell density. There are several phenomena that may increase ComA-P levels in the cell. These include increased cell density factor production, more efficient response to cell density factors, some combination of these two, or some factor-independent means of activating ComA.

The rest of this chapter is divided into three parts. First, I discuss how the \textit{ups} mutations affect carbon and energy metabolism and describe experiments that show that the effect of the
Figure 3-1. The expression of srfA-lacZ is induced at lower cell density in ups mutants than in wild type cells. Strains containing the srfA-lacZ Ω682 fusion were grown in defined minimal medium and samples taken at the indicated cell densities for determination of β-galactosidase activity. In each panel expression in wild type (TMH280) is marked with open circles while expression in the indicated ups mutant is marked with closed circles: A. ups144 (PHR321), B. ups150 (PHR318), C. ups157 (PHR319), D. ups159 (PHR234); E. ups164 (PHR320).
ups mutations on the quorum response can be recapitulated by growing wild type cells under conditions that support slow growth. This is followed by the presentation of experiments that demonstrate that glcR, glcT, ptsG, and pnc mutations (and poor growth conditions) affect ComA activation, and therefore the quorum response, by altering extracellular signaling. The final section outlines the role of the alternate sigma factor sigma-H in mediating the effect of the ups mutations.

THE ups MUTATIONS MAP TO GENES INVOLVED IN CARBON AND ENERGY METABOLISM

The 5 ups mutations were mapped by a combination of generalized co-transduction and/or co-transformation and plasmid rescue. Each of the ups mutations maps to a gene involved in carbon and energy metabolism (Table 3-1), demonstrating a connection between cellular physiology and the quorum response.

Four ups mutations were mapped to three genes affecting the function of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). ups150 (now called ptsG150) was mapped to ptsG, which encodes one of at least three glucose transporters (Gonzy-Treboul et al., 1991; Zagorec and Postma, 1992; Paulsen et al., 1998). ups144 (now called glcT144) and ups157 (now called glcT157) were mapped to gcT, which encodes an antiterminator required for ptsG expression (Bachem and Stulke, 1998; Paulsen et al., 1998). ups164 (now called glcR164) was mapped to glcR, which encodes a putative positive regulator of ptsG expression (Kunst et al., 1997).

The remaining ups mutation was mapped to an operon encoding enzymes involved in the pyridine nucleotide cycle, which regulates the amount of NAD in the cell. ups159 (now called pncB159) was mapped to the pncAB operon and most likely affects pncB.

Each of the ups mutants has a growth defect when grown on glucose. In defined minimal medium containing glucose as a carbon source, the ups mutants have doubling times that range from 68 minutes to 86 minutes compared with a doubling time of approximately 60 minutes for wild type cells (Table 3-2). The slow growth of glcR164, glcT144, glcT157, and ptsG150
Table 3-1. The *ups* mutations map to genes involved in carbon and energy metabolism.

<table>
<thead>
<tr>
<th>gene(s)</th>
<th>allele(s)</th>
<th>function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ptsG</em></td>
<td><em>ups150</em></td>
<td>glucose transporter of the phosphotransferase system</td>
</tr>
<tr>
<td><em>glcT</em></td>
<td><em>ups144, ups157</em></td>
<td>antiterminator required for <em>ptsG</em> expression</td>
</tr>
<tr>
<td><em>glcR</em></td>
<td><em>ups164</em></td>
<td>putative transcription factor that activates expression of <em>ptsG</em></td>
</tr>
<tr>
<td><em>pncB</em></td>
<td><em>ups159</em></td>
<td>nicotinate phosphoribosyltransferase required for NAD recycling</td>
</tr>
</tbody>
</table>
Table 3-2. The *ups* mutations cause slow growth in defined minimal medium containing glucose as a carbon source.

<table>
<thead>
<tr>
<th>strain</th>
<th><em>ups</em> mutation</th>
<th>doubling time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMH280</td>
<td>wild type</td>
<td>56</td>
</tr>
<tr>
<td>PHR320</td>
<td><em>glcR</em>164</td>
<td>70</td>
</tr>
<tr>
<td>PHR321</td>
<td><em>glcT</em>144</td>
<td>74</td>
</tr>
<tr>
<td>PHR319</td>
<td><em>glcT</em>157</td>
<td>68</td>
</tr>
<tr>
<td>PHR318</td>
<td><em>ptsG</em>150</td>
<td>69</td>
</tr>
<tr>
<td>PHR234</td>
<td><em>pncB</em>159</td>
<td>86</td>
</tr>
</tbody>
</table>
mutants indicates that a decreased rate of glucose uptake decreases growth rate, while the slow growth of the \textit{pncB159} mutant indicates that changes in NAD levels limit growth.

The \textit{ptsG150, glcT144, glcT157, and glcR164} mutations are loss of function alleles. To determine whether the \textit{ups} mutations are gain of function or loss of function mutations, the growth and expression of \textit{srfA-lacZ} in glucose of the \textit{ups} mutants was compared to the growth and expression of \textit{srfA-lacZ} of strains containing null mutations in the genes affected by the \textit{ups} mutations. Null mutations in \textit{ptsG (\Delta ptsG::tet), glcT (\Delta glcT::erm)}, and \textit{glcR (glcR::spc)} affect the expression of \textit{srfA-lacZ} (Fig. 3-2) and growth (data not shown) in a manner similar to the \textit{ups} mutations. These EMS-induced \textit{ups} mutations are, therefore, loss of function mutations.

The \textit{pncB159} allele appears to be a partial loss of function allele. Strains carrying the \textit{pncB159} mutation grow very slowly on defined minimal medium containing glucose as a carbon source (Table 3-2). An allele created by the integration of pTH28, which deletes the last 14 amino acids of PncB and replaces them with 16 vector-encoded amino acids, causes an even more severe growth defect on solid medium than does \textit{pncB159} (data not shown). This allele makes the strain genetically unstable as evidenced by rapid spawning of fast-growing colonies from the slow-growing colonies. Finally, as described below, an attempt to make a deletion-insertion mutation into \textit{pncB} failed. Taken together these observations suggest that \textit{pncB159} is a partial loss of function mutation.

\textbf{PncB is essential for cell viability in \textit{B. subtilis}.} YueJ and YueK are the \textit{B. subtilis} proteins most similar to PncA (pyrazinamidase/nicotinamidase) and PncB (nicotinate phosphoribosyltransferase). Specifically, YueJ is 32% identical (43% similar) to pyrazinamidase/nicotinamidase of \textit{Mycobacterium tuberculosis} encoded by \textit{pncA}, while YueK is 21% identical (40% similar) to nicotinate phosphoribosyltransferase of \textit{E. coli} encoded by \textit{pncB}. Therefore, it is likely that YueJ is the \textit{B. subtilis} PncA and YueK is the \textit{B. subtilis} PncB. These enzymes are required for the recycling of NAD via the pyridine nucleotide cycle (PNC) and
Figure 3-2. Null mutations in \( \text{ptsG} \), \( \text{glcT} \), and \( \text{glcR} \) cause an early induction of the quorum response. Strains containing the \( \text{srfA-lacZ} \) \( \Omega682 \) fusion were grown in defined minimal medium and samples taken at the indicated cell densities for the determination of \( \beta \)-galactosidase activity. wild type, TMH280, open circles; \( \Delta \text{ptsG::tet} \), TMH545, closed circles; \( \Delta \text{glcR::spc} \), TMH589, open squares; \( \Delta \text{glcT::erm} \), TMH598, closed squares.
regulate the level of NAD inside the cell [reviewed in (Foster and Moat, 1980; Penfound and Foster, 1996)].

In *Salmonella typhimurium*, PncB activity is dispensable in strains which can synthesize NAD de novo (Foster et al., 1979). Though *B. subtilis* can synthesize NAD de novo, in *B. subtilis* *pncB* appears to be essential for cell viability. Attempts to make a strain completely lacking PncB activity failed. A null mutation in *pncB* (Δ*pncB::tet*) could only be introduced when *pncB* was duplicated (data not shown). In *B. subtilis*, PncB may be a bifunctional protein with roles in both NAD recycling (supported by high similarity to PncB) and another essential process (demonstrated by its indispensable nature). Another possibility is that in *B. subtilis* PncB plays a much greater role (an essential role) in regulating the concentration of NAD. Perhaps without PncB to break down NAD to nicotinic acid when NAD levels climb too high and recycle nicotinic acid, nicotinamide, and nicotinamide mononucleotide when NAD levels fall too low the cell cannot survive. It is probable that disruption of PNC function decreases NAD levels in the cell, critically impairing energy metabolism.

**THE PTS AND PNC AFFECT CARBON AND ENERGY METABOLISM**

The phosphoenolpyruvate:sugar phosphotransferase system is involved in **energy metabolism**. The PTS system is responsible for the concomitant import and phosphorylation of sugars in bacterial systems [reviewed in (Postma et al., 1993)]. There are five components of bacterial PTSs: enzyme I (EI), HPr (histidine protein), enzyme IIA (EIIA), enzyme IIB (EIIB), and enzyme IIC (EIIC) (Fig. 3-3). Enzyme I and Hpr are utilized in the transport of all PTS-imported sugars, while enzymes IIA, IIB, and IIC are specific to a particular sugar or group of sugars. In *B. subtilis*, enzyme I is encoded by *ptsI* (Gonzy-Treboul and Steinmetz, 1987), HPr by *ptsH* (Gonzy-Treboul and Steinmetz, 1987), and enzymes IIA, IIB, and IIC for glucose transport by *ptsG* (Gonzy-Treboul et al., 1991; Zagorec and Postma, 1992), which encodes a single peptide containing all three activities (Gonzy-Treboul et al., 1991). The elements of the PTS system compose a phosphotransfer relay in which phosphate from phosphoenolpyruvate (PEP) is
Figure 3-3. The phosphoenolpyruvate:sugar phosphotransferase system

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is a phosphorelay through which phosphate is transferred from phosphoenolpyruvate to a sugar concomitant with sugar import. A. General PTS. In general, a PTS is composed of 5 elements. EI and HPr are distinct proteins, while EIIA, EIIB, and EIIC may be found in one or more proteins. B. PTS system for glucose transport in *B. subtilis*. In *B. subtilis* EIIA, EIIB, and EIIC are domains of a single protein.
transferred first to enzyme I, then to HPr, then to enzyme IIA, then to enzyme IIB, and finally to the sugar during transport by enzyme IIC (Fig. 3-3). Upon transport, glucose is converted to glucose-6-phosphate, which is fed into glycolysis to generate ATP and energy for growth and metabolism.

