Characterization of a Competence Pheromone in *Bacillus subtilis*

by

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Abstract

Under permissive nutrient conditions, competence in *Bacillus subtilis* is induced by a cell density signal. This density signal is mediated by the accumulation of two genetically and biochemically distinct peptide factors that I call competence pheromone and competence stimulating factor (CSF).

Two genes, *comQ* and *comX* are required for the production of competence pheromone. Competence pheromone is a modified 9 or 10 amino acid peptide derived from the C-terminus of the deduced 55 amino acid product of *comX*. ComQ is required for the production of active competence pheromone and may be involved in the modification or proteolytic processing of the (deduced) pre-pro-pheromone.

The pleiotropic regulatory genes *spo0H* and *spo0A* are required for the production of CSF. CSF appears to be a small peptide of approximately 5 to 7 amino acids. Both CSF and pheromone are required for the development of competence.

The *spo0K* oligopeptide permease and the *comP*/*comA* two-component regulatory system are required for the response to the density signals, but not for the production of those signals. Jonathan Solomon has shown that Spo0K is required specifically for the response to CSF and that ComP is required specifically for the response to the competence pheromone.

These two signaling pathways converge to induce expression of *srfA*, a locus that is required for the production of surfactin, the development of competence and for efficient sporulation.

Density signals in general may be important for the coordination of social strategies. Competence-inducing density signals may also play a role in speciation and in the selection of sexual partners.

Thesis Supervisor: Dr. Alan D. Grossman
Title: Associate Professor of Biology

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List of Abbreviations:

Abbreviation:	**Term:**
Ab.	absorbance
Bgal. S.A.	ß-galactosidase Specific Activity
BGSC	Bacillus Genetic Stock Center
CM	Conditioned Medium
CSF	competence stimulating factor
MM	minimal media
O.D.	optical density
S.A.	specific activity
Tnf.	transformants
TFA	triflouroacetic acid

Acknowlegements

I especially thank Alan Grossman, without whom this work would not have been possible; my fellow students, who freely shared their time and their ideas; and Rich Losick whose unrelenting enthusiam, like the proverbial light at the end of the tunnel, has guided me through some dark moments in this project.

Much of the following material has been published (Magnuson et al., 1994); and additional material presented here has been submitted for publication (Magnuson et al., 1995; Solomon et al., 1995). In the work on CSF, Jonathan Solomon was especially instrumental in emphasizing the rescuability of *spo0H*, and thus in redirecting attention to the role of the *spo0* genes in the production of extracellular factors. Jonathan was also the first to show extracellular effects on expression of *comG*. Finally, Jonathan's work on the sensing of extracellular factors has been crucial to the development of the models presented here.

I am indebted to Paul Matsudaira for his advice and for the use of his HPLC and mass spectrometry equipment; to Jack Prior and C. Cooney and others for their help with fermentations; to K. Jaacks Siranosian, David Rudner, and Nereus Gunther for excellent technical assistance; to Joseph Zaia and K. Biemann of the MIT Mass Spectrometry Facility for additional mass spectral data, and to R. Cook and other members of the MIT Biopolymers lab for advice, peptide sequencing and amino acid analysis.

Chapter 1:
Introduction to Genetic Competence in *Bacillus subtilis*

In this thesis, I explore a very simple developmental event: the development of competence in the gram-positive soil bacterium *Bacillus subtilis*. Competence (for genetic transformation) is perhaps best thought of as a form of sex that, by permitting the exchange of genetic material, favors the long term survival of the organism. Since *Bacillus subtilis* is not always competent, it is possible to explore competence as a developmental event; to ask when and how *Bacillus subtilis* becomes competent. In this work, I present evidence that density signals initiate the development of competence, and I investigate the production of, the nature of, and the sensing of these density signals. I describe how a simple density signal can give rise to temporal patterns of gene expression. Finally, I discuss the utility of density signals in the coordination of social strategies and the possible role of competence-inducing density signals in the selection of sexual partners, and thus, on the maintenance and creation of bacterial species.

Discovery of Competence

Genetic markers can be transferred from one strain of bacteria to another by natural transformation. Although it is not ubiquitous, natural transformation occurs in more than 20 phylogenetically diverse genera (Wojciechowski, 1992). Transformation was first described in *Streptococcus pneumoniae* (previously called *Pneumococcus* or *Diplococcus pneumoniae*) (Griffith, 1928). This gram-positive organism is the most common cause of bacterial pneumonia. The capsule of this bacterium is an important pathogenic and immunogenic determinant, both in man and in mouse models. Griffith was studying the variability and stability of capsular types in

Streptococcus pneumoniae when he discovered that avirulent, R form (rough colony) bacteria could be "transformed" into a virulent, S form (smooth colony) bacteria when mixed, in the mouse, with (heat killed) S-form bacteria (Griffith, 1928). This effect was reproduced *in vitro* (without the mouse) (Dawson and Sia, 1931) and with extracts of S form bacteria (Alloway, 1932). The ability to detect the presence or absence of genetic information in cell extracts permitted an investigation into the chemical nature of genetic information. The purification and identification of this "transforming principle" as DNA (Avery et al., 1944; McCarty and Avery, 1946a; McCarty and Avery, 1946b) is one of the seminal events in the history of molecular biology.

Regulation of Competence

Streptococcus pneumoniae is "competent" for transformation for only a brief period in mid-exponential growth. The dramatic and well-coordinated development of competence in *Streptococcus pneumoniae* is mediated by an (uncharacterized) extracellular competence factor (Tomasz and Hotchkiss, 1964). Some non-competent strains of *Streptococcus sanguis* are made competent by the extracellular factors produced by closely related competent strains (Pakula and Walczak, 1963). Similar extracellular effects on competence are observed in other gram-positive bacteria such as *Bacillus subtilis* (Akrigg and Ayad, 1970; Akrigg et al., 1967; Charpak and Dedonder, 1965; Joenje et al., 1972; Magnuson et al., 1994), *Bacillus stearothermophilus* (Streips and Young, 1971), and *Bacillus cereus* (Felkner and Wyss, 1964).

The development of competence in the gram-negative bacterium *Haemophilus influenzae* is induced by internal (and external) concentrations of cAMP and occurs primarily in stationary phase (Barnhart, 1967; Chandler, 1992; Dorocicz et al., 1993). *Haemophilus influenza* selectively takes up DNA containing a certain 11 base pair sequence that is primarily found in the

genome of *Haemophilus influenzae* (Danner et al., 1980). Similarly, the constitutively competent gram-negative organism *Neisseria gonnorhoeae* selectively takes up DNA containing a certain 10 base pair sequence (Goodman and Scocca, 1988). In contrast to these gram-negative bacteria, competent gram-positive bacteria, such as *Bacillus subtilis* and *Streptococcus pneumoniae*, take up DNA nonselectively. In all cases, segments of DNA homology are required for efficient incorporation into the chromosome.

Possible Functions of Competence

Three hypotheses, ascribing three different functions to competence, have been proposed to explain the existence of competence. These hypotheses are not mutually exclusive. A combination of explanations might apply in any particular case; and no single explanation need apply in all cases.

The first hypothesis is that competence is essentially a feeding behavior, and that genetic transformation is an accidental byproduct of this feeding. However, since the uptake of DNA is species specific in some cases, and since competence only occurs in a subfraction of the population in other cases, it seems unlikely that competence is a general feeding strategy, although it could conceivably be a specialized type of autophagy (Hudson and Michod, 1992).

The second hypothesis is that competence is a mechanism of DNA repair (Bernstein et al., 1985). This hypothesis is consistent with the fact that the UV resistance of competent *Bacillus subtilis* is enhanced by the ability of the cells to take up and be transformed by exogenous DNA (Michod et al., 1988). It nonetheless seems unlikely that competence is an acute response to DNA damage, as there is no evidence in any species that DNA damage is necessary or sufficient to induce the development of competence. However, the development of competence does give cells the opportunity to correct

mutations caused by errors in the repair or replication of DNA. In this nonacute form, the DNA repair hypothesis begins to resemble the third hypothesis.

The third hypothesis is that competence is a form of genetic exchange. Genetic exchange, by promoting the recombination and reassortment of alleles in a population, leads to greater genetic diversity. Without genetic exchange, eight mutations occurring among the various progeny of a single bacterium, yields only eight new genotypes, or nine genotypes in all. With genetic exchange, 2^8 or 256 genotypes can be generated. Thus, genetic exchange generates a large, combinatorial increase in genetic diversity. Genetic exchange also permits each genetic variant to be judged on its own merits, without regard to the particular background in which it arose, and without competing with favorable mutations that arose in other lines. Thus, sex results in an increased efficiency in selecting for beneficial mutations (Fisher, 1930; Muller, 1932) and in an increased efficiency in selecting against mildly deleterious mutations (Muller, 1964). These evolutionary effects of sex are beneficial to the long term survival of an organism. Refinements and simulations of this basic model (reviewed by Felsenstein, 1985) indicate that sexuality may be especially advantageous in the face of varying environmental conditions or in the face of the varying challenges posed by innovative competitors, parasites and predators (Bell, 1987; Bremermann, 1980; Haldane, 1937; Hamilton, 1980; Hamilton, 1983; Jaenike, 1978; Levin, 1975; Rice, 1983; Sturtevant and Mather, 1938; van Valen, 1973).

The supposed advantages of sex have been tested by mathematical simulation. These models might also be tested by head to head competition between competent bacteria and isogenic noncompetent mutants in a chemostat or in serial culture. The short generation time of bacteria, the

dispensability of competence, and the independence of competence and reproduction, might make bacteria a good system in which to test the evolutionary consequences of genetic exchange.

Evidence of genetic exchange in competent bacteria

If the function of competence is to promote genetic exchange, then evidence of such genetic exchange should exist in the wild in populations of transformable bacteria. For the most part, this appears to be the case. It is perhaps a poorly appreciated fact that cultures of competent bacteria may contain free DNA; and thus, that genetic exhange can take place without the addition of purified DNA. In fact, genetic exchange is observed in test mixtures of *Bacillus subtilis* in liquid culture (Ephrati-Elizur, 1968) and in soil (Graham and Istock, 1979). The exchange (in the wild) of medically significant determinants for resistance to penicillin and for capsular serotype is documented in clinical isolates of *Streptococcus pneumoniae* (Coffey et al., 1991). In a less anecdotal manner, the statistical association or lack of association between (neutral) genetic variants at different loci can be used to measure genetic exchange (sex) within a population. This measure is stringent, but imperfect, since evidence of sexuality can be obscured by the differential success of certain variants or by the inclusion of geographically (and therefore sexually) isolated populations. *Haemophilus influenzae*, although transformable, is not highly sexual by this measure. However, other competent species such as *Bacillus subtilis*, *Neisseria gonnorhoeae,* and *Neisseria meningiditis* are highly sexual by this measure and various noncompetent species, such as *Escherichia coli* and *Salmonella* species, are not sexual by this measure (Istock et al., 1992; Smith et al., 1993; Vazquez et al., 1993). Thus, there appears to be a fair correlation between transformability in the lab and genetic exchange in the wild. The existence of

efficient genetic exchange in certain bacteria suggests that these bacteria qualify as true species. (A species is often defined as a group of related individuals that exchange genetic information). It will be interesting to learn more about what promotes and what limits the genetic flux between microbes. The genetic exchange observed in the wild in transformable species is presumed to be mediated by transformation, although in no case has this been absolutely proved.

Mechanism of Competence

In minimal glucose medium, a sub-population of cells in a culture of *Bacillus subtilis* differentiate to become competent. Competent cells are metabolically less active (Dooley et al., 1971) and more buoyant than the bulk of the non-competent cells (Cahn and Fox, 1968). The development of competence requires a network of regulatory genes and results in the production of specialized proteins that bind and take up DNA independent of nucleotide sequence. Competent *Bacillus subtilis* cells bind and cut double-stranded DNA at the surface of the cell. It appears that one strand of the DNA enters the cell while the complementary strand is degraded (Figure 1). The single-stranded DNA within the cell recombines efficiently with homologous sequences to yield stable recombinant or transformed cells. The average size of an incorporated fragment is about 10,000 bases, but an individual fragment may be much larger or much smaller (Fornili and Fox, 1977), (reviewed by Smith and Danner, 1981).

The *comG* operon encodes proteins that may be directly involved in the uptake of DNA (Albano and Dubnau, 1989). Several of these proteins are

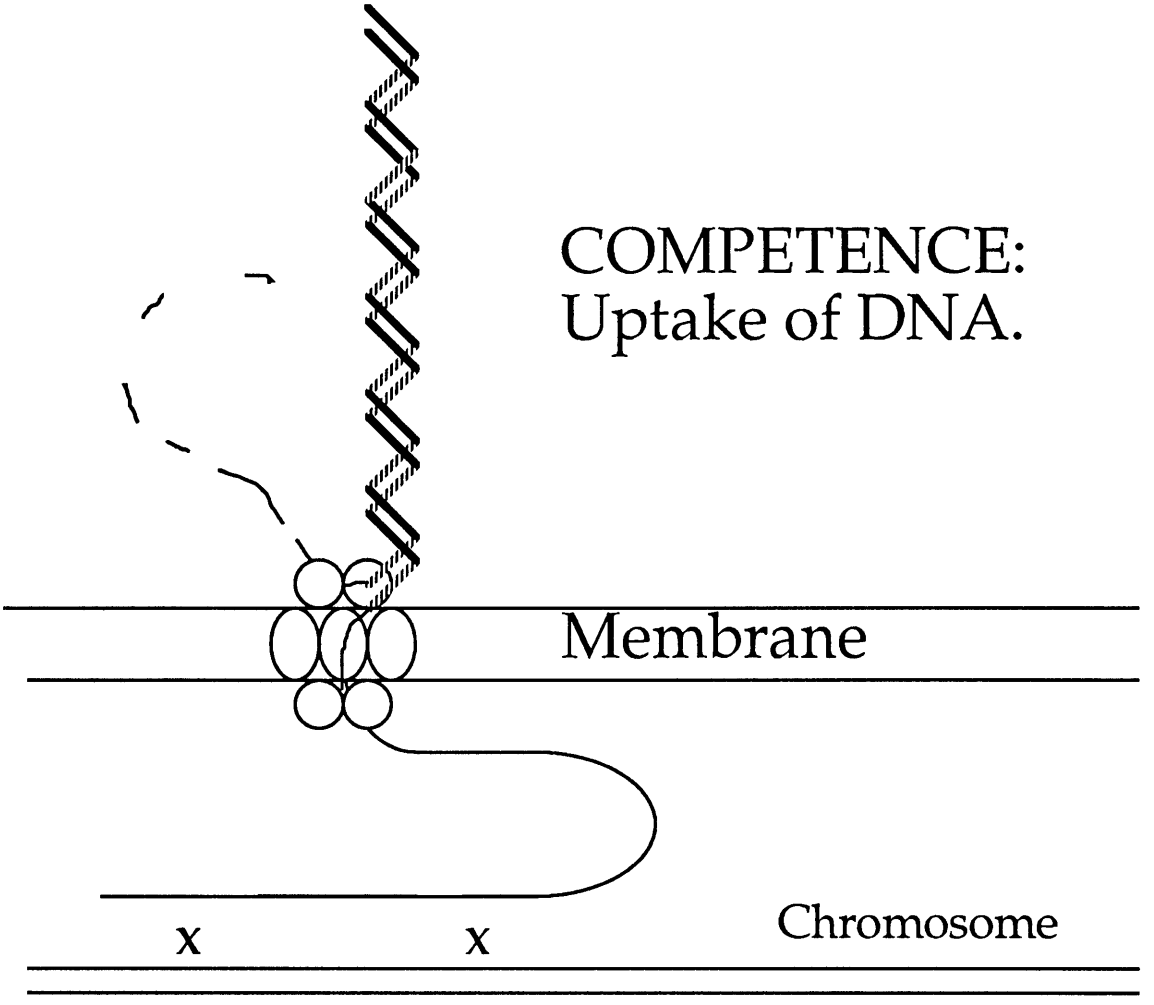


Figure 1. Model of transformation in *Bacillus subtilis*. Double-stranded DNA is bound and cut at the surface of the cell. As DNA is taken up, it is protected from the action of exogenously added DNase. The products of *comG, comC, comDE, comF, comM, comN* and *comO,* represented here by a generic multisubunit complex, may be directly involved in taking up DNA (reviewed by Dubnau, 1993). Uptake of one strand appears to be associated with the degradation of the complementary strand. DNA that has just been taken up is in a single-stranded or "eclipse" form. This ssDNA recombines efficiently with homologous DNA (reviewed by Smith and Danner, 1981).

similar to pilin proteins that are required for pilin formation and for competence in *Neisseria gonorrhoeae* (Seifert et al., 1990). Thus, although the regulation of competence and the specificity of DNA uptake is different in gram-negative and gram-positive organisms, it seems that some of the basic machinery of competence is conserved. It is also tempting to speculate that the uptake of DNA during competence might be mechanistically similar to other DNA transfer processes, such as conjugation.

Regulation of competence in *Bacillus subtilis*

A temporal and regulatory sequence of gene expression controls the development of competence in *Bacillus subtilis* (Figure 2). Several regulatory genes, including *spo0K, comP, comA*, and *comQ*, are required for expression of *srfA* (Hahn and Dubnau, 1991; Magnuson et al., 1994; Nakano et al., 1991; Nakano and Zuber, 1989; Weinrauch et al., 1991). *srfA* is required, in turn, for expression of ComK (van Sinderen et al., 1990). ComK, a transcription factor, is required to increase its own expression and the expression of the late competence operons (*comC, E, F, G*) (van Sinderen et al., 1994). The products of these late competence operons may be directly involved in DNA uptake (reviewed by Dubnau, 1991).

Transcription of *srfA* and development of competence are greatly reduced in *spo0K*, *comP*, *comA*, and *comQ* mutants, but expression of *srfA* from an IPTG-inducible promoter bypasses the requirement of these genes for competence (Hahn and Dubnau, 1991; Nakano and Zuber, 1991). These results suggest that *srfA* is the major, and perhaps the only, competence-related target of Spo0K, ComP, ComA, and ComQ.

Transcription of *srfA* is also reduced in *spo0H* and *spo0A* mutants. However, the competence defect of these mutants is only partially restored by the ectopic expression of *srfA* from an IPTG-inducible promoter.

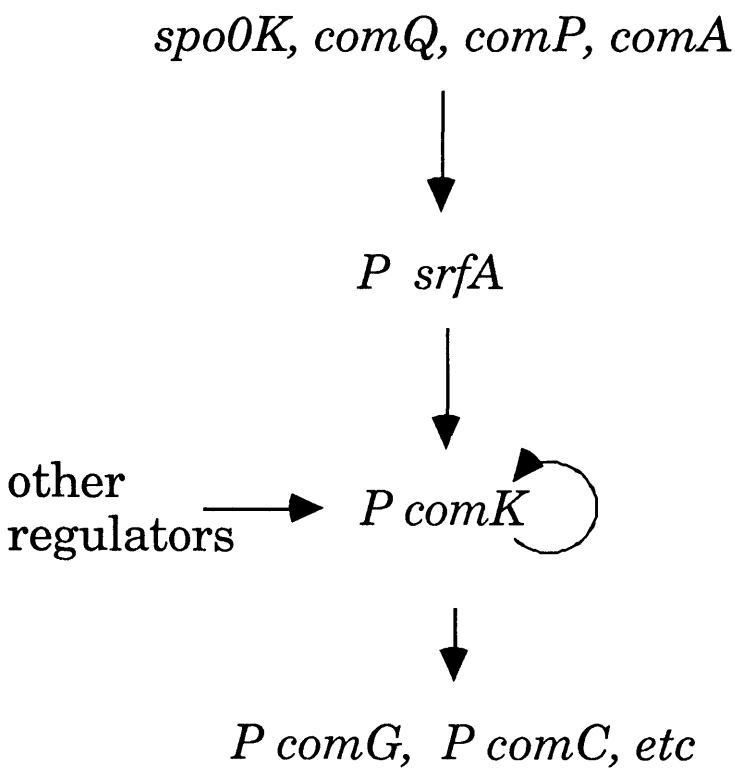


Figure 2. A transcriptional pathway regulates competence in *Bacillus subtilis*. The promoter, (P), is indicated for genes that are transcriptionally regulated. Spo0K, ComQ, ComP and ComA are required for the expression of *srfA* (Hahn and Dubnau, 1991; Nakano and Zuber, 1991). SrfA is required for the expression of *comK*. ComK is required in turn for its own expression and for the expression of *comG, comC* and other late competence genes (van Sinderen and Venema, 1994; van Sinderen et al., 1990), (reviewed by Dubnau, 1993).

Furthermore, the development of competence in *srfA spo0H* and *srfA spo0A* double mutants is more defective than in the single mutants, suggesting that *spo0A* and *spo0H* must affect another aspect of competence, in addition to affecting expression of *srfA* (Hahn and Dubnau, 1991). The product of *spo0A* is a transcription factor of the two-component family and is required for sporulation, for competence, and for the production of extracellular antibiotics and proteases (Ferrari et al., 1985; Kudoh et al., 1985). *spo0H* encodes a sigma factor, which modulates the promoter specificity of RNA polymerase and is required for the intiation of sporulation and for the development of comptence (Carter and Moran, 1986; Dubnau et al., 1988).

The DNA binding proteins encoded by *sin* and *degU* and *abrB* (other regulators, Figure 2) are not required for expression of *srfA* but are required for expression of *comK* (Hahn et al., 1994). Thus, these regulators appear to act on or before expression of *comK,* but after expression of *srfA*. The direct targets of these regulators have not been identified. Two negative regulators of competence, MecA and MecB, also appear to act between *srfA* and *comK*, as loss of function mutations in either *mecA* or *mecB* permit expression of *comK* in *srfA, spo0K, comP* and *comA* mutants (Roggiani et al., 1990). MecA and MecB might act by sequestering or proteolytically cleaving ComK (Kong and Dubnau, 1994; Msadek et al., 1994).