**B. subtilis** has at least two, and probably 3 or more, glucose transporters (Gonzy-Treboul et al., 1991; Zagorec and Postma, 1992; Paulsen et al., 1998). PtsG is the enzyme II component of the PTS system for glucose transport (Gonzy-Treboul et al., 1991; Zagorec and Postma, 1992). Since ptsG mutants continue to grow on glucose, there must be at least one more glucose transporter. The characterization of a second glucose transporter, GlcP, was reported while this work was in progress (Paulsen et al., 1998). Of these two transporters, PtsG appears to be the major glucose transporter supporting growth on glucose under laboratory conditions. A ptsG mutant grows much more slowly on solid medium containing glucose than does a glpP mutant (Paulsen et al., 1998). A ptsG glcP mutant still takes up glucose (Paulsen et al., 1998) demonstrating that there is at least one additional way for cells to take up glucose.

The expression of ptsGHI is regulated by glucose availability (Fig. 3-4). In the presence of glucose the operon is expressed, while in its absence it is not (Stulke et al., 1997). Noting the presence of a terminator-like sequence between the ptsG promoter and the ptsG ORF, Stulke hypothesized and then demonstrated that GlcT, a member of the BglG family of antiterminators, is required for ptsG expression and that this requirement depends upon the presence of the terminator-like sequence (Stulke et al., 1997). In the presence of glucose, GlcT binds to a site which overlaps the terminator-like sequence and prevents termination of ptsG transcription, while in absence of glucose GlcT is inactivated by phosphorylation (Stulke et al., 1997; Bachem and Stulke, 1998).

GlcR is likely to regulate the expression of ptsG. It is highly similar to members of the DeoR family of transcriptional regulators (Kunst et al., 1997). Its closest homolog in the non-redundant protein database is another putative transcriptional regulator, YulB, of **B. subtilis**. GlcR and YulB are 35% identical (54% similar). It is also highly similar to the *E. coli* transcriptional
Figure 3-4. Regulation of *ptsG* expression in *B. subtilis* The expression of *ptsG* is regulated by GlcT and GlcR. GlcT is an antiterminator that, in the presence of glucose, allows transcription to proceed through a terminator-like sequence upstream of the *ptsG* open reading frame. The activity of GlcT is inhibited in the absence of glucose. GlcR is hypothesized to be an activator of *ptsG* expression activated by glucose~P. The structure of the *ptsGHI* operon is as shown. Open reading frames are indicated by open boxes, terminators by stem-loops, and promoters by arrows.
repressor GlpR (30% identical and 52% similar), which represses the transcription of genes involved in glycerol-3-phosphate uptake (Schweizer et al., 1985), and the *E. coli* transcriptional activator FucR (30% identical and 50% similar), which activates transcription of genes involved in L-fucose metabolism (Chen et al., 1984; Chen et al., 1987).

*glcR* was identified in the sequencing of the *B. subtilis* genome. Though no mutations in *glcR* have been reported, in the genome project GlcR was proposed to be a repressor involved in the expression of the phosphotransferase system (Kunst et al., 1997). Based on the comparison of strains containing null mutations in *glcR* or *ptsG*, GlcR appears to be an activator of PtsG expression or activity. Both *glcR* and *ptsG* nulls grew poorly and had an *ups* phenotype in minimal medium containing glucose as a carbon source (Fig. 3-2). This suggests that the *glcR* null is defective in *ptsG* function and supports a role for GlcR in the activation of PtsG expression or activity. By analogy with the activation of the *fuc* operon by FucR, it is likely that GlcR activates *ptsG* transcription in the presence of glucose by binding near the *ptsG* promoter and serving as a transcription factor (Fig. 3-4). It is also possible that GlcR represses the transcription of a gene that encodes a negative regulator of PtsG expression or activity.

**The pyridine nucleotide cycles affects metabolism.** The pyridine nucleotide cycles (PNCs) are responsible for the intracellular recycling and extracellular scavenging of compounds which can be used to make nicotinamide adenine dinucleotide (NAD) [reviewed in (Foster and Moat, 1980; Penfound and Foster, 1996)]. NAD is essential for life. It is used as an enzymatic co-factor in catabolic reactions, while NADP, which is produced upon phosphorylation of NAD, is used as a co-factor in anabolic reactions. Together NAD and NADP play a key role in metabolism, directly or indirectly affecting almost every pathway in the cell. As an enzymatic co-factor, NAD is involved in over 300 oxidation-reduction reactions (Foster and Moat, 1980). It also serves as a substrate for ADP-ribosylation of proteins and nucleotides and in the repair of DNA by DNA ligase (Foster and Moat, 1980).

Multiple pathways for the biosynthesis and recycling of NAD have evolved. Most organisms can synthesize NAD de novo. Since NAD is essential for life, organisms that cannot
make NAD de novo must be provided with nicotinic acid, nicotinamide, or nicotinamide mononucleotide [reviewed in (Foster and Moat, 1980; Penfound and Foster, 1996)]. These compounds are taken up by the cell and recycled to make NAD. Although the PNCs are not essential in cells that can make NAD de novo, mutants that cannot synthesize NAD de novo depend upon the PNCs to survive.

The products of the **nad** genes are responsible for de novo biosynthesis of NAD. Although the pathway has not been thoroughly characterized in *B. subtilis*, it is believed to be very similar to the pathway in *E. coli* and *Salmonella typhimurium* (Fig. 3-5) [reviewed in (Penfound and Foster, 1996)]. In these organisms the biosynthesis of NAD from aspartate occurs in five steps catalyzed by the products of five **nad** genes: NadB, NadA, NadC, NadD, and NadE. If cells are missing NadB, NadA, or NadC they require one of the supplements listed above. In addition, **nadB** and **nadA** mutants can synthesize NAD if provided with quinolinate. NadD and NadE, however, are absolutely essential for viability since they are involved in both de novo synthesis of NAD and NAD recycling (Fig. 3-5).

The pyridine nucleotide cycles (PNCs) are the pathways by which NAD is recycled from nicotinic acid, nicotinamide, nicotinamide mononucleotide, nicotinamide riboside, or (extracellular) NAD. Cells will preferentially recycle these compounds before they resort to de novo biosynthesis [reviewed in (Foster and Moat, 1980; Penfound and Foster, 1996)]. There are three PNCs - named PNC IV, PNC V, and PNC VI for the number of steps in the cycle (Fig. 3-5). PNCs IV and V are involved in the intracellular recycling of compounds generated by the breakdown of NAD [reviewed in (Penfound and Foster, 1996)], while PNC VI is involved in the scavenging of nicotinic acid, nicotinamide, nicotinamide mononucleotide, and NAD (after extracellular degradation to NMN) [reviewed in (Penfound and Foster, 1996)]. Since PncB is involved in both PNC IV and PNC VI, it plays a key role in both internal NAD recycling and external scavenging.

Close regulation of NAD levels is critical to cellular physiology. The activity of the PNCs helps regulate the amount of NAD in the cell. Since the reaction carried out by PncB is reversible,
Figure 3-5. Synthesis and recycling of NAD. De novo synthesis of NAD from aspartate is depicted from top to bottom. The three pyridine nucleotide cycles (PNCs) are depicted in the lower half of the figure: PNC V to the left, PNC IV in the center, and PNC VI to the right. The gene product responsible for each reaction is indicated if known. Abbreviations are as follows: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; FAD, flavin adenine dinucleotide; FADH₂, reduced flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; P, inorganic phosphate; PP, inorganic pyrophosphate; PRPP, phosphoribosyl pyrophosphate.
the cell secretes nicotinic acid upon degradation of NAD when intracellular NAD concentrations are too high. When NAD levels fall, the cell is able to scavenge the nicotinic acid and regenerate NAD via PNC VI [reviewed in (Foster and Moat, 1980; Penfound and Foster, 1996)]. An increase in PncB activity has also been shown to increase intracellular NAD concentrations (Wubbolts et al., 1990).

**GROWTH CONDITIONS MODULATE THE QUORUM RESPONSE**

**Mutations that affect the PTS and PNC have pleiotropic effects on the cell.**
The ups mutations have multiple effects on the cell. In addition to causing an early induction of the quorum response, they affect growth and catabolite repression. They cause poor growth on minimal medium containing glucose as a carbon source (Table 3-2), while (except for ups159) having little effect on growth rate in rich medium such as LB (data not shown). ups mutations cause the relief of glucose catabolite repression. The relief of catabolite repression in ptsG mutants is well documented (Frisby and Zuber, 1994; Paulsen et al., 1998). Since glcT is required for ptsG expression (Stulke et al., 1997) and glcR is hypothesized to be required for ptsG expression, both glcT and glcR mutations should lead to the relief of catabolite repression. This was confirmed by assaying for catabolite resistant sporulation in the ups mutants. All 5 ups mutants sporulated at a high frequency in sporulation medium containing glucose (data not shown), conditions under which sporulation is normally repressed (Schaeffer et al., 1965). The same phenomenon was observed in the pncB mutant (data not shown), indicating that catabolite repression is also relieved in this mutant. At this time it is not clear whether the ups phenotypes are due to slow growth, the relief of catabolite repression, or some combination of the two.