Signals for the development of competence in *Bacillus subtilis*

The development of competence and the expression of late competence genes in *Bacillus subtilis* requires glucose. These nutritional effects are bypassed by *mec* (medium-independent expression of competence) mutations or by other conditions leading to the ectopic expression of *comK* (reviewed by Dubnau, 1993). In permissive nutrient conditions, the development of competence is still temporally regulated, due to the requirement for cell density

signals. As shown in this thesis, these density signals regulate expression of *srfA* and, as a consequence, also regulate the expression of *comG* and the development of competence. This thesis also presents experiments concerning the number and chemical nature of these cell density signals and a genetic analysis of genes involved in the production and sensing of these cell density signals.

Even under optimal nutritional and cell density conditions, not all cells become competent. Competence develops in only a fraction of the population. Competent cells can be physically separated from the bulk of the noncompetent cells by virtue of their altered buoyant density (Cahn and Fox, 1968). *comK* and the late competence genes are expressed exclusively in the competent fraction of a bacterial culture. In contrast, expression of *srfA* is similar in both competent and noncompetent fractions (Hahn et al., 1994). Thus, although expression of *srfA* is necessary for expression of *comK*, it is not sufficient, since *srfA* appears to be expressed in all cells while *comK* is expressed in a fraction of the cells. Possibly, a second signal, present in some cells but not others, is required for the expression of *comK*. Alternatively, stochastic fluxations in the level of ComK, together with a positive feedback loop, may be sufficient to explain the fact that *comK* is expressed in only a fraction of the population.

ComK has a positive effect on its own expression (van Sinderen and Venema, 1994), thus, ComK may be a natural switch. Once some low, critical threshold level of ComK is reached, the positive autoregulatory effect may lead to full expression of *comK*. It is possible to imagine that expression of *srfA* might bring the level of ComK to this threshold, and that stochastic fluxuations might put ComK past this threshold in a fraction of the cells. It is not known how *comK* is turned off, or how cells return to a noncompetent state.

Functions of regulators of *srfA*

The *spo0K* operon encodes an oligopeptide permease (Perego et al., 1991; Rudner et al., 1991) that is required for competence, for sporulation, and for the transport of oligopeptides. The operon contains five genes. The five genes encode a ligand binding protein, two transmembrane proteins, and two ATPases (Rudner et al., 1991; Sadaie and Kada, 1983). Similar permeases are required for the opine induced conjugation of Ti plasmids in *Agrobacterium tumefaciens* (Valdivia et al., 1991; Zanker et al., 1992). Proteins homologous to the ligand binding protein of Spo0K are required for the binding and sensing of plasmid conjugation-inducing pheromones in *Enterococcus faecalis* (Ruhfel et al., 1993; Tanimoto et al., 1993) and for the development of competence in *Streptococcus pneumoniae* (Pearce et al., 1994).

The *comP* and *comA* gene products are members of the large family of two-component regulatory systems found in prokaryotes (Albright et al., 1989; Bourret et al., 1991), and recently in eukaryotes (Chang et al., 1993; Ota and Varshavsky, 1993) (Alex and Simon, 1994; Swanson and Simon, 1994). These two-component systems are involved in the regulation of diverse functions such as virulence, nitrogen assimilation, chemotaxis and adaptation to changes in osmolarity. ComP is homologous to histidine protein kinases (Weinrauch et al., 1990) that autophosphorylate on a histidine residue. ComA is homologous to response regulators (Nakano and Zuber, 1989; Weinrauch et al., 1989) and is phosphorylated, probably on an aspartate in the conserved N-terminal domain (Roggiani and Dubnau, 1993). By analogy, it is expected that ComP autophosphorylates and transfers its phosphate group to ComA, which then binds to DNA and activates transcription of *srfA* (Nakano et al., 1991; Roggiani and Dubnau, 1993) and several other genes not required for

competence (Msadek et al., 1991; Mueller et al., 1992). ComQ is required to generate the signal for ComP (Magnuson et al., 1994).

In the following chapters, I present the characterization of the *srfA* locus, and the evidence that *srfA* and competence are regulated by a density signal. I present the purification and genetic characterization of two peptides that mediate the density signal. Finally, I describe the genetic analysis of the cellular response to these two peptides.

Chapter 2:
Characterization of *srfA*, A Locus Required for the Development of Competence and for the Production of Surfactin

Abstract

The *csh293::*Tn*917lac* insertion is in the *srfA* operon. Insertions in the 5' end of *srfA* caused a defect in the production of surfactin and, under some conditions, a defect in sporulation. Insertions in the 3' end of this locus caused an additional defect in the development of competence. Sequence of regions upstream of the *csh293::*Tn*917lac* showed significant homology to subunits of gramicidin S synthetase. Thus, it appeared that the *srfA* locus encoded surfactin synthetase, and that only a portion of the *srfA* locus was required for the development of competence.

Introduction

The *csh293::*Tn*917lac* insertion was isolated in a screen for *spo0H*-dependent genes. Expression of *csh293::*Tn*917lac* is reduced in a *spo0H* mutant. Furthermore, this insertion mutation causes a roughly one hundred-fold defect in transformation and causes a slight defect in sporulation under some conditions (Jaacks et al., 1989). The sporulation defect is observed when sporulation was induced with decoyinine. (The addition of decoyinine, an inhibitor of GTP synthetase, causes a drop in the level of GTP, and thus mimics many of the effects of starvation.) Other insertions were independently identified in this locus in screens for insertions that caused defects in production of the lipopeptide antibiotic, surfactin (Nakano et al., 1988), or in competence (van Sinderen et al., 1990). This chapter presents the

results of a preliminary characterization of the *csh293::*Tn*917lac* insertion mutant.

Results

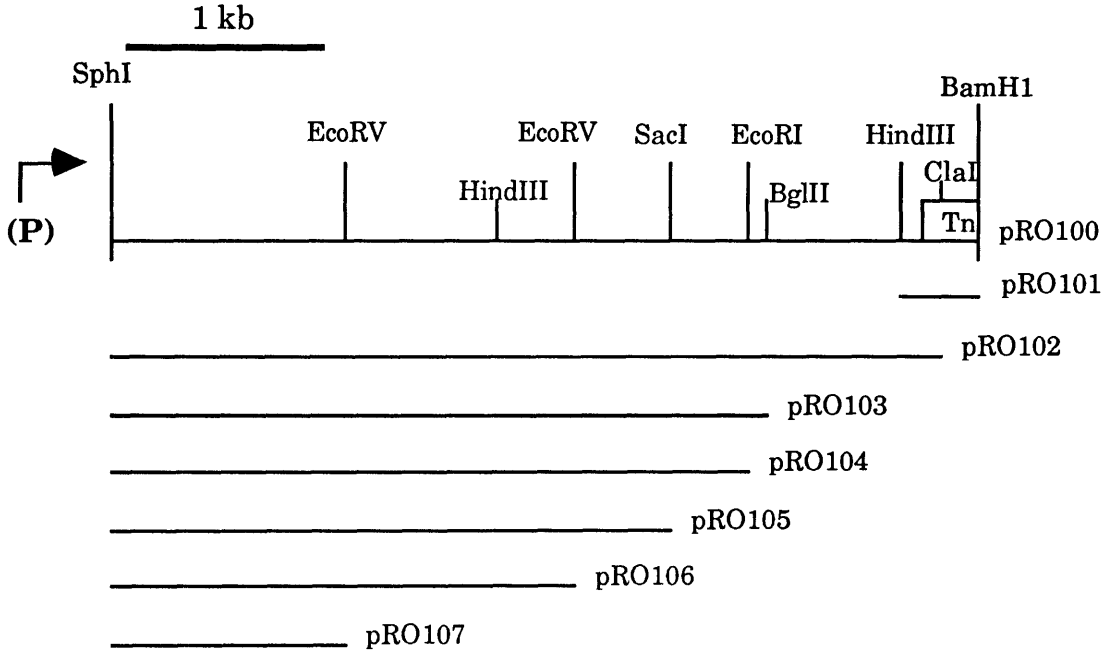
csh293 homologous to Gramicidin Synthetase

To characterize the *csh293::*Tn*917lac* insertion, a 4 kb fragment upstream of *csh293::*Tn*917lac* was cloned. An insertion-duplication of this region caused a defect in competence, indicating that the 4 kb fragment did not encompass the promoter for this locus. Preliminary sequencing yielded 4 nonoverlapping segments of sequence from this 4 kb fragment. Each of these four segments contained an open reading frame with significant homology to gramicidin S synthetase (Figure 3).

Gramicidin S, a nonribosomally synthesized cyclic peptide antibiotic containing several nonstandard amino acid residues, is produced by *Bacillus brevis.* Gramidicin S synthetase is required for the production of Gramicidin S. "Gramicidin" (a mixture of several peptides) and tyrocidine (another cyclic peptide antibiotic) were among the first natural antibiotics to be discovered and characterized (Hotchkiss and Dubos, 1940).

csh293 is allelic to *srfA*

Since *csh293::*Tn*917lac* disrupts a locus with homology to Gramicidin S synthetase, it seemed likely that this locus was involved in the production of a peptide antibiotic. Surfactin, the most powerful known biosurfactant, is a nonribosomally synthesized cyclic lipopeptide antibiotic from *Bacillus subtilis* that contains several nonstandard amino acid residues and a hydrophobic modification. The hydrophobic fatty acid, 3-hydroxy-13-methyl-tetradecanoic acid, is attached through an amide bond to the N-terminus of the seven amino acid peptide moiety. The molecule is circularized by an ester bond



Sequence segments: seq6 seq5 seq4 seq3 (junction)

csh293 sequence segment	Length of segment	% Identity to GrsA	% Similarity to GrsA	% Identity to GrsB	% Similarity to GrsB
seq6	96 aa	16%	46%	43%	61%
seq5	90 aa	38%	61%	36%	53%
seq4	97 aa	39%	61%	46%	68%
seq3	91 aa	50%	71%	46%	69%

Figure 3. Csh293 ORFS are homologous to Gramicidin S synthetase of *Bacillus brevis*. Four segments of *csh293* were sequenced (Top Panel). Homology to Gramicidin S synthetase was initially detected by comparing candidate csh293 ORFs to the protein database using FASTA. The best ORF of each segment was then compared to GrsA or GrsB (subunits of gramicidin S synthetase from *Bacillus brevis)* using the Bestfit computer program (Bottom Panel). The Bestfit alignments were done using the standard gap weight of 3.0 and length weight of 0.1. Sequence of GrsA is from accession number M29703 (Kraetzschmar et al., 1989). Sequence of GrsB is from accession number P14688 (Turgay et al., 1992). The *csh293* insertion is after nucleotide 10,125 of the published sequence of *srfA*, accession number X70356 (Cosmina et al., 1993).

between the C-terminus of the peptide and the hydroxyl group of the fatty acid (reviewed by Ratledge, 1986). Nakano and Zuber isolated three loci involved in the production of surfactin. One of these, *srfA*, maps by transduction to the same approximate chromosomal position (between *aroI* and *dal*) as the insertion in *csh293*. The second gene, *srfB,* is allelic with *comA*. Expression of *srfA,* like expression of *csh293*, depended upon *comA, spo0A* and *spo0H* (Nakano et al., 1991; Nakano and Zuber, 1989). These considerations led to the hypothesis that *srfA* and *csh293* were allelic, in spite of the fact that the original *srfA* insertion mutation did not cause a competence defect.