**The effect of ups mutations on the quorum response is due growth under conditions that support slow growth.** To look at the effect of growth conditions on the quorum response, I compared the expression of srfA-lacZ in defined minimal medium containing a poor carbon source, succinate, to srfA-lacZ expression in defined minimal medium containing a good carbon source, glucose. Since growth is slow and catabolite repression is relieved in succinate, growth in succinate mimicks growth of the ups mutants, while growth in glucose is
‘wild type’ (fast growth, active catabolite repression). The induction of srfA-lacZ expression in succinate (doubling time \( \sim 2 \) hours) occurs at a much lower cell density than in glucose (doubling time \( \sim 1 \) hour) (Fig. 3-6A). The effects on doubling time and srfA-lacZ expression mimic those seen when one compares an ups mutant to wild type in minimal glucose medium (Fig. 3-1, Table 3-2). This demonstrates that growth in a poor carbon source is sufficient to cause an early induction of the quorum response. (Since succinate does not repress the expression of catabolite repressed genes, this experiment does not separate effects on growth rate from effects on catabolite repression.)

I also measured the expression of srfA-lacZ in wild type cells grown on either of two sugars which support the same doubling time and have the same effect on the expression of catabolite repressed genes. In the presence of either glucose or glycerol catabolite repression is active and wild type B. subtilis grows with a doubling time of approximately one hour. The pattern of srfA-lacZ expression in glucose is virtually indistinguishable from that in glycerol (Fig. 3-6B). This demonstrates that a simple change of carbon source is not sufficient to cause an early induction of the quorum response.

The induction of the quorum response in wild type cells at lower cell density under conditions that support slow growth leads to the prediction that ups mutants should exhibit a wild type quorum response if grown under conditions where they grow at the same rate as wild type cells. To test this, wild type and ptsG150 strains were grown in a defined minimal medium containing glycerol as a carbon source. In glycerol, both strains grew with a doubling time of approximately one hour. Under these conditions wild type and ptsG150 strains have virtually indistinguishable patterns of srfA-lacZ expression (Figure 3-7). This confirms that, under conditions where catabolite repression is active, restoring normal growth rate is sufficient to relieve the ups phenotype.

It is not clear whether growth rate, catabolite repression, or some combination of the two affect the quorum response. There are four possibilities: (1) slow growth sensitizes the quorum response; (2) the relief of catabolite repression sensitizes the quorum response; (3) both slow
Figure 3-6. Conditions that support an extended doubling time and the relief of catabolite repression cause an early induction of the quorum response. Wild type cells containing the srfA-lacZ Ω682 fusion (TMH280) were grown in defined minimal medium containing the indicated carbon source and samples taken at the indicated cell densities for determination of β-galactosidase activity. A. srfA-lacZ expression in carbon sources that support different doubling times and the relief of catabolite repression. glucose, doubling time ~60', open circles; succinate, doubling time ~120', closed circles. B. srfA-lacZ expression in carbon sources which support the same doubling time (~60') and cause catabolite repression. glucose, open circles; glycerol, closed circles.
Figure 3-7. A *ptsG150* mutant behaves like wild type in defined minimal medium containing glycerol as a carbon source. Strains containing the *srfA-lacZ* Ω682 fusion were grown in defined minimal medium containing glycerol as a carbon source and samples taken at the indicated cell densities for the determination of β-galactosidase activity. wild type, TMH280, open circles; *ptsG150*, PHR318, closed circles.
growth and the relief of catabolite repression contribute to the early quorum response in ups mutants; or (4) neither slow growth nor the relief of catabolite repression sensitizes the quorum response. To discriminate among these possibilities, one must assay the quorum response under conditions that separate the effects on growth from the effects on catabolite repression.

Elimination of the catabolite repressor protein CcpA causes both a relief of catabolite repression and a growth defect on glucose, but some recently reported alleles of B. megaterium ccpA separate these two effects (Henkin et al., 1991; Kuster et al., 1999). If these alleles have the same phenotypes in B. subtilis, they may help elucidate whether the relief of catabolite repression and/or a growth defect cause an early induction of the quorum response. To do this one would compare the expression (in defined minimal medium containing glucose as a carbon source) of srfA-lacZ in the following strains: wild type, ccpA, ccpA<sup>+</sup> (affecting catabolite repression only), and ccpA<sup>+</sup> (affecting growth only). If ccpA and ccpA<sup>+</sup> strains have identical (and early) induction of srfA-lacZ expression, then one can conclude that the early induction of the quorum response is due primarily to effects on growth rate. If ccpA and ccpA<sup>+</sup> strains have identical (and early) induction of srfA-lacZ expression, then one can conclude that the early induction of the quorum response is due primarily to effects on catabolite repression. If ccpA<sup>+</sup> and ccpA<sup>+</sup> strains exhibit the induction of the quorum response at a higher cell density than a ccpA strain (but a lower cell density than a wild type strain), then one can conclude that both growth rate and catabolite repression affect the induction of the quorum response. If ccpA and wild type strains have identical expression of srfA-lacZ, one can conclude that neither slow growth rate or the relief of catabolite repression (at least CcpA-mediated catabolite repression) are responsible for the early induction of the quorum response.

**pts and pnc mutations affect multiple aspects of the quorum response**

As mentioned above, there are several conditions that could increase the expression of ComA-controlled, quorum-regulated genes: increased production of one (or more) of the signaling peptides, hypersensitivity to one (or more) of the signaling peptides, some combination of factor
over-production and hyper-response, or even a factor-independent effect. The ups mutants cause increased production of CSF which in turn sensitizes the response to ComX pheromone. In this section I detail the experiments which demonstrate that CSF is a modulatory factor which integrates information on both cell density and growth conditions to fine-tune the quorum response.

**ups mutations cause increased production of a cell-cell signaling peptide.** Both ComX pheromone and CSF stimulate srfA-lacZ expression and accumulate at high cell density (Magnuson et al., 1994; Solomon et al., 1995; Solomon et al., 1996). Increased accumulation of either factor at low cell density could account, at least partially, for the early activation of the quorum response in the ups mutants. The ups mutants were tested for production of both CSF and ComX pheromone in minimal medium. The ups mutants produce more CSF during exponential growth than do wild type cells (Fig. 3-8A, Table 3-3). There is no corresponding increase in the production of ComX pheromone (data not shown).

To look at CSF production, conditioned medium (growth medium from which the cells have been removed) was harvested at various time points throughout the growth of wild type and ups mutants. After separation into CSF-containing and ComX pheromone-containing fractions, the amount of CSF and ComX pheromone present at each time point was quantified. The ups mutants have increased CSF production. Compared to wild type cells, ups mutants produce much more CSF during early exponential growth (Figure 3-8A, Table 3-3). In contrast, the ups mutations had no effect on ComX pheromone production (data not shown).

Elevated CSF production can be induced in wild type cells by growing them under nutrient conditions that support poor growth. Wild type cells were grown in defined minimal medium containing either glucose or succinate as a carbon source and samples were taken for measurement of CSF and ComX pheromone as described above. As expected, wild type cells grown under poor growth conditions (succinate-grown cells) made more CSF during exponential growth than did wild type cells grown under good growth conditions (glucose-grown cells) (Fig. 3-8B). No effect was observed on ComX pheromone production (data not shown).
Figure 3-8. Production of CSF is elevated under conditions that support poor growth. A. Strains were grown in defined minimal medium containing glucose as a carbon source and samples taken at the indicated cell densities for the determination of extracellular CSF concentration. Wild type, JH642, open circles; ptsG150, PHR344, closed circles. B. Wild type cells (JH642) were grown in defined minimal medium containing the indicated carbon source and samples taken at the indicated cell densities for the determination of extracellular CSF concentration. Glucose, open circles; succinate, closed circles.
Table 3-3. CSF accumulation is greater in *ups* mutants than in wild type cells during early exponential growth.

<table>
<thead>
<tr>
<th>genotype</th>
<th>CSF concentration at 0.3 OD&lt;sub&gt;600nm&lt;/sub&gt;</th>
<th>fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.2 nM</td>
<td>1.0</td>
</tr>
<tr>
<td><em>glcR164</em></td>
<td>2.2 nM</td>
<td>1.8</td>
</tr>
<tr>
<td><em>glcT157</em></td>
<td>2.5 nM</td>
<td>2.1</td>
</tr>
<tr>
<td><em>ptsG150</em></td>
<td>3.6 nM</td>
<td>3.0</td>
</tr>
<tr>
<td><em>pncB159</em></td>
<td>7.5 nM</td>
<td>6.3</td>
</tr>
</tbody>
</table>
By analogy with the effect of increased ComX pheromone production on the expression of srfA-lacZ (Chapter 2), an increase in CSF production could account for the early induction of the quorum response in the ups mutants. To test this, the effect of ups mutations on expression of srfA-lacZ in a wild type background was compared to that in a phrC (CSF) background. Although the ups mutations cause an early induction of the quorum response in a wild type background, the ups mutations have no effect on the expression of srfA-lacZ in cells that are unable to make CSF (phrC background) (Figure 3-9). Therefore, the ups phenotype depends upon the production of CSF.

**ups mutations sensitize the cell to the major cell density signaling peptide.** Although the ups mutants produce wild type amounts of ComX pheromone, they should respond more vigorously to ComX pheromone than do wild type cells (Fig. 3-10). As described in Chapter 1, CSF inhibits the activity of the (putative) ComA–P phosphatase RapC (Solomon et al., 1996; Lazazzera et al., 1997). Since ups mutants make excess CSF during exponential growth, there should be even less RapC activity during exponential growth of ups mutants than of wild type cells. With little ComA–P phosphatase activity, whatever ComA–P is generated by the activity of ComP in response to ComX pheromone should be more stable. If ComA–P generated in response to ComX pheromone is more stable, even a small amount of ComX pheromone should result in increased srfA-lacZ transcription.

As predicted, ups mutations or conditions that support poor growth lead to a hyper-response to ComX pheromone. To measure the response to ComX pheromone a strain that cannot make ComX pheromone (a comQ mutant) but which carries lacZ fused to a quorum-responsive gene is grown to low cell density and mixed with samples containing (partially-purified) ComX pheromone. The amount of srfA-lacZ induction in the presence of ComX pheromone is compared to that without any added factor (Solomon et al., 1995). About half as much ComX pheromone must be added to an ups comQ mutant than to comQ cells to see a given fold response (Figure 3-11A). This indicates that the response to ComX pheromone is more
Figure 3-9. The early induction of \textit{srfA-lacZ} expression in an \textit{ups} mutant depends upon the production of CSF. Strains containing the \textit{srfA-lacZ} \textit{Ω682} fusion were grown in defined minimal medium and samples taken at the indicated cell densities for the determination of β-galactosidase activity. wild type, TMH280, open circles; \textit{ptsG150}, PHR318, closed circles; \textit{phrC}, TMH335, open squares; \textit{ptsG150 phrC}, PHR356, closed squares.
Figure 3-10. Model predicting a hyper-response to ComX pheromone resulting from an increase in CSF production
Figure 3-11. An ups mutation causes hyper-response to both ComX pheromone and CSF. A. Response to partially purified ComX pheromone. Strains containing the srfA-lacZ Ω682 fusion were grown to low cell density, mixed with the indicated amounts of partially purified ComX pheromone, and the fold increase in β-galactosidase activity determined after a 70' incubation. comQ, TMH281, open circles; ptsG150 comQ, PHR352, closed circles. B. Response to synthetic CSF. Strains containing the srfA-lacZ Ω682 fusion were grown to low cell density, mixed with the indicated concentration of synthetic CSF, and the fold increase in β-galactosidase activity determined after a 70' incubation. phrC, TMH335, open circles; ptsG150 phrC, PHR356, closed circles.
vigorous in ups comQ mutants than in comQ cells. It is also more vigorous in comQ cells grown in succinate than in comQ cells grown in glucose (data not shown).

Since the hyper-response to ComX pheromone was predicted as a consequence of increased CSF production, it was necessary to determine whether the hyper-response to ComX pheromone is dependent upon CSF production. Given that an ups mutation has no effect on the expression of srfA-lacZ in a phrC background (Fig. 3-6), one would predict that there would be no hyper-response to ComX pheromone in the absence of CSF production. The ComX pheromone response in ups comQ mutants was compared to that in ups phrC comQ mutants, which do not make CSF. In the absence of CSF production the ups mutations have no effect on the response to ComX pheromone (data not shown).

**ups mutations also affect the response to the modulatory cell density signaling peptide.** The assay for CSF response is similar to the assay for ComX pheromone response, except synthetic CSF is used to stimulate the expression of srfA-lacZ and the indicator cells are unable to make CSF due to a phrC mutation. phrC ups mutants also exhibit a hyper-response to CSF (Fig. 3-11B). Also, phrC cells grown in succinate respond more vigorously to CSF than do phrC cells grown in glucose (data not shown). These results are surprising because all previous experiments indicated that the effect of the ups mutations on the quorum response was relieved by a phrC mutation. The hyper-response to CSF in ups phrC mutants (which are unable to make CSF) suggests that an additional signal, perhaps another signaling peptide, plays a minor role in modulating the quorum response (see next section).

**ALL EFFECTS OF THE ups MUTATIONS ON THE QUORUM RESPONSE DEPEND UPON THE ALTERNATE SIGMA FACTOR SIGMA-H**

*B. subtilis* has a host of alternate sigma factors dedicated to reprogramming gene expression under special conditions. One of the alternate sigma factors, sigma-H, reprograms gene expression under conditions of nutrient stress (Healy et al., 1991; Weir et al., 1991). Sigma-H is required for both competence development and sporulation (Weir et al., 1984; Albano et al.,
1987). Frisby was the first to report that mutations which cause poor glucose utilization by affecting the PTS system lead to increased sigma-H activity and increased expression of \(srfA-lacZ\) (Frisby and Zuber, 1994).

The elevation of sigma-H activity by \(pts\) mutations leads to the prediction that the expression of the CSF precursor gene, \(phrC\), should be elevated in \(ups\) mutants since it is under the control of two promoters, one of which is sigma-H dependent (Solomon et al., 1996; Lazazzera et al., 1999). This would account, at least in part, for increased CSF production. Since increased CSF production is the primary means by which the \(ups\) mutations affect the quorum response, increased expression from the sigma-H dependent \(phrC\) promoter suggests that effects on sigma-H activity may be the means by which information on growth conditions is integrated into the quorum response. The experiments in this section demonstrate that both of these predictions are met.

**\(ups\) mutations cause increased expression of the CSF precursor gene \(phrC\).** The gene which encodes the CSF precursor, \(phrC\), is transcribed from two promoters. The first (P1), which drives expression of both \(rapC\) and \(phrC\), is ComA-dependent, while the second (P2), which drives expression of only \(phrC\), is sigma-H-dependent (Chapter 1, Fig. 3-12A) (Solomon et al., 1996; Lazazzera et al., 1999). Consistent with an increase in CSF production, the expression of the CSF precursor gene, \(phrC\), from each of these promoters is elevated in an \(ups\) mutant. Expression from ComA-controlled P1 was measured by looking at expression of \(\beta\)-galactosidase from a fusion that puts \(lacZ\) under the control of P1 (but not P2) at the non-essential locus \(amyE\). The expression of \(rapC-lacZ\) is induced at a lower cell density in the \(ups\) mutants than in wild type cells (Fig. 3-12B). This is reminiscent of the effect of these mutations on \(srfA-lacZ\) and is as expected since both promoters are ComA-dependent. Expression of \(\beta\)-galactosidase from a \(phrC-lacZ\) fusion that puts \(lacZ\) under the control of P2 (but not P1) at \(amyE\) was used to measure activity of P2. Expression from sigma-H-dependent P2 is greatly elevated in the \(ups\) mutants relative to wild type (Fig. 3-12C). This is likely to account, at least partially, for the increase in CSF production in these mutants.
Figure 3-12. The expression of the CSF precursor gene, \textit{phrC}, is elevated in an \textit{ups} mutant. A. Structure of the rapC phrC operon. The two promoters, P1 and P2, are indicated by arrows. The ComA box upstream of P1 is indicated by the small black box. The transcription terminator is indicated by a stem-loop. B. Expression of rapC-lacZ. Strains containing the rapC-lacZ fusion \( \Omega_1 \) were grown in defined minimal medium and samples taken at the indicated cell densities for the determination of \( \beta \)-galactosidase activity. wild type, IRN216, open circles; \textit{ptsG150}, PHR424, closed circles. C. Expression of phrC-lacZ. Strains containing the phrC-lacZ fusion \( \Omega_2 \) were grown in defined minimal medium and samples taken at the indicated cell densities for the determination of \( \beta \)-galactosidase activity. wild type, IRN235, open circles. \textit{ptsG150}, PHR429, closed circles.
As described above, the effect of the ups mutations on several aspects of the quorum response depends upon the production of CSF. Given that sigma-H is required for CSF production and that ups mutations have an effect on the activity of sigma-H, I hypothesize that effects on sigma-H may be the primary mechanism by which growth conditions modulate the quorum response.

**Sigma-H is required for the early induction of the quorum response.** To look at the effect of an ups mutation on the quorum response in the absence of sigma-H, the expression of srfA-lacZ in a spo0H mutant was compared to that in ups spo0H double mutants. The pattern of srfA-lacZ induction was virtually identical in the spo0H and ups spo0H strains (Fig. 3-13A). Therefore, the effect of the ups mutations on the final output of the quorum response, the expression of ComA-controlled genes, is dependent upon sigma-H.

**Sigma-H is required for the hyper-response to ComX pheromone.** The response to ComX pheromone was compared in comQ spo0H and ups comQ spo0H strains. As expected, in a spo0H background the ups mutations have little effect on the response to ComX pheromone (Figure 3-13B). This is entirely consistent with the inability of the ups mutants to hyper-respond to ComX pheromone if they are unable to make CSF.

**Sigma-H is required for the hyper-response to CSF.** The response to synthetic CSF was compared in spo0H and ups spo0H strains. Although the ups mutations cause a hyper-response to CSF in a phrC background, there is no hyper-response in a spo0H background (Fig. 3-13C).