Nakano and Zuber cloned >20 kb of DNA upstream from their insertion before finding the promoter of *srfA*. A comparison of restriction maps indicated that the 4 kb fragment that I had cloned lay roughly in the middle of the *srfA* locus that they had cloned. In a collaboration, Nakano established by southern blot hybridization that the *csh293* insertion was located within the *srfA* operon and furthermore established that although the entire locus was required for the production of surfactin and for efficient decoyinine-induced sporulation, only the 5' portion of the locus was required for competence.

Discussion

Since our efforts in this area, considerable progress has been made by other research groups. Our results showed that *csh293* was allelic to *srfA* and was likely to be in the structural operon for the surfactin synthetase. Since then, the entire *srfA* locus has since been sequenced (Cosmina et al., 1993). The *csh293* insertion is in the 3' end of the first *orf* of the operon, after nucleotide 10,125. The N-terminal sequence of the subunits of surfactin synthetase have been matched to the deduced products of *srfA* (Vollenbroich et al., 1994).

srfA plays an intermediate role in the development of competence, since *spo0K* and early *com* genes are required for the expression of *srfA* and since *srfA* is required for the expression of late *com* genes (van Sinderen et al., 1990). The ability of ectopic expression of *srfA* to suppress the effects of mutations in *spo0K* and early *com* genes has been examined in three different labs (Hahn and Dubnau, 1991; Nakano and Zuber, 1991; van Sinderen et al., 1993). These results support the general notion that *srfA* is the major and probably the sole competence related target of these genes. Nakano and Zuber characterized the promoter of *srfA*. They propose that ComA binds to two sites of dyad symmetry centered at - 65 and - 109; and that binding of ComA causes a DNA loop to form between these two sites (Nakano et al., 1991). In support of this model, they show that the alteration of the putative sites or of the phasing between the sites destroys promoter activity (Nakano and Zuber, 1993). Roggiani and Dubnau show that ComA binds to these sites *in vitro* and that phosphorylated ComA binds more tightly than nonphosphorylated ComA. Furthermore, as predicted by the DNA looping model, some DNase hypersensitivity is observed between the ComA binding sites (Roggiani and Dubnau, 1993).

Our results indicated that only the 5' portion of *srfA* was required for the development of competence, while the entire operon was required for efficient (decoyinine-induced) sporulation and for the production of surfactin. Subsequent work has shown that only a small portion of *srfA* is required for the development of competence. This portion of the *srfA* operon contains a large *orf* encoding part of the surfactin synthetase and also contains a small *orf* in a different and overlapping reading frame that encodes the competence related determinant now called *comS* (D'Souza et al., 1994). It is still not clear by what mechanism the product of *comS* affects the expression of late

competence genes. ComS might directly stimulate transcription of *comK* or might interact with MecB or MecA or ComK.

The nature of the defect in sporulation has not been further investigated. In *Bacillus brevis,* mutations that block the production of the cyclic peptide antibiotic tyrocidine cause, under some conditions, a mild sporulation defect that can be rescued by the addition of exogenous tyrocidine (Modest et al., 1984). Similarly, it is possible that the spore defect of *srfA* is due to the lack of some product of the *srfA* operon. In principle, this proposition is testable for *Bacillus subtilis* although the experiment is made somewhat difficult by the weak and specialized nature of the sporulation phenotype and by the fact that the normal lab strains do not actually make surfactin, due to a defect in another locus, *sfp* (Nakano et al., 1992).

Surfactin itself is not necessary for the expression of competence genes, but some *lacZ* fusions are dependent on *sfp* and can be rescued extracellularly, presumably by surfactin (Marahiel et al., 1993). A homolog of *sfp* has been implicated in the formation of heterocysts in *Anabena* (Black and Wolk, 1994). Heterocyst formation, like competence, is believed to be regulated at least in part by diffusible signals. The diffusion of these signals is believed to be important in establishing the regular spacing of the heterocysts in filaments of *Anabena.* Thus, it is possible that homologs of *srfA* and related genes may be involved in cell-cell communication in a variety of microorganisms.

Chapter 3:
Genetic and Biochemical Characterization of a Competence Pheromone

Abstract

The purification and characterization of a modified peptide pheromone is described. The pheromone accumulates in culture medium as *Bacillus subtilis* grows to high density and is required for the development of genetic competence. When added to cells at low density, the pheromone induces the premature development of competence. The peptide moiety of the pheromone matches 9 of the last 10 amino acids predicted from a 55 codon open reading frame, *comX*. *comX* and *comQ*, the gene immediately upstream of *comX*, are required for production of the pheromone. Since *comQ* cells are rescued extracellularly by the addition of crude or purified pheromone, it appears that the production of pheromone is the one and only role of *comQ* in the development of competence.

Introduction

Cell-cell signaling can provide key environmental, temporal, or positional cues for the initiation and coordination of cellular differentiation. A full understanding of a cell-cell signaling pathway requires knowledge of the biochemical nature of the signal and of the mechanisms by which cells produce and respond to that signal. In this chapter, I present the biochemical and genetic characterization of a competence pheromone in *Bacillus subtilis*.

Previous reports indicated that competence for transformation in *B. subtilis* is affected by extracellular signaling factors (Akrigg and Ayad, 1970; Akrigg et al., 1967; Charpak and Dedonder, 1965; Joenje et al., 1972). By

analyzing the expression of competence genes, I have extended this work and have shown that the initiation of competence in *B. subtilis*, as defined by the expression of *srfA*, is induced by a competence pheromone that accumulates in culture medium as cells grow to high density. Furthermore, I have purified and characterize the pheromone and to identify two genes, *comQ* and *comX*, that are required for the production of this pheromone. As expected, the pheromone is required for the development of competence and the addition of the pheromone to cells at low density precociously induces the development of competence. Under some conditions, the competence pheromone also stimulates sporulation.

Results

Regulation of *srfA* by density signals

The level of *srfA* expression, as monitored by a *lacZ* fusion, increased during exponential growth in minimal medium (Figure 4). On solid media, expression of *srfA*, as visualized with Xgal, was most pronounced in the center of large colonies. This "fried egg" pattern of gene expression may be the spatial equivalent of the density-dependent pattern of gene expression observed in culture. To explain these observations, I hypothesized that some extracellular density signal might be regulating the expression of *srfA*. To test this idea, conditioned medium was prepared by growing wild-type cells to a high density, clarifying the culture by centrifugation, and sterilizing the supernatant by filtration. This "conditioned medium" was tested for its ability to (precociously) induce the expression of *srfA-lacZ* at low density. As hypothesized, the addition of conditioned medium resulted in the strong, rapid, and precocious expression of *srfA-lacZ* (Figure 5).

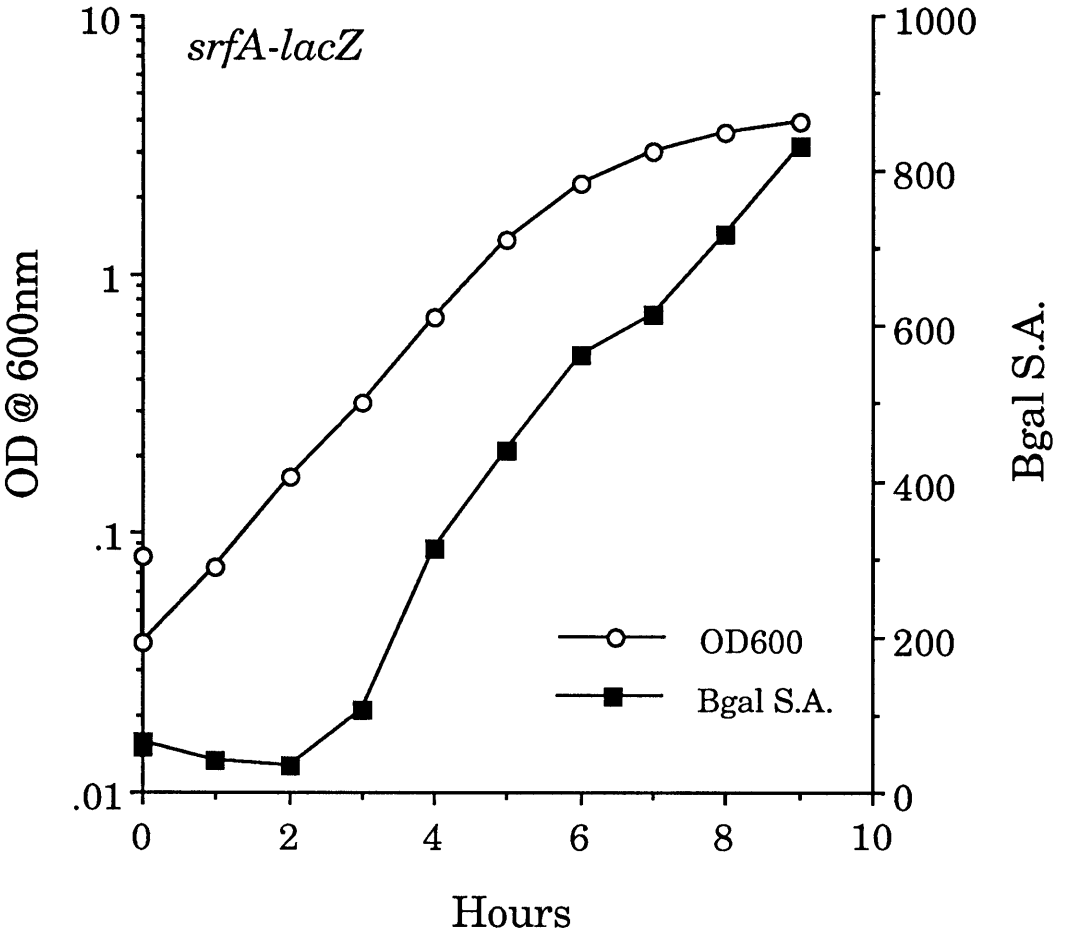


Figure 4. Expression of *srfA-lacZ* increases with cell density.
Cells containing a *srfA-lacZ* fusion (JRL293) were grown in minimal medium for three generations before the start of the experiment. At the indicated times, samples were taken and the optical density (open circles) and the ß-galactosidase specific activity (filled squares) were determined. ß-galactosidase specific activity is ß-galactosidase activity divided by optical density at 600 nm.