As described above, the hyper-response to CSF persists even in the absence of CSF production. Since the hyper-response disappears in a spo0H mutant, the additional input must be Sigma-H-dependent. Multiple phr genes have sigma-H promoters (Chapter 1). It is intriguing to speculate that one or more of these gene products give rise to a peptide that plays a minor role in modulating the response to CSF. Perhaps this peptide inhibits a second phosphatase that has very minor (and perhaps not even physiologically relevant) phosphatase activity towards ComA–P.
Figure 3-13. Sigma-H is required to mediate the effect of an ups mutation on the quorum response. A. Expression of srfA-lacZ. Strains containing the srfA-lacZ Ω682 fusion were grown in defined minimal medium and samples taken at the indicated cell densities for determination of β-galactosidase activity. spo0H, TMH283, open circles; ptsG150 spo0H, PHR447, closed circles. B. Response to partially purified ComX pheromone. Strains containing the srfA-lacZ Ω682 fusion were grown to low cell density, mixed with the indicated amounts of partially purified ComX pheromone, and the fold increase in β-galactosidase activity determined after a 70' incubation. spo0H comQ, TMH617, open circles; ptsG150 spo0H comQ, PHR451, closed circles. C. Response to synthetic CSF. Strains containing the srfA-lacZ Ω682 fusion were grown to low cell density, mixed with the indicated amounts of synthetic CSF, and the fold increase in β-galactosidase activity determined after a 70' incubation. spo0H, TMH283, open circles; ptsG150 spo0H, PHR447, closed circles.
The work in this chapter demonstrates that growth conditions modulate the activity of the alternate sigma factor sigma-H and, therefore, the production of the extracellular cell density factor CSF, which results in modulation of the quorum response in *B. subtilis*. It raises questions about the regulation of sigma-H activity and the modulation of the quorum response by growth conditions in other systems. These ideas are discussed in the next chapter.
Chapter 4

Summary and Perspectives
GROWTH CONDITIONS MODULATE THE QUORUM RESPONSE

In *B. subtilis* the quorum response is activated by two extracellular peptides: ComX pheromone and competence and sporulation factor (CSF). These two peptides are at low extracellular concentration at low cell density and higher concentration at higher cell density. Specifically, ComX pheromone accumulates during exponential growth (Chapter 2), while CSF accumulates slowly during exponential growth and more markedly as the cells enter stationary phase (Lazazzera et al., 1999b). Consistent with its accumulation in rough parallel with cell density, ComX pheromone is the primary signal of cell density. In contrast, CSF is a modulatory factor and mainly influences the timing of the quorum response (Chapter 3).

ComX pheromone and CSF activate convergent signaling pathways to trigger the quorum response (Solomon et al., 1996). ComX pheromone activates the membrane-bound histidine protein kinase ComP that in turn allows phosphorylation and activation of the response regulator ComA (Solomon et al., 1996). After transport into the cell by the oligopeptide permease (Opp), CSF inhibits the activity of the (putative) ComA-P phosphatase RapC (Solomon et al., 1996; Lazazzera et al., 1997). In this way the signals which affect ComX pheromone and CSF production are integrated at the level of ComA activation.

The quorum response has far-reaching effects in *B. subtilis*. Active ComA (ComA–P) binds to a site upstream of and activates transcription from multiple promoters allowing expression of genes involved in competence development, sporulation, extracellular enzyme production, and antibiotic production (Roggiani and Dubnau, 1993; Yang et al., 1986; Nakano et al., 1991; D'Souza et al., 1994; Perego et al., 1994; Hamoen et al., 1995; Perego et al., 1996; Solomon et al., 1996; Kunst et al., 1997). Putative ComA boxes appear upstream of at least 12 other promoters (driving expression of at least 17 other genes). Members of this putative ComA regulon include genes involved in translation (*infA, tsf*), extracellular enzyme production (*pelA*), and ribonucleotide metabolism (*deoD, yurl*) (Lazazzera et al., 1999a). Factors that regulate the expression of known ComA-dependent genes, such as high cell density and growth conditions...
(Chapter 3), are likely also to affect the expression of the putative ComA-regulated genes and the processes in which these gene products act.

As presented in this thesis, information on growth conditions is integrated into the quorum response via the regulation of CSF production by the alternate sigma factor sigma-H (Chapter 3) (Fig. 4-1). When growth is impaired (due either to a mutation that affects the efficiency of carbon metabolism or to growth on a poor carbon source) sigma-H activity is elevated. The expression of the gene that encodes the CSF precursor, phrC, is under the control of two promoters, one of which is dependent upon sigma-H (Solomon et al., 1996; Lazazzera et al., 1999b). Therefore, increased sigma-H activity results in increased expression of phrC and increased production of CSF (Chapter 3). Increased production of CSF results in a hyper-response to ComX pheromone (Chapter 3). In this way sigma-H allows the quorum response to be activated at a lower cell density under poor growth conditions than good growth conditions.

SIGMA-H IS MORE THAN JUST ANOTHER SPORULATION SIGMA FACTOR

Sigma-H is one of multiple alternate sigma factors found in B. subtilis. B. subtilis has a host of alternate sigma factors that are responsible for reprogramming gene expression in response to physiological, developmental, or stress conditions. At least 9 of the sigma factors encoded in the B. subtilis genome have been characterized in some detail [reviewed in (Haldenwang, 1995)]. Sigma-A is the housekeeping sigma, while each of the others is responsible for altering the specificity of promoter recognition by RNA polymerase in response to a particular signal, developmental cue, or set of conditions. There are alternate sigmas involved in regulation of chemotaxis, degradative enzyme production, competence, sporulation, and general stress responses [reviewed in (Haldenwang, 1995)]. Five of the alternate sigma factors are involved in sporulation gene expression. Sigma-H acts early in sporulation, sigma-E and sigma-F act after septation, and sigma-G and sigma-K act even later in sporulation [reviewed in (Losick and Stragier, 1992)].
Figure 4-1. Growth conditions modulate the quorum response in *B. subtilis*. 
Although sigma-E, sigma-F, sigma-G, and sigma-K appear to be dedicated to sporulation, sigma-H is more than just another sporulation sigma factor. Since sigma-H regulates the expression of multiple sporulation genes (including $kinA$, $spoOF$, $spoOA$, $spoIIA$, and $spoVG$) it is obviously a sporulation sigma factor [reviewed in (Haldenwang, 1995)]. However, through its effects on the quorum response, sigma-H is involved in competence development (Albano et al., 1987; Lazazzera et al., 1999b). It is also involved in the expression of several metabolic enzymes (Price et al., 1989; Tatti et al., 1989; Wray et al., 1997). In addition to these roles, sigma-H-dependent promoters are found upstream of several genes which are regulated by sigma-B in response to general stresses, suggesting that sigma-H may also play a role in the general stress response (Varon et al., 1996; Drzewiecki et al., 1998; Gaidenko and Price, 1998).

**Sigma-H is required for the effect of ups mutations on the quorum response.** It was previously reported that mutations that affect the PTS lead to increased sigma-H activity (Frisby and Zuber, 1994). Study of the $ups$ mutants confirmed that $ptsG$ mutations affect sigma-H activity and expanded the range of mutations known to affect sigma-H activity to include those in a gene involved in the pyridine nucleotide cycles ($pncB$) and in genes involved in the expression of $ptsG$ ($glcT$ and $glcR$). As described in Chapter 3, the $ups$ mutations have no effect on the quorum response in strains lacking sigma-H. Specifically, sigma-H is required for (1) the production of CSF (Solomon et al., 1996; Lazazzera et al., 1999b), (2) the effect of $ups$ mutations on the response to ComX pheromone, (3) the effect of $ups$ mutations on the response to CSF, and (4) the effect of $ups$ mutations on the timing of the expression of $srfA-lacZ$. The simplest model to explain these observations is that growth conditions regulate sigma-H activity, which in turn affects the production of CSF (and perhaps another signaling peptide) and, therefore, quorum-regulated gene expression.

**Sigma-H is regulated transcriptionally and post-transcriptionally.** Although sigma-H was initially characterized as a sporulation gene (Weir et al., 1991) and then as a sporulation sigma factor (Dubnau et al., 1988), it should actually be considered more of an 'nutrient-sensing' sigma factor since it is regulated by growth conditions (Chapter 3) (Frisby and
Zuber, 1994) and the ultimate nutrient deprivation, starvation (Weir et al., 1991). The regulation of sigma-H is complex and occurs at multiple levels: at the level of transcription of the gene encoding sigma-H (spoOH), at the level of sigma-H stability, and at the level of sigma-H activity (Fig. 4-2).

The transcription of sigma-H is regulated by the transition state regulator AbrB. AbrB represses the transcription of spoOH during exponential growth (Dubnau et al., 1987; Healy et al., 1991; Weir et al., 1991). Relief of AbrB repression occurs when abrB expression is downregulated by Spo0A-P, so conditions that contribute to Spo0A activation also contribute to the activation of spoOH transcription (Perego et al., 1988). However, in an abrB mutant, where spoOH is transcribed constitutively, the amount of sigma-H in the cell is still regulated by starvation, indicating that there is post-transcriptional control of sigma-H (Healy et al., 1991; Weir et al., 1991)

The proteases LonA and LonB and the regulatory ATPase ClpC are responsible for the post-transcriptional control of the amount of sigma-H protein in the cell. LonA and LonB are activated by low pH (acid stress) (Liu et al., 1999). When LonA or LonB is absent or acid stress is relieved, sigma-H accumulates (Liu et al., 1999). (Whether LonA and/or LonB act directly on sigma-H remains to be determined.) This increase in sigma-H levels is not sufficient to increase sigma-H-dependent gene expression unless ClpX is also present, indicating that ClpX stimulates sigma-H activity (see below) (Liu et al., 1999). ClpC also affects the stability of sigma-H. clpC mutants accumulate sigma-H at high temperature, suggesting that ClpC stimulates sigma-H degradation (Nanamiya et al., 1998). Since the effect of ClpC on sigma-H levels is notable only after the transition to stationary phase, it is hypothesized that ClpC acts in the normal shut-off of Sigma-H activity after sporulation is well underway (Nanamiya et al., 1998).

Finally, the activity of sigma-H is also regulated. As mentioned above, although sigma-H accumulates in lon mutants, sigma-H activity is not elevated unless ClpX is present (Liu et al., 1999). Therefore, ClpX must regulate the activity of sigma-H. ClpX is activated (transcriptionally or post-transcriptionally remains to be seen) by starvation, allowing starvation to regulate sigma-H
Figure 4-2. Regulation of sigma-H activity. Transcriptional regulation, regulation of protein stability, and regulation of protein activity each contribute to the level of sigma-H-dependent transcription in the cell. Transcription of $spoOH$ is repressed by the transition phase regulator AbrB. Protein stability is regulated by the proteases LonA and LonB and the regulatory ATPase ClpC. Protein activity is regulated by the regulatory ATPase and, most likely, growth conditions.
expression both transcriptionally (via AbrB) and post-transcriptionally (via ClpX) (Carter and Moran, 1986; Dubnau et al., 1987; Weir et al., 1991; Liu et al., 1999). The mechanism by which ClpX affects sigma-H activity is unknown. Perhaps it regulates the proteolysis of a sigma-H inhibitor or facilitates sigma-H/RNA polymerase interactions.