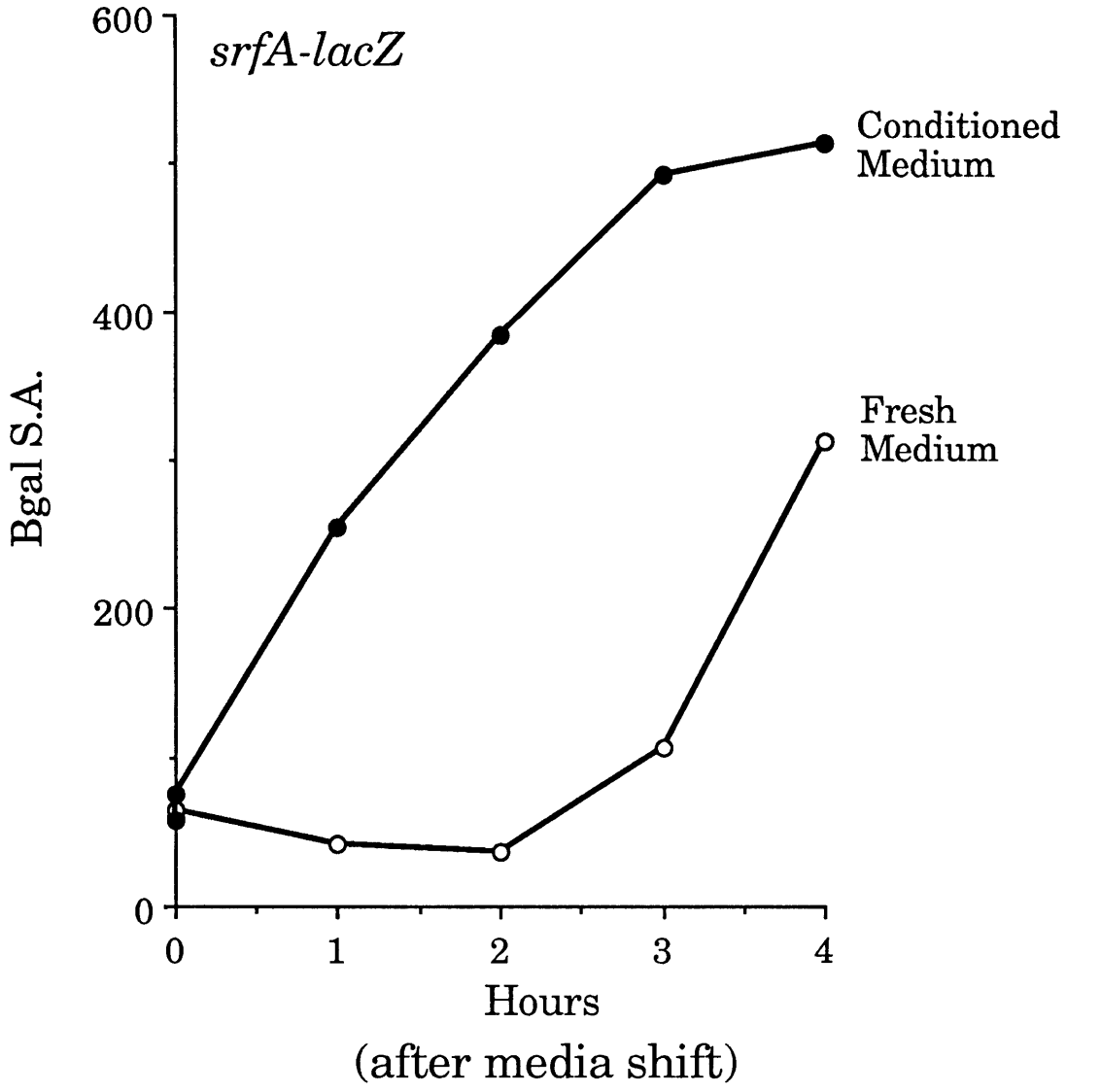


Figure 5. Conditioned medium induces the precocius expression of *srfA-lacZ*. Cells containing a *srfA-lacZ* fusion (JRL293), were grown for three doublings in minimal medium to an O.D. of 0.081 at 600 nm and were then mixed with an equal volume of fresh or conditioned medium. Cultures were sampled hourly and the optical density at 600 nm and the ß-galactosidase activity of the samples were determined (Materials and Methods). ß-galactosidase specific activity is ß-galactosidase activity divided by the optical density at 600 nm. Conditioned medium was prepared from ROM119 (prototroph).

Assay for *srfA*-inducing activity

To further characterize the nature of the *srfA*-inducing activity, a quick and quantitative assay of the *srfA*-inducing activity was developed. This assay measured the ability of a sample, relative to fresh medium, to cause an increase in the ß-galactosidase specific activity 70 minutes after the samples were mixed with aliquots of a low density culture of cells containing a *srfA-lacZ* fusion. These conditions were chosen so as to obtain a large change in gene expression in a minimal amount of time. Serial two-fold dilutions of each sample were made using fresh medium as a diluent. Each sample was then mixed with an equal volume of a fresh, low density culture containing the *srfA-lacZ* fusion, incubated for 70 minutes, and assayed for ß-galactosidase activity (Materials and Methods).

The induction of ß-galactosidase specific activity was roughly proportional to the concentration of conditioned medium over a 10-fold range in the concentration of conditioned medium (Figure 6). The maximal induced ß-galactosidase specific activity at 70 minutes, under these conditions, was 10 to 20 times the background ß-galactosidase specific activity.

Conditioned medium probably affects the rate of transcription of *srfA*. The response to conditioned medium was seen with a variety of transcriptional and translational fusions to *srfA*. Thus, the response to conditioned medium cannot involve translational control. If conditioned medium were to affect mRNA stability, then the mRNA stability determinants would have to be within the first 140 nucleotides of *srfA* mRNA, since fusions containing as little as 140 nucleotides of *srfA* mRNA (such as the fusion in JMS374) respond to conditioned medium. It appears most likely that conditioned medium acts on the promoter of *srfA* to affect the rate of transcription of *srfA*.

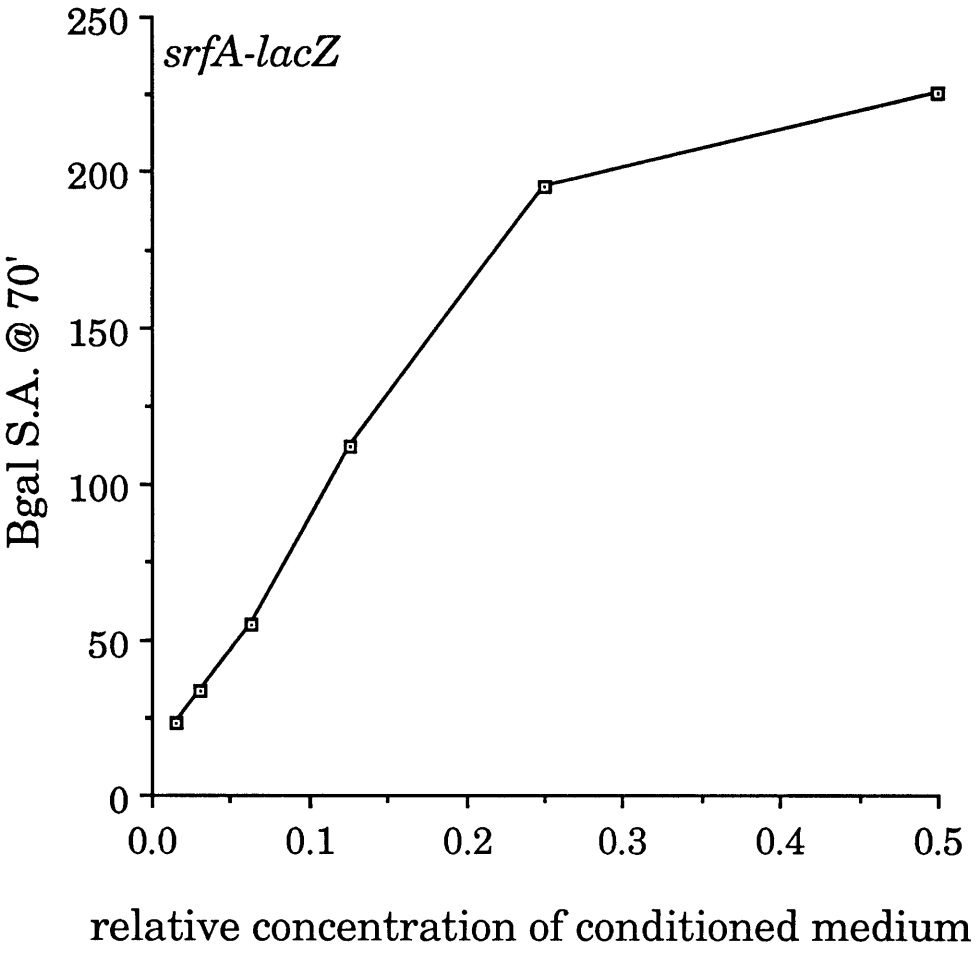


Figure 6. The expression of *srfA* increases with the concentration of conditioned medium. Conditioned medium was serially diluted into fresh medium. One volume of each dilution was mixed with an equal volume of low density culture (0.086 O.D. at 600 nm) containing a *srfA-lacZ* fusion (JRL293), incubated for 70 minutes at 37°C, and assayed for ß-galactosidase as described in Materials and Methods. Conditioned medium was prepared from ROM100 (*trp, phe*). The dose-response was approximately linear over a 10-fold range of the concentration of conditioned medium. In quantitive assays, *srfA*-inducing activity was calculated by choosing a sample in the linear range and multiplying the induced ß-galactosidase specific activity at 70 minutes by the dilution factor of the sample. (Induced ß-galactosidase specific activity is the ß-galactosidase specific activity of cells mixed with sample, minus the ß-galactosidase specific activity of cells mixed with fresh medium.)

Conditioned media harvested at different optical densities was tested for its ability to induce expression of *srfA*. The strength of the *srfA*-inducing activity increased in direct proportion to the density of the culture from which the conditioned medium was harvested (Figure 7). Thus, the *srfA*-inducing activity appears to be a true density signal, in that the strength of the signal increases with the density of the culture. Furthermore, the strength of the signal corresponds well with the expression of *srfA*. That is, the conditioned medium not only has the activity to induce expression of *srfA*, but it is also present at appropriate times and concentrations to be responsible for the normal developmental expression of *srfA*. Total *srfA*-inducing activity is well below half-maximal at 0.04 O.D. at 600 nm, roughly half maximal at 0.40 O.D. at 600 nm, and well above half-maximal at 4.0 O.D. at 600 nm (Figure 7). Similarly, expression of *srfA* is low at 0.04 O.D. at 600 nm, increasing rapidly at 0.40 O.D. at 600 nm, and highly expressed at 4.0 O.D. at 600 nm (Figure 4). Thus, the temporal regulation of *srfA* (Figure 4) appears to be a natural and direct consequence of the density-dependent accumulation of *srfA*-inducing activity (Figure 7).

Purification and characterization of the competence pheromone

The ability of crude or fractionated conditioned medium to induce expression of *srfA-lacZ* in cells at low density was used as an assay to characterize and purify a competence pheromone. Preliminary characterization of crude conditioned medium indicated that the *srfA*-inducing activity was, at least in part, a small peptide. The *srfA*-inducing activity was protease sensitive (pronase, trypsin), heat stable (80°C, 12 hours), and passed through filters (Centricon) with a nominal molecular mass cut-off of 10,000 daltons.