**Further study is needed to determine how growth conditions affect sigma-H activity.** Potentially growth conditions could affect the transcription of spoOH, the level of sigma-H, and/or the activity of sigma-H. Further study is needed to determine how growth conditions effect sigma-H activity, but I suspect that growth conditions modulate the activity of sigma-H, as opposed to its stability or transcription of spoOH.

It is unlikely that growth conditions contributes significantly to the regulation of spoOH transcription. As described in the introduction, many conditions contribute to the activation of Spo0A. The most important in light of the work presented in this thesis are starvation and cell density. Since growth conditions modulate the production of CSF and CSF affects the activation of Spo0A-P, one would predict that alterations in growth conditions may affect the transcription of spoOH as regulated by AbrB. However, the effect, if there is one at all, appears to be very small (Frisby and Zuber, 1994). This is not very surprising given that cell density is just one of the many inputs into the phosphorelay leading to Spo0A activation and abrB repression.

The pH-dependent regulation of sigma-H levels by LonA and LonB is unlikely to account for the effect of ups mutations on sigma-H-dependent gene expression. Under some conditions mutations affecting the PTS lead to a maintained elevated pH and result in increased Sigma-H activity (Cosby and Zuber, 1997). In these situations the inhibition of LonA and LonB at the elevated pH may be sufficient to explain the effect of pts mutations on Sigma-H activity. However, the effect of ups mutations on sigma-H is not completely explained by the effect of these mutations on extracellular pH. Under the conditions used for the experiments presented in this thesis the extracellular pH of wild type cultures is indistinguishable from ups cultures (data not shown), yet sigma-H activity is most certainly elevated. This leaves regulation of sigma-H activity...
as the most likely avenue by which growth conditions may modulate sigma-H-dependent gene expression.

Several lines of evidence suggest that sigma-H activity is regulated by growth conditions and starvation. First, as described above, ClpX positively regulates sigma-H activity. ClpX is activated by starvation. It would be interesting to see if the ups phenotype persists in a clpX mutant. If it did not one may conclude that the regulation of sigma-H activity by growth conditions is dependent upon ClpX. However, if the phenotype did persist one would have to conclude that there is another mechanism for the regulation of sigma-H activity. Work by Asai demonstrated that sigma-H activity (as opposed to protein level or transcription) is regulated by carbon and nitrogen limitation (Asai et al., 1995). In studying the sigma-H-dependent transcription of kinA they found that even when sigma-H levels were low (i.e. in a spo0A mutant) activity was induced by starvation. They hypothesized the existence of a transcriptional activator of kinA that would facilitate sigma-H-dependent transcription. Another regulator of sigma-H activity is also plausible. Since the effect of nutrient limitation is seen at both the kinA and phrC promoters, it seems entirely possible that whatever regulates sigma-H activity under the conditions used by Asai may contribute to the regulation of sigma-H by the ups mutants.

GROWTH CONDITIONS AND THE QUORUM RESPONSE IN OTHER SYSTEMS

The use of a second cell-cell signaling peptide to integrate information on growth conditions into the quorum response of B. subtilis answers the question ‘Why two factors?’ and raises the question ‘Who else does this?’.

Two AHL signaling systems operate in the induction of virulence in the human pathogen P. aeruginosa (Passador et al., 1993; Latifi et al., 1996). Recent work demonstrates that the two systems act in a linear order with the first (LasR/LasI) activating expression of the second (RhlR/RhlI) and the second activating expression of the virulence factors encoded by the quorum-activated genes (Latifi et al., 1996). An expanded signaling pathway such as this is reminiscent of
the convergent quorum responses seen in *B. subtilis* and leads one to ponder why there are two cell-density factors in yet another system. Perhaps, like in *B. subtilis*, one of the factors is modulatory. There is a catabolite repressor protein (CRP) binding site upstream of the *lasR* gene and CRP acts as an activator of *lasR* expression (Albus et al., 1997). In this case the autoinducer made by LasI would be modulatory not because its production is modulated by growth conditions but because the response to it is (if that is indeed the case). To date no one has reported studies of the impact of growth conditions, specifically carbon source, on the response to the autoinducer sensed by LasR. In light of what is now known about the regulation of the quorum response of *B. subtilis* by growth conditions, this may be worth closer examination.

Growth conditions modulate the quorum response in multiple organisms where even less is known about how growth conditions impinge upon cell-cell signaling. For example, little is known about how carbon source affects the regulation of the expression of the cell division genes *ftsQA* in *E. coli* and the regulation of nisin production in *Lactococcus lactis*. However, both are regulated by cell-cell signaling and both are regulated by carbon source (van der Meer et al., 1993; Sitnikov et al., 1996). The idea that growth conditions can modulate cell-cell signaling should lead to a reexamination of carbon source regulation of genes and processes known to be regulated by cell-cell signaling.
Chapter 5

Materials and Methods
**Strains.** Strains used are listed in Table 5-1. Each is derived from *B. subtilis* strain JH642 and contains the *trpC2* and *pheA1* mutations (Perego et al., 1988). Strains were constructed by transformation of plasmid or chromosomal DNA using standard protocols (Harwood and Cutting, 1990).

Mutant alleles used include: Δ*aphrC::erm*, Δ*spoOH::cat*, Δ*comQ::spc*, Δ*spo0K::erm*, and Δ*comP::cat*. The creation of Δ*ptsG::tet*, Δ*gicR::spc*, and Δ*glcT::erm* alleles is described below. The isolation of *ptsG150* (*ups150*), *gicT144* (*ups144*), *gicT157* (*ups157*), *pncB159* (*ups159*), and *glcR164* (*ups164*) is also described below.

**Construction of *ptsG*, *gicT*, and *glcR* mutations.** The Δ*ptsG::tet* deletion-insertion replaced 1102 bp internal to *ptsG* with the tetracycline resistance cassette. The plasmid pTH76 contains Δ*ptsG::tet*. pTH76 was transformed into *B. subtilis* and tetracycline-resistant transformants were selected. Transformants were screened for chloramphenicol sensitivity to identify those in which Δ*ptsG::tet* had recombined by double crossover.

The Δ*glcT::erm* deletion-insertion mutation deleted 449 bp from *glcT* and replaced them with the erythromycin resistance cassette. The plasmid pTH102 contains Δ*glcT::erm*. pTH102 was transformed into *B. subtilis* and transformants resistant to MLS (macrolide-lincosamide-streptogramin B antibiotics) were selected. Transformants were screened for chloramphenicol sensitivity to identify those in which Δ*glcT::erm* had recombined by double crossover.

The Δ*gicR::spc* insertion mutation was made by inserting the spectinomycin resistance cassette 162 bp downstream of the start of *gicR*. The plasmid pTH99 contains Δ*gicR::spc*. pTH99 was transformed into *B. subtilis* and spectinomycin-resistant transformants were selected. Transformants were screened for chloramphenicol sensitivity to identify those in which Δ*gicR::spc* had recombined by double crossover.

**Plasmids.** Plasmids used are listed in Table 5-2. Plasmid construction is described in Table 5-2.

**Media.** Defined minimal medium containing S7 salts was prepared as described (Vasantha and Freese, 1980), except that MOPS buffer was present at 50 mM (Jaacks et al., 1989). Defined
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRN216</td>
<td>amy::(rapC-lacZ Ω1 neo)</td>
</tr>
<tr>
<td>IRN235</td>
<td>amy::(phrC-lacZ Ω2 neo)</td>
</tr>
<tr>
<td>JH642</td>
<td>trpC2 pheA1</td>
</tr>
<tr>
<td>JMS751</td>
<td>ΔphrC::erm</td>
</tr>
<tr>
<td>TMH144</td>
<td>amy::(srfA-lacZ Ω374 neo)</td>
</tr>
<tr>
<td>TMH145</td>
<td>pTH2 amy::(srfA-lacZ Ω374 neo)</td>
</tr>
<tr>
<td>TMH146</td>
<td>pH13 amy::(srfA-lacZ Ω374 neo)</td>
</tr>
<tr>
<td>TMH149</td>
<td>ΔcomQ::spc amy::(srfA-lacZ Ω374 neo)</td>
</tr>
<tr>
<td>TMH280</td>
<td>amy::(srfA-lacZ Ω682 neo)</td>
</tr>
<tr>
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<td>ΔcomQ::spc amy::(srfA-lacZ Ω682 neo)</td>
</tr>
<tr>
<td>TMH283</td>
<td>spo0H::cat amy::(srfA-lacZ Ω682 neo)</td>
</tr>
<tr>
<td>TMH333</td>
<td>sigB::cat dal amy::(srfA-lacZ Ω682 neo)</td>
</tr>
<tr>
<td>TMH335</td>
<td>ΔphrC::erm amy::(srfA-lacZ Ω682 neo)</td>
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<td>TMH500</td>
<td>amy::(comQX-lacZ Ω500 neo)</td>
</tr>
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<td>TMH545</td>
<td>ΔptsG::tet amy::(srfA-lacZ Ω682 neo)</td>
</tr>
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<td>TMH589</td>
<td>glcR::spc amy::(srfA-lacZ Ω682 neo)</td>
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<td>TMH598</td>
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<td>TMH617</td>
<td>ΔcomQ::spc Δspo0H::cat amy::(srfA-lacZ Ω682 neo)</td>
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<td>PHR234</td>
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<td>ptsG150 (ups150) amy::(srfA-lacZ Ω682 neo)</td>
</tr>
<tr>
<td>PHR319</td>
<td>glcT157 (ups157) amy::(srfA-lacZ Ω682 neo)</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------</td>
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<tr>
<td>PHR320</td>
<td>glcR164 (ups164) amy::(srfA-lacZ O682 neo)</td>
</tr>
<tr>
<td>PHR321</td>
<td>glcT144 (ups144) amy::(srfA-lacZ O682 neo)</td>
</tr>
<tr>
<td>PHR344</td>
<td>ptsG150 (ups150) amy::cat</td>
</tr>
<tr>
<td>PHR352</td>
<td>ΔcomQ::spc ptsG150 (ups150) amy::(srfA-lacZ O682 neo)</td>
</tr>
<tr>
<td>PHR356</td>
<td>ΔphrC::erm ptsG150 (ups150) amy::(srfA-lacZ O682 neo)</td>
</tr>
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<td>PHR424</td>
<td>ptsG150 (ups150) amy::(rapC-lacZ Ω1 neo)</td>
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<tr>
<td>PHR429</td>
<td>ptsG150 (ups150) amy::(phrC-lacZ Ω2 neo)</td>
</tr>
<tr>
<td>PHR447</td>
<td>Δspo0H::cat ptsG150 (ups150) amy::(srfA-lacZ O682 neo)</td>
</tr>
<tr>
<td>PHR451</td>
<td>ΔcomQ::spc Δspo0H::cat ptsG150 (ups150) amy::(srfA-lacZ O682 neo)</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
</tr>
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</tr>
<tr>
<td>pDG1513</td>
<td>amp, tet, source of tetracycline resistance cassette</td>
</tr>
<tr>
<td>pDG795</td>
<td>amp, cat, used to clone DNA into thr locus of <em>B. subtilis</em></td>
</tr>
<tr>
<td>pGEMcat</td>
<td>amp, cat, integrative vector</td>
</tr>
<tr>
<td>pH13</td>
<td>cat, mls, multicopy vector</td>
</tr>
<tr>
<td>pJPM8</td>
<td>amp, mls, source of erythromycin resistance cassette</td>
</tr>
<tr>
<td>pKB1</td>
<td>amp, cat, pDG795 containing thr: degQ comQX comP'</td>
</tr>
<tr>
<td>pNG11</td>
<td>amp, pGEMcat containing from 110 bp upstream of the 3' end of degQ to 111 bp into comX</td>
</tr>
<tr>
<td>pNG15</td>
<td>amp, cat, pGEMcat containing from 100 bp upstream of the 3' end of degQ to 111 bp into comX</td>
</tr>
<tr>
<td>pNG17</td>
<td>cat, mls, pH13 containing from 110 bp upstream of the 3' end of degQ to 111 bp into comX</td>
</tr>
<tr>
<td>pTH1</td>
<td>cat, mls, pH13 containing the 3' end of comQ, all of comX, and most of comP created by deleting the 1.6 kb Sacl-XbaI fragment containing the 3' end of comP, all of comA, and the 5' end of orfII from pNG12</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pTH2</td>
<td>cat, mls, PHP13 containing the 3' end of <em>degQ</em>, all of <em>comQ</em>, all of <em>comX</em>, and most of <em>comP</em>&lt;br&gt;created by cloning the 1383 bp HindIII-HindIII fragment of pTH1 into the HindIII site of pNG17</td>
</tr>
<tr>
<td>pTH8</td>
<td>amp, cat, pGEMcat containing from 110 bp upstream of the 3' end of <em>degQ</em> to 281 bp into <em>comQ</em>&lt;br&gt;created by cloning the 579 bp MfeI-AccI fragment of pNG11 into pGEMcat</td>
</tr>
<tr>
<td>pTH15</td>
<td>amp, cat, pGEMcat containing ~800 bp upstream of the 3' end of <em>pncB</em> to 281 bp into <em>comQ</em>&lt;br&gt;created by walking to the SphI site upstream of the insert in pTH8</td>
</tr>
<tr>
<td>pTH28</td>
<td>amp, cat, pGEMcat containing ~800 bp internal to <em>pncB</em>&lt;br&gt;created by subcloning an 800 bp fragment (BamHI-SphI) from pTH15 into pGEMcat</td>
</tr>
<tr>
<td>pTH32</td>
<td>amp, cat, pGEMcat containing ~1600 bp internal to <em>pncAB</em>&lt;br&gt;created by walking to the SacI site upstream of the pTH28 insert.</td>
</tr>
<tr>
<td>PTH33</td>
<td>amp, cat, pGEMcat containing ~2800 bp extending from upstream of <em>pncA</em> to just before the 3' end of <em>pncB</em>&lt;br&gt;created by walking to the KpnI site upstream of the pTH28 insert</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pTH38</td>
<td>amp, cat, pGEMcat containing ~1300 bp extending from ~800 bp upstream of the 3’ end of <em>pncB</em> to ~360 bp into <em>yuxH</em></td>
</tr>
<tr>
<td>pTH43</td>
<td>amp, cat, pGEMcat containing ~2000 bp extending from ~500 bp before the 3’ end of <em>pncB</em> to ~1500 bp downstream of the 3’ end of <em>pncB</em></td>
</tr>
<tr>
<td>pTH60</td>
<td>amp, neo, pKS2 containing from 110 bp upstream of the 3’ end of <em>degQ</em> to 112 bp into <em>comX</em></td>
</tr>
<tr>
<td>pTH68</td>
<td>amp, cat, pGEMcat containing from 94 bp upstream of <em>ptsH</em> to 31 bp into <em>ptsI</em></td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pTH69</td>
<td>amp, cat, pGEMcat containing a fragment extending from 26 bp upstream of <em>ptsG</em> (downstream of the <em>ptsG</em> promoter) to 31 bp into <em>ptsI</em> created by walking to the <em>SpeI</em> site upstream of the insert in pTH68</td>
</tr>
<tr>
<td>pTH76</td>
<td>amp, cat, tet, pGEMcat containing Δ<em>ptsG::tet</em> created by cloning the tetracycline resistance cassette (StuI-NaeI) from pDG1513 into pTH69 from which 1102 bp of <em>ptsG</em> (NdeI-RsrII) had been deleted</td>
</tr>
<tr>
<td>pTH87</td>
<td>amp, cat, pGEMcat containing the 3' end of <em>ykvZ</em> and all of <em>glcT</em> created by cloning an 1165 EcoRI-EcoRI fragment of a PCR product into the EcoRI site of pGEMcat</td>
</tr>
<tr>
<td>pTH94</td>
<td>amp, cat, pGEMcat containing from 230 bp downstream of the 5' end of <em>glcR</em> (in <em>ywpJ</em>) to 123 bp downstream of <em>glcR</em> (in <em>ywpH</em>) created by cloning the 1128 bp THP42-THP43 PCR product into the HincII site of pGEMcat THP43 is a primer (5' GCTGATTGACACCCCTGCT 3') that amplifies from 230 bp upstream of <em>glcR</em> (in <em>ywpJ</em>) THP42 is a primer (5' CCACCACCTTCAATGCCGT 3') that amplifies to 123 bp downstream of <em>glcR</em> (in <em>ywpH</em>)</td>
</tr>
<tr>
<td>pTH99</td>
<td>amp, cat, spc, pGEMcat containing <em>glcR::spc</em> created by cloning the 1.5 kb spectinomycin cassette (NdeI-BamHI) from pUS19 into the EcoR47III site 162 bp downstream of the start of <em>glcR</em></td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>pTH102</td>
<td>amp, cat, MLS, pGEMcat containing ΔglcT::erm created by cloning the erythromycin cassette (EcoRI-HindIII) of pJPM8 into pTH87 from which 487 bp of glcT (starting at 28 bp downstream of the start of glcT) had been deleted (PpuMI-BspEI)</td>
</tr>
<tr>
<td>pUS19</td>
<td>amp, cat, spc, pUC19 containing a 1.1 kb ClaI-NdeI spectinomycin resistance cassette in the NarI-NdeI sites of pUC19</td>
</tr>
</tbody>
</table>
minimal medium contained required amino acids (40 μg/ml), glutamate (0.1%), and glucose (1%), glycerol (1%), or succinate (1%). The hunt for mutants with altered expression of srfA-lacZ was done on SpII competence media (Dubnau and Davidoff-Abelson, 1971). All plate work was done on media solidified with 15 g of agar per liter.

Supplements were used at the following concentrations: Chloramphenicol at 5 μg/ml, neomycin 5 μg/ml, MLS at 0.5 μg/ml erythromycin and 12.5 μg/ml lincomycin, spectinomycin at 100 μg/ml, tetracycline at 12.5 μg/ml, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) at 30 μg/ml.

**Isolation of mutants with altered expression of srfA-lacZ** Ethyl methanesulfonate (EMS) mutagenized cultures of strain TMH280 were generated as essentially described (Harwood and Cutting, 1990). After outgrowth, mutagenized cultures were frozen at −80°C. To screen for changes in the expression of srfA-lacZ mutagenized cultures were diluted and plated at low colony density (<150 colonies per plate) on SpII containing 30 μg/ml Xgal and incubated overnight at 30°C.

EMS-induced mutations were backcrossed into TMH333 by congression with dal⁺. Briefly, chromosomal DNA from each mutant was transformed into TMH333. dal⁺ transformants were selected on LB and screened for chloramphenicol sensitivity (loss of sigB::cat) and altered expression of srfA-lacZ on SpII containing 30 μg/ml Xgal.