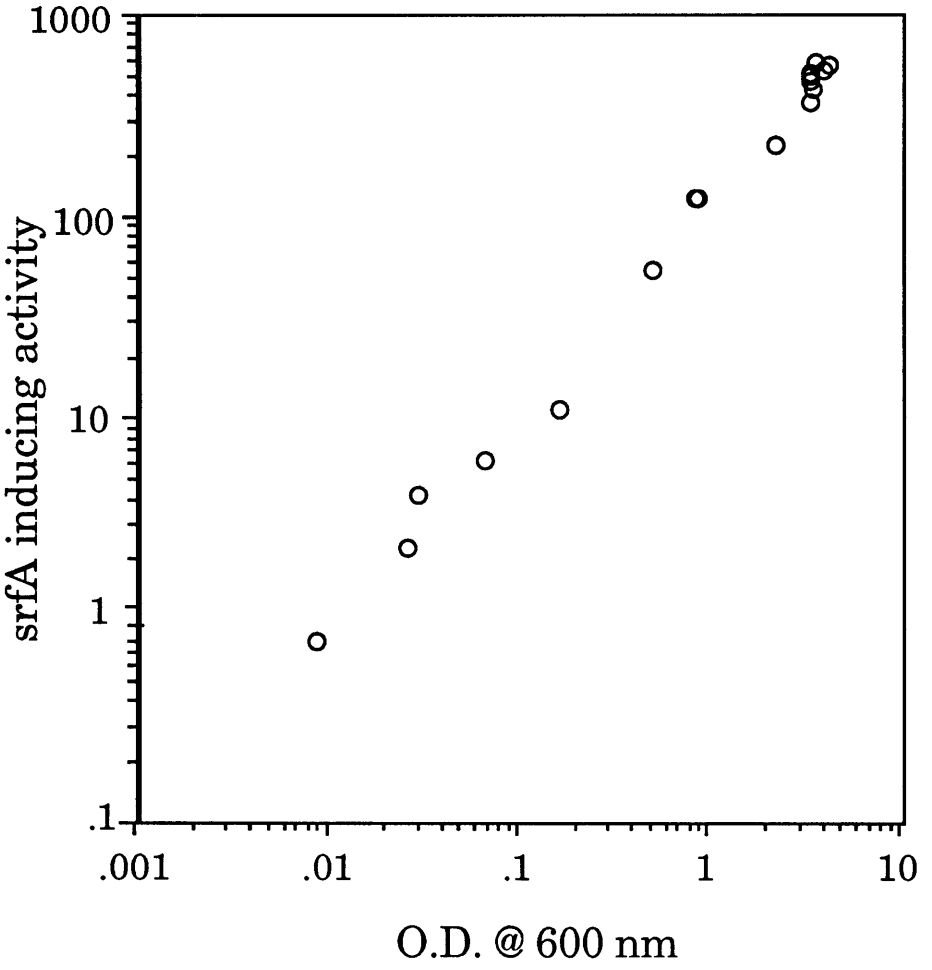


Figure 7. *srfA*-inducing activity increases with cell density. The *srfA*-inducing activity of conditioned medium was plotted versus the cell density at which the conditioned medium was harvested. The *srfA*-inducing activity was determined by testing the conditioned media at several dilutions (as described in Figure 6), choosing a sample in the linear range of the dose-response, and multiplying the induced ß-galactosidase specific activity at 70' by the dilution factor of the sample. ROM178 (*abrB, trp, phe*) was grown in minimal medium at 37°C with agitation. Conditioned medium was harvested, by centrifugation and filtration, at various times during growth and stationary phase. The conditioned medium was then quantitatively tested, at various dilutions for *srfA*-inducing activity, as described in Figure 6, except that ROM102 (*csh293::*Tn*917lac, trp, phe)* was used as an indicator strain. The maximum response (ß-galactosidase specific activity) to conditioned medium in this experiment was about 80. Thus, the *srfA*-inducing activity of the culture was half-maximal at approximately 0.4 O.D. at 600 nm.

Preliminary fractionation of conditioned medium by reverse phase chromatography revealed that two distinct species stimulated expression of *srfA-lacZ*. The species eluting at low acetonitrile concentration (≤5%) stimulated expression of *srfA-lacZ* only 2- to 3-fold, even at very high doses. (See Chapter 4). The species eluting at relatively high acetonitrile concentration (~50%), like crude conditioned medium, stimulated expression of *srfA-lacZ* 10- fold to 20-fold in 70 minutes. The purification and analysis of this species, which I call the competence pheromone, is described in detail below.

To purify the competence pheromone, conditioned medium was loaded onto a Waters C-18 Sep-Pak cartridge in 20% methanol and eluted with a linear gradient from 20% to 80% methanol. Fractions with pheromone activity were pooled, concentrated, loaded onto a C-18 HPLC column and eluted with a gradient of acetonitrile (0.4% per minute). In some cases, this step was repeated. Fractions with pheromone activity were again pooled, concentrated, rerun on the C-18 HPLC column, and eluted with a shallower gradient of acetonitrile (0.08% per minute).

The gradient elution yielded a doublet of pheromone activity and a corresponding doublet of ultraviolet absorbance (Figure 8). Peptide sequence analysis of the trailing peak of our most pure preparations indicated the presence of two peptides that differed only in their N-terminal residue:

ala-asp-pro-ile-thr-arg-gln-(blank cycle)-gly-asp

and asp-pro-ile-thr-arg-gln-(blank cycle)-gly-asp

Total amino acid analysis was consistent with the above sequence: all of the amino acids were detected in the proper molar ratios, and no additional amino acids were detected. The trypsin-sensitivity of the pheromone activity in crude conditioned medium was consistent with the presence of arginine in the pheromone.

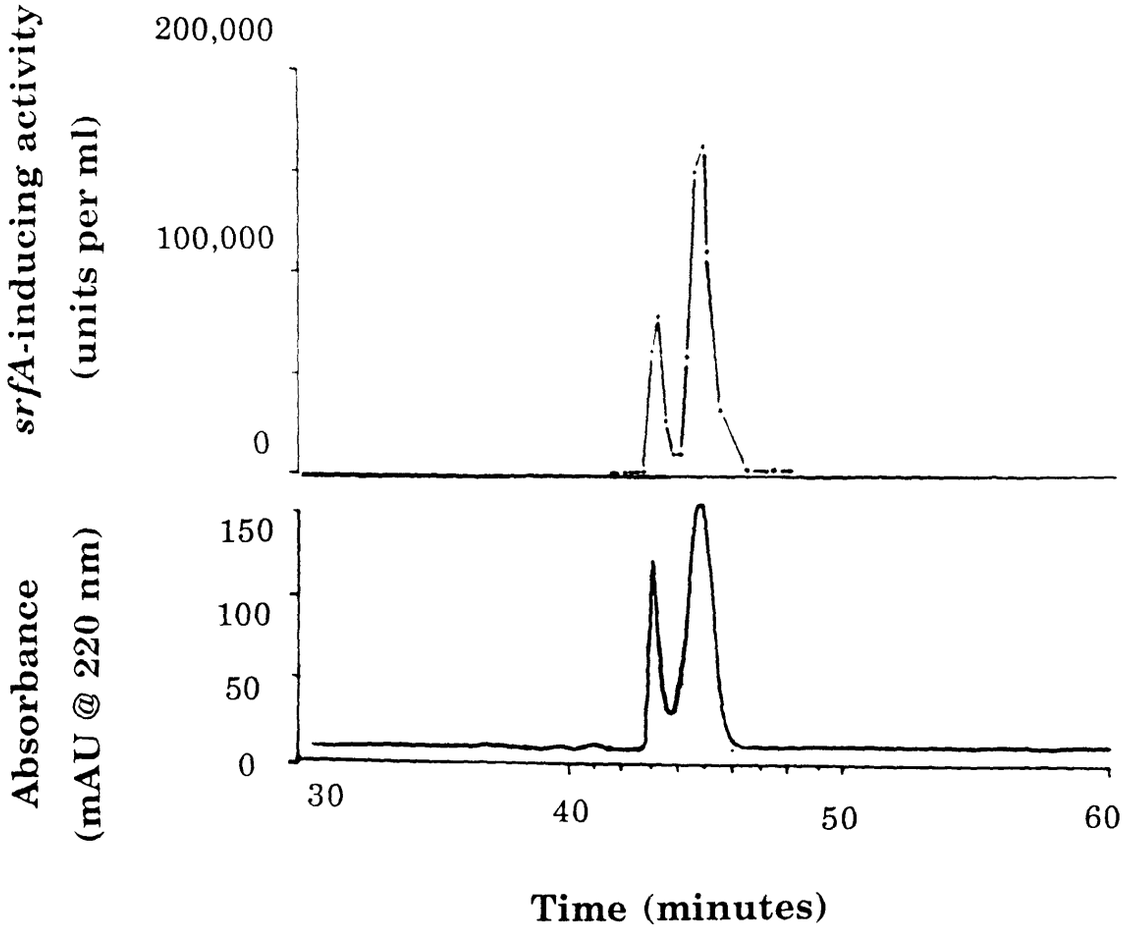


Figure 8. Purification of Pheromone. The *srfA*-inducing activity (Top) and the absorbance at 220 nm (Bottom) are shown for highly purified pheromone eluting from an HPLC C18 column in a linear gradient of acetonitrile in 0.1% TFA. A doublet of activity and of absorbance were observed. The specific activity of this material (activity divided by absorbance at 220 nm) was approximately 10,000-fold greater than the specific activity of conditioned medium. The yield was approximately 6%.

Analysis of purified pheromone by mass spectrometry detected two species of approximately 1,363 and 1,292 daltons in the trailing peak, corresponding to the two peptides indicated by microsequencing. Two species of similar or identical mass were also detected in the leading peak, thus four potentially active species were present. Rechromatography of each peak gave a mixture of the two original peaks, although not necessarily in the original ratio. The chromatographic doublet may be due to a slow isomerization or change in conformation. The difference between the chromatographic peaks does not involve any significant change in mass. Since both peaks were active, it was clear that both chromatographic forms were active or were easily converted to an active form. The two mass species have not been separated, thus it has not been possible to determine whether both mass species are active, or if only one mass species is active.