**Plate assay for ComX pheromone production** The ability to produce extracellular ComX pheromone was assayed on indicator plates consisting of SpII medium (Dubnau and Davidoff-Abelson, 1971) containing 30 μg/ml Xgal and solidified with 15 ml agar. The indicator strain (TMH281) was suspended in 1X TSS salts (Harwood and Cutting, 1990) containing 1 mM MgSO₄ (1 colony per ml) and then plated on pre-warmed indicator plates (100 μl per plate). Plates were incubated at 37°C for approximately 30 minutes before mutants were patched on them. Plates were then incubated at 37°C for 16-18 hours. Since extracellular ComX pheromone can stimulate the expression of srfA-lacZ in TMH281, extracellular ComX pheromone activity was indicated by the development of a blue halo around the patch of mutant cells. TMH280 served as a positive
control and TMH281 as a negative control. Up to 8 strains could be patched onto each plate without evident cross talk between strains.

**β-galactosidase assays** β-galactosidase was expressed from the following fusions: amy::(srfA-lacZ ∆374 neo) (Solomon et al., 1995), amy::(srfA-lacZ ∆682 neo), amy::(rapA-lacZ erm) (Mueller et al., 1992), amy::(rapC-lacZ ∆1 neo) (Lazazzera et al., 1999b), amy::(phrC-lacZ ∆2 neo) (Lazazzera et al., 1999b), or amy::(comQX-lacZ ∆500 neo). amy::(srfA-lacZ ∆682) neo is exactly the same fusion as amy::(srfA-lacZ ∆374 neo), except that it is integrated into the chromosome by a double crossover (JMS and ADG, unpublished data).

The transcriptional amy::(comQX-lacZ ∆500 neo) fusion was constructed by cloning a comQX fragment upstream of lacZ and integrating the fusion into the B. subtilis chromosome at amyE. The comQX fragment extended from 298 bp upstream of the start codon of comQ (in the 5' end of degQ) to 112 bp downstream of the start codon of comX. pTH60, containing the amy::(comQX-lacZ ∆500 neo) fusion, was transformed into JH642. Neomycin-resistant transformants were screened for loss of amylase activity as described (Harwood and Cutting, 1990) to identify transformants in which the fusion had integrated by double crossover.

β-galactosidase specific activity was measured essentially as described (Miller, 1972; Magnuson et al., 1994) and is presented as (ΔA_{420} per min per ml culture per OD_{600}) x 1000.

**Mapping of ups mutations** ups144, ups150, ups157 and ups164 mutations were mapped relative to known loci using PBS1 generalized transduction essentially as described (Harwood and Cutting, 1990). ups159 was found to be ~40% linked to comAB by cotransformation, in an area of the chromosome ~4 kb upstream of comQ. Fine mapping was done by plasmid rescue and complementation. glcT144 (ups144) and glcT157 (ups157) were complemented by pTH87, which contains the 3' end of ykvZ and all of glcT, indicating that these mutations are in glcT. ptsG150 (ups150) was rescued (frequency = 23%, 22/96 transformants) by pTH69, which contains the region upstream of ptsG (lacking the promoter), all of ptsG, all of ptsH, and the 5' end of ptsL. ptsG150 (ups150) was not rescued by pTH68, which contains all of ptsH and the 5' end of ptsL. Taken together these two pieces of data indicate that ptsG150
(ups150) is a mutation in ptsG. glcR164(ups164) was complemented by pTH94 which contains the 3' end of ywpH, all of glcR, and the 5' end of ywpJ.

pncB159 (ups159) was mapped to pncB in several steps. First, it was found to be ~40% linked to comAB::spc by co-transformation. A three-factor co-transformation cross gave the gene order ups159 comQ comAB and suggested that the ups159 mutation was approximately 4 kb upstream of comQ. pTH15, which contains a DNA fragment that starts within the pncB open reading frame and ends in the comQ reading frame did not rescue the ups159 mutation. This suggested that the ups159 mutation lay in pncB or an upstream gene. Another three-factor cross indicated that the ups mutation was just upstream of the end of this plasmid, most likely within the pncAB operon. Integration of either of two plasmids (pTH28 or pTH32) containing DNA internal to the pncAB operon caused an ups phenotype, while integration of either of two plasmids (pTH33 or pTH43) containing DNA extending beyond either end of the operon did not. This demonstrated that a pnc mutation causes an ups phenotype and suggest that ups159 is in the pncAB operon. Since pTH33 contains all of pncA yet did not complement pncB159 (ups159), I concluded that pncB159 (ups159) is a mutation in pncB. I was unable to clone the entire pncB locus in E. coli, presumably because high levels of PncB are toxic. Therefore, I was unable to test for complementation of the pncB159 (ups159) mutation.

**Preparation of conditioned medium** Conditioned medium was prepared essentially as described (Grossman and Losick, 1988; Magnuson et al., 1994). Cells were grown in defined minimal medium and cultures harvested at an OD600 of 2.5 – 3.0 for the preparation of partially purified ComX pheromone for use in factor response assays and at various time points during growth for construction of factor production time courses. After harvest, cells were removed by centrifugation and filter sterilized. Conditioned media was stored at 4°C until use.

**Partial purification of ComX pheromone** ComX pheromone was partially purified by passage over a Sep-pak C-18 cartridge (Waters). 90 ml of conditioned medium from strain JH642 was combined with 22.5 ml acetonitrile (ACN) and 113 µl trifluoracetic acid (TFA) to a final concentration of 20% ACN and 0.1% TFA and then centrifuged. Conditioned medium at
20% ACN 0.1% TFA was applied to a 0.3g Sep-pak C-18 cartridge (Waters) equilibrated with 30 ml 80% ACN 0.1% TFA followed by 10 ml 20% ACN 0.1% TFA. After sample application the column was washed with 5 ml 20% ACN 0.1% TFA. A step-elution with 5 ml volumes was done to collect material eluting at 40% ACN 0.1% TFA, 60% ACN 0.1% TFA, and 80% ACN 0.1% TFA. Samples were split in thirds and dried in a speed-vac in low binding micro centrifuge tubes (Marsh). Dried samples were stored at -20°C until use. Samples were resuspended in defined minimal medium containing 100 μg/ml bovine serum albumin (BSA) and combined. Samples were then re- aliquoted and stored at -20°C until use in response assays.

Separation of CSF and ComX pheromone  CSF and ComX pheromone were partially purified (and separated from each other) by passage over Sep-pak C-18 cartridges essentially as described (Lazazzera et al., 1999b). 7 ml of conditioned medium was adjusted to pH 2 with TFA and 5 ml applied to a 0.3 g Sep-pak C-18 cartridge that had been equilibrated with 30 ml 80% ACN 0.1% TFA and 10 ml aqueous 0.1% TFA. After sample application, the column was washed with 3 ml aqueous 0.1% TFA. CSF was eluted with 3 ml 11% ACN 0.1% TFA. ComX pheromone was eluted with 3 ml 80% ACN 0.1% TFA. Samples were dried in a speed vac, stored at -20°C until use, and resuspended in 500 μl minimal medium containing 100 μg/ml BSA. Resuspended samples were either used immediately or stored at -20°C. Before being used in CSF and ComX pheromone factor assays a series of 1:2 serial dilutions into fresh medium containing 100 μg/ml BSA was prepared.

CSF and ComX pheromone factor assays  The activity of CSF and ComX pheromone was assayed essentially as described previously (Magnuson et al., 1994; Solomon et al., 1996). Cells containing a srfA-lacZ fusion were grown to low cell density and 250 μl of culture was mixed with 250 μl of sample. For CSF assays the indicator strain contained a phrC mutation to eliminate CSF production and cells were grown to an OD 
600 of ~0.1. For ComX pheromone assays the indicator strain contained a comQ mutation to eliminate ComX pheromone production and cells were grown to an OD 
600 of ~0.25. After mixing samples were incubated at 37°C for 70 minutes and frozen at -20°C until being assayed for β-galactosidase activity.
Time course of ComX pheromone production in a phrC mutant Conditioned medium from JMS751 was harvested at various time points throughout growth in minimal medium, prepared, and assayed for srfA-inducing activity as described above. The activity in a sample in which the indicator strain (TMH149) was mixed with fresh medium containing 100 μg/BSA was subtracted from each sample. The remaining (induced) activity in each sample was plotted against the volume of partially purified ComX pheromone in each sample. A point in the linear portion of the curve was chosen to calculate the units of srfA-inducing activity per ml conditioned medium (Miller units induced per ml CM). This number was expressed as a percentage of the maximum activity per ml obtained in the experiment and this relative ComX pheromone activity plotted versus time to generate the curve shown in Figure 2-2.

Time course of CSF production wild type cells and ups mutants Conditioned medium was harvested at various time points throughout growth in minimal medium, prepared, fractionated, and assayed for srfA-inducing activity as described above. CSF production time-courses were prepared essentially as described (Lazazzera et al., 1999b).

CSF response curves CSF response curves were constructed essentially as described (Solomon et al., 1996), except response was quantified in terms of fold increase in srfA-lacZ expression in the presence of (partially purified) CSF as compared to in the absence of CSF. Briefly, for each strain to be tested, a series of 1:2 serial dilutions of synthetic CSF (Solomon et al., 1996) (in minimal medium containing 100 μg/ml BSA) containing from 97 pM to 100 nM CSF and a sample of minimal medium were mixed 1:1 with the strain to be tested as described above for CSF factor assays, β-galactosidase activity was determined, and the fold response to CSF (specific activity in the presence of CSF/specific activity in the absence of CSF) plotted versus concentration of CSF.

ComX pheromone response curves ComX pheromone response curves were constructed in a manner analogous to the way in which CSF response curves were generated. Briefly, for each strain to be tested, a series of 1:2 serial dilutions of partially purified ComX pheromone (in minimal medium containing 100 μg/ml BSA) containing 4 μl to 250 μl of the
ComX pheromone preparation and a sample of minimal medium were mixed 1:1 with the strain to be tested as described above for ComX pheromone factor assays, β-galactosidase activity was determined, and the fold response to partially purified ComX pheromone (specific activity in the presence of partially purified ComX pheromone/specific activity in the absence of partially-purified ComX pheromone) plotted versus relative ComX pheromone concentration.
References


144