The ß-galactosidase specific activity, 70 minutes after the addition of pheromone to indicator cells at low density, is a rough measure of the rate of synthesis of ß-galactosidase and thus, of the rate of transcription of *srfA*. As might be expected, the rate of transcription of *srfA* (as indicated by the ß-galactosidase specific activity) increases with the concentration of pheromone, up to a maximum (Figure 9). The saturation of the response presumably reflects the saturation of a pheromone receptor. The half-maximal response corresponds to an (initial) pheromone concentration of approximately 3-5 nM (Figure 9). If pheromone is present in excess (so that the free concentration of pheromone is approximately equal to the initial concentration of pheromone), and if the response (ß-galactosidase specific activity) is directly proportional to the concentration of the pheromone-receptor complex (so that the response can serve as a direct measure of pheromone-receptor complex), then the concentration of pheromone sufficient to give a half-maximal response, 3-5 nM,

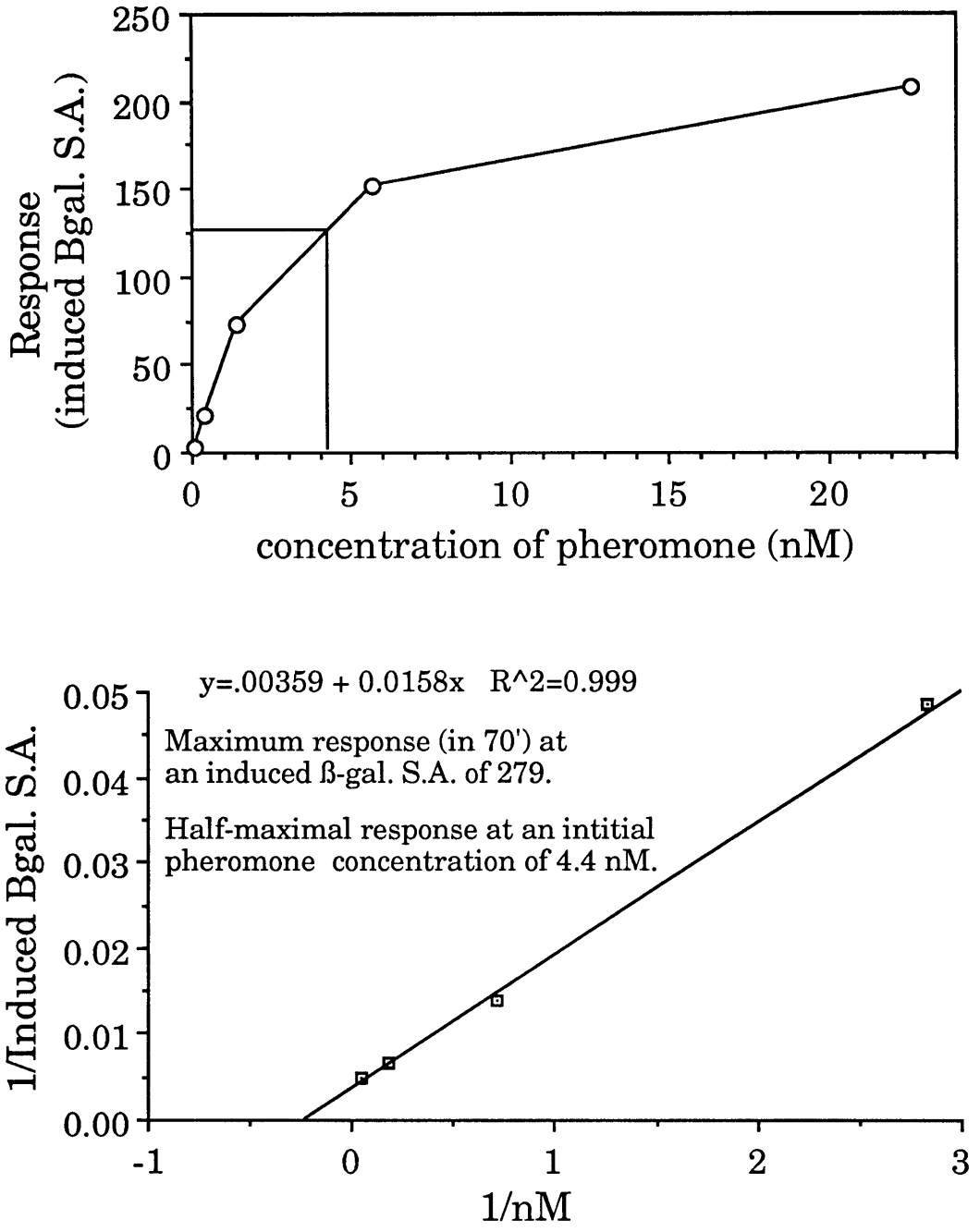


Figure 9. Quantitative Dose-Response of Purified Pheromone.
Pure pheromone, quantitated by amino acid analysis, was tested for *srfA*-inducing activity. The half-maximal response was at 3-5 nM. This preparation of pheromone contained the 9 and 10 amino acid peptides in a ratio of approximately 1:2. BSA was included in all assay samples at a final concentration of 50 ug/ml in order to prevent the nonspecific loss of pheromone.

will also be the dissociation constant of the pheromone-receptor complex. It will be interesting to see if this estimate of the dissociation constant can be confirmed.

If all of the activity in crude conditioned medium is due to pheromone, then the pheromone concentration is roughly 30- 40 nM (or approximately 10 times the half-maximal concentration) for a culture at 4.0 O.D. at 600 nm. This may be an overestimate, since other components of conditioned medium make a contribution to the induction of *srfA* (Chapter 4).

A search of the translated gene databases with the pheromone sequence gave a precise match to 9 of the last 10 amino acids of a small open reading frame from *B. subtilis* (Figure 10). This reading frame is between *comQ* and *comP*, two competence genes that are required for expression of *srfA* (Hahn and Dubnau, 1991). In fact, the 3' end of *comQ* overlaps with the putative 5' end of the open reading frame that I named *comX* (X for extracellular). The *comQ* open reading frame was originally identified by DNA sequencing (Msadek et al., 1991; Weinrauch et al., 1991). Experiments described below demonstrate that the primary, and perhaps only, role of *comQ* in competence is in the production of the pheromone.

The *comX* open reading frame predicts a tryptophan in the position of the blank cycle from the peptide sequencing. The expected mass of the pheromone, based on the predicted amino acid sequence of the last 10 amino acids of ComX, was 1,157 daltons (or 1,086 daltons for the 9 amino acid species). However, the masses observed in preparations of purified pheromone, 1,363 and 1,292 daltons, were significantly larger than the expected masses. The 71 dalton difference between the masses of the two observed species was consistent with the difference in the N-termini of the

A.

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TAAAGAAAAAAAAGAGCTAATAAAGGAATGCTTATTAAGTTATACAAAGGGGGATACAAG

ATGCAAGACCTAATTAACTACTTTTTAAATTATCCTGAGGCTTTAAAAAAATTGAAAAAT
M  Q  D  L  I  N  Y  F  L  N  Y  P  E  A  L  K  K  L  K  N

AAAGAAGCCTGCCTTATAGGTTTTGATGTGCAAGAAACTGAAACAATAATTAAAGCTTAT
K  E  A  C  L  I  G  F  D  V  Q  E  T  E  T  I  I  K  A  Y

AATGATTATTATCTGGCTGATCCAATAACCCGTCAATGGGGTGATTAATAGGTGGATTAA
N  D  Y  Y  L  A  D  P  I  T  R  Q  W  G  D  *
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B.

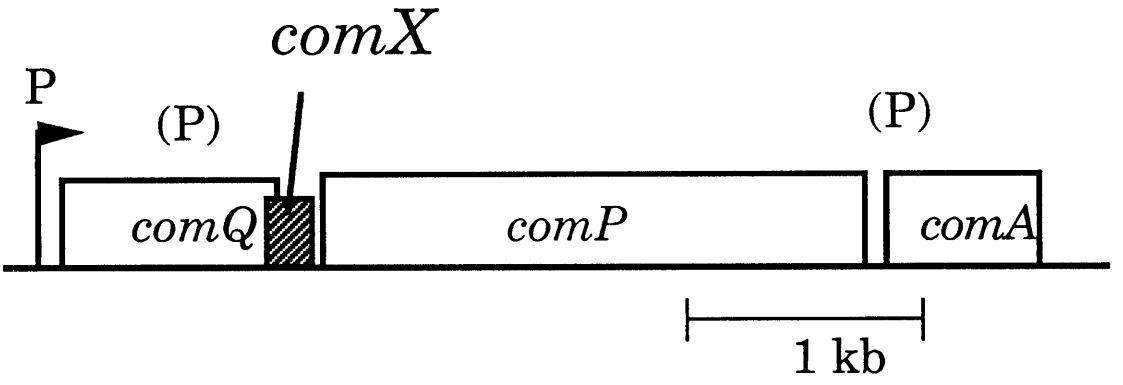

Figure 10.
(A) DNA sequence and predicted amino acid sequence of *comX*. DNA sequence of *comX* (extracellular) is from published work (Weinrauch et al., 1991). The putative start codon is located downstream of an apparently strong ribosome binding site. The bases in the ribosome binding site that are complementary to the 3' end of 16S rRNA are underlined. The C-terminal 10 amino acids (except for tryptophan) are found in the mature pheromone and are indicated in bold. The 5' end of *comX* overlaps with the 3' end of *comQ*, and the *comQ* stop codon, TAA, is underlined. The HindIII site towards the 3' end of *comX* is underlined and was the site used to construct the *comX*::*spc* mutation.
(B) Map of the competence regulatory region . The *comQ* and *comX* reading frames have a 13 bp overlap. The *comX* and *comP* reading frames are separated by 14 bp. *comQ* and *comX* are required for production of the competence pheromone. The downstream genes, *comP* and *comA*, encode, respectively, a histidine protein kinase and a response regulator (transcription factor) that are needed for the cellular response to the competence pheromone. Postulated promoters are indicated in parentheses (P).

peptides, as detected by peptide sequencing. However, the observed masses of the purified pheromone were 206 daltons greater than the expected masses based on the sequence of *comX*. This difference indicated that the peptide moiety of the pheromone was modified in some way. The modification appeared to be important for pheromone activity since synthetic 9 and 10 amino acid peptides (containing tryptophan at positions 7 and 8, respectively) had no pheromone activity.

Further analysis suggested that only the tryptophan residue was modified. Fragmentation mass spectrometry showed that the N-terminal 7 amino acids were unmodified, but that the next largest fragment, (corresponding to the entire 10 amino acid pheromone less the carboxyl groups of the C-terminal aspartate residue), contained the excess mass. This effectively limited the position of the modification to the eighth and ninth residues of the 10 amino acid pheromone. Since the ninth residue is a glycine, it seems that the excess mass must be exclusively associated with the eighth residue, where the tryptophan was expected. Synthetic 9 and 10 amino acid peptides had the expected, characteristic tryptophan ultra-violet absorbance at 280 nm, but purified pheromone had no significant absorbance in this region of the spectrum. Furthermore the peptide sequence analysis and total amino acid analysis did not indicate that any of the other amino acids were modified. Thus, it appears that the eighth residue, where the sequence predicts a tryptophan, is a modified, non-standard amino acid. Possibly, a nonstandard amino acid is incorporated into this position when the pheromone is synthesized. More likely, a tryptophan residue is incorporated at this position and is later modified. Only a few tryptophan modifications have been described. Serotonin, a decarboxylated tryptophan with a 3' hydroxyl group, is perhaps the most familar modified tryptophan. The recently discovered

cofactor of methylamine dehydrogenase, tryptophan tryptophylquinone, consists of two cross-linked tryptophan residues (McIntire et al., 1991). Two additional examples of modified tryptophans are found in the peptide antibiotics nosiheptide and thiostrepton (Mocek et al., 1993a; Mocek et al., 1993b). The masses and the chemical and spectral properties of these modified tryptophans do not make them likely candidates for the competence pheromone. Nonetheless, they illustrate the biological possibility of modifying a tryptophan residue.

comQ and *comX* are required for production of pheromone

The *comX* region was cloned by PCR using oligonucleotides based on the published DNA sequence (Weinrauch et al., 1991; Weinrauch et al., 1990). A cassette conferring spectinomycin resistance was cloned into the HindIII site of *comX*. This mutant allele was then introduced, by transformation, into *Bacillus subtilis* (Materials and Methods). The *comX*::*spc* mutation drastically reduced the production of *srfA*-inducing activity (Figure 11). *comP* mutations had no detectable effect on the production of *srfA*-inducing activity. Thus, the effects on the *comX* mutation on the production of *srfA*-inducing activity were not due to polarity on *comP*.

A null mutation (deletion/insertion) in *comQ* was constructed by standard techniques (Materials and Methods). As described previously (Weinrauch et al., 1991), *comQ* mutations cause a defect in expression of *srfA*, in expression of *comG*, and in the development of competence. The *comQ* mutation, like the *comX* mutation, drastically reduced the production of *srfA*-inducing activity (Figure 11). Fractionation of conditioned media from *comX* and *comQ* strains showed that the residual *srfA*-inducing activity produced by these strains was due to CSF and not to pheromone (Chapter 4, Table 4).

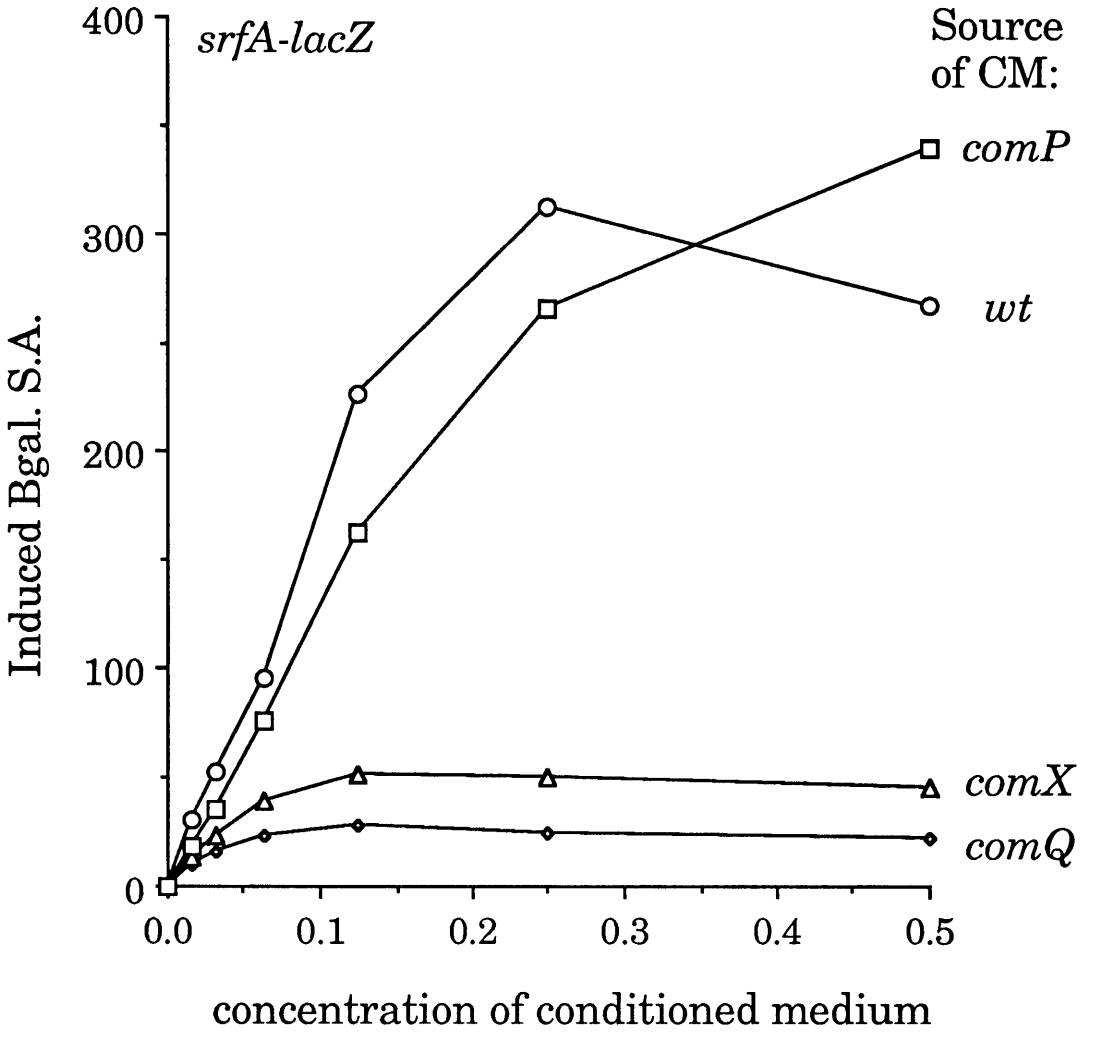

Figure 11. *srfA*-inducing activity of conditioned media. Strains with *comX* and *comQ* mutations were defective in the production of *srfA*-inducing activity. Fractionation showed that the residual activity of conditioned medium from these strains was due to CSF and not to pheromone. Conditioned medium (CM) was harvested from *wt* (ROM100), *comQ* (ROM238), *comX* (ROM230), and *comP* (AG1082) cultures grown in minimal medium to approximately 4.0 O.D. at 600 nm. JRL293 (*amyE::(srfA-lacZ, cat), trp, phe*) was the indicator strain. The background ß-galactosidase specific activity in this experiment was 14.

The defect in expression of *srfA* in a *comQ* mutant (Figure 12, top panel), was completely rescued by the addition of conditioned medium containing the pheromone (Figure 12, bottom panel). Thus, *comQ* was required for the production of pheromone, but was not required for the response to pheromone.

Similarly, the defect in expression of *comG* in a *comQ* mutant (Weinrauch et al., 1991) (Figure 13, top panel), was completely rescued by the addition of conditioned medium containing pheromone (Figure 13, bottom panel). Expression of *comG-lacZ* in a *comQ* mutant was also rescued in cross-streak experiments, by wild-type, *comP* and various other strains (which produce pheromone), but not by *comX* or *comQ* strains (which do not produce pheromone) (Table 1). When the *comQ*$^+$ allele (without *comX*) was provided in trans, pheromone production and competence were restored (Materials and Methods), indicating that the *comQ* mutation was not polar on *comX*.

In *comQ* strains, expression of *srfA*, expression of *comG,* and transformability were restored by the addition of purified pheromone as well as by crude conditioned medium (Table 2). Since the *comQ* mutation abolished the production of the competence pheromone and was rescued by an exogenous source of pheromone, it appeared that the only role of ComQ in the development of competence was in the production of pheromone.

The cell density signals present in conditioned medium were not only necessary for the development of competence, but, given permissive nutrient conditions, the addition of conditioned medium was sufficient to induce the development of competence, as indicated by expression of *comG* (first shown by Jonathan Solomon) (Figure 13, top and bottom panels) or as indicated by transformation (Table 2) (Charpak and Dedonder, 1965; Joenje et al., 1972; Magnuson et al., 1994).

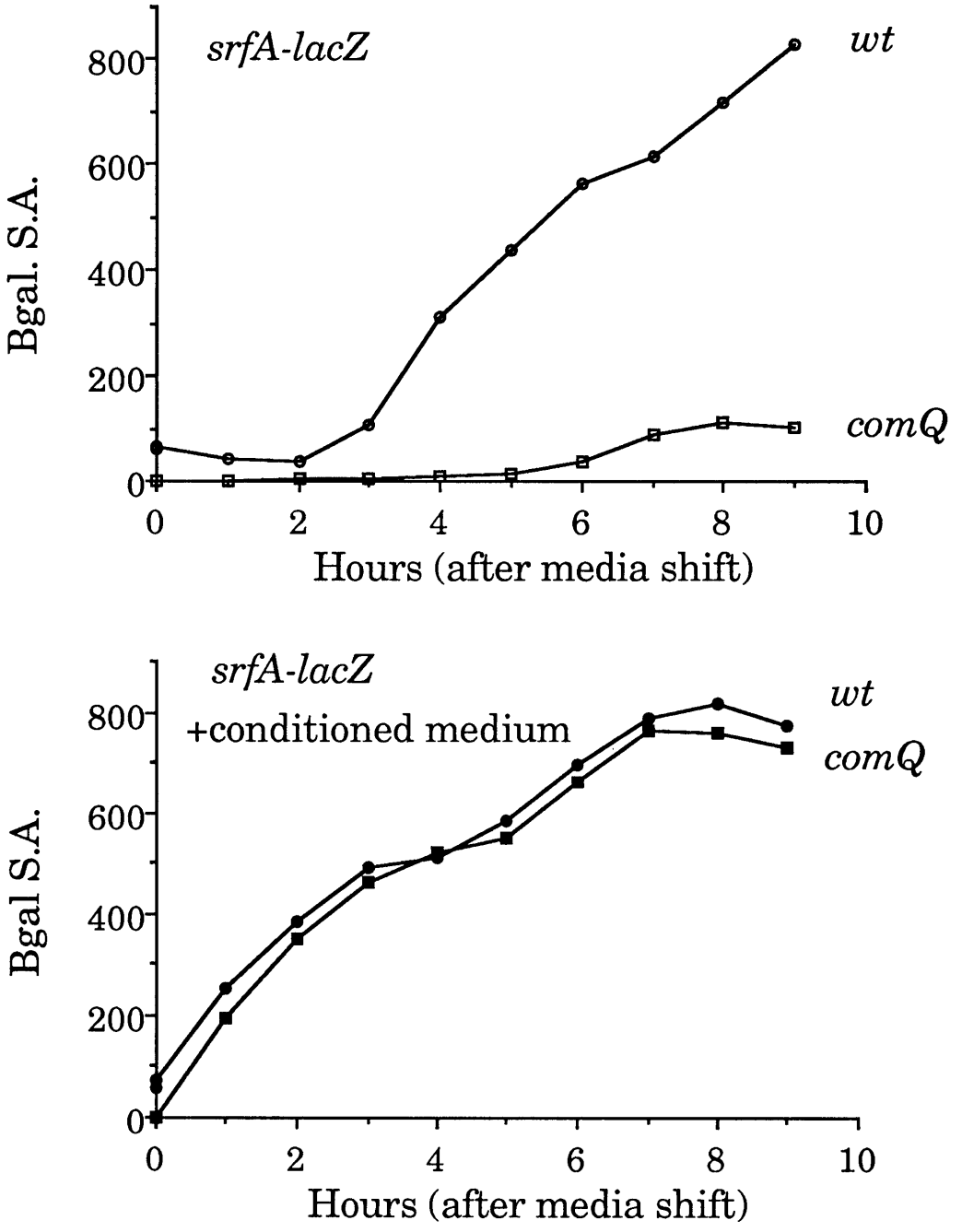

Figure 12. (Top panel) *comQ* is required for expression of *srfA-lacZ*. (Bottom panel) The *comQ* defect in expression of *srfA-lacZ* is rescued by conditioned medium. *comQ*$^+$ (JRL293, circles) and *comQ*$^-$ (JMS323, squares) cells containing a *srfA-lacZ* fusion were grown for three doublings (to 0.080 to 0.120 O.D. at 600 nm) in minimal medium and were then mixed with an equal volume of fresh medium (open symbols, top panel) or conditioned medium (filled symbols, bottom panel). Conditioned medium was prepared from ROM119.

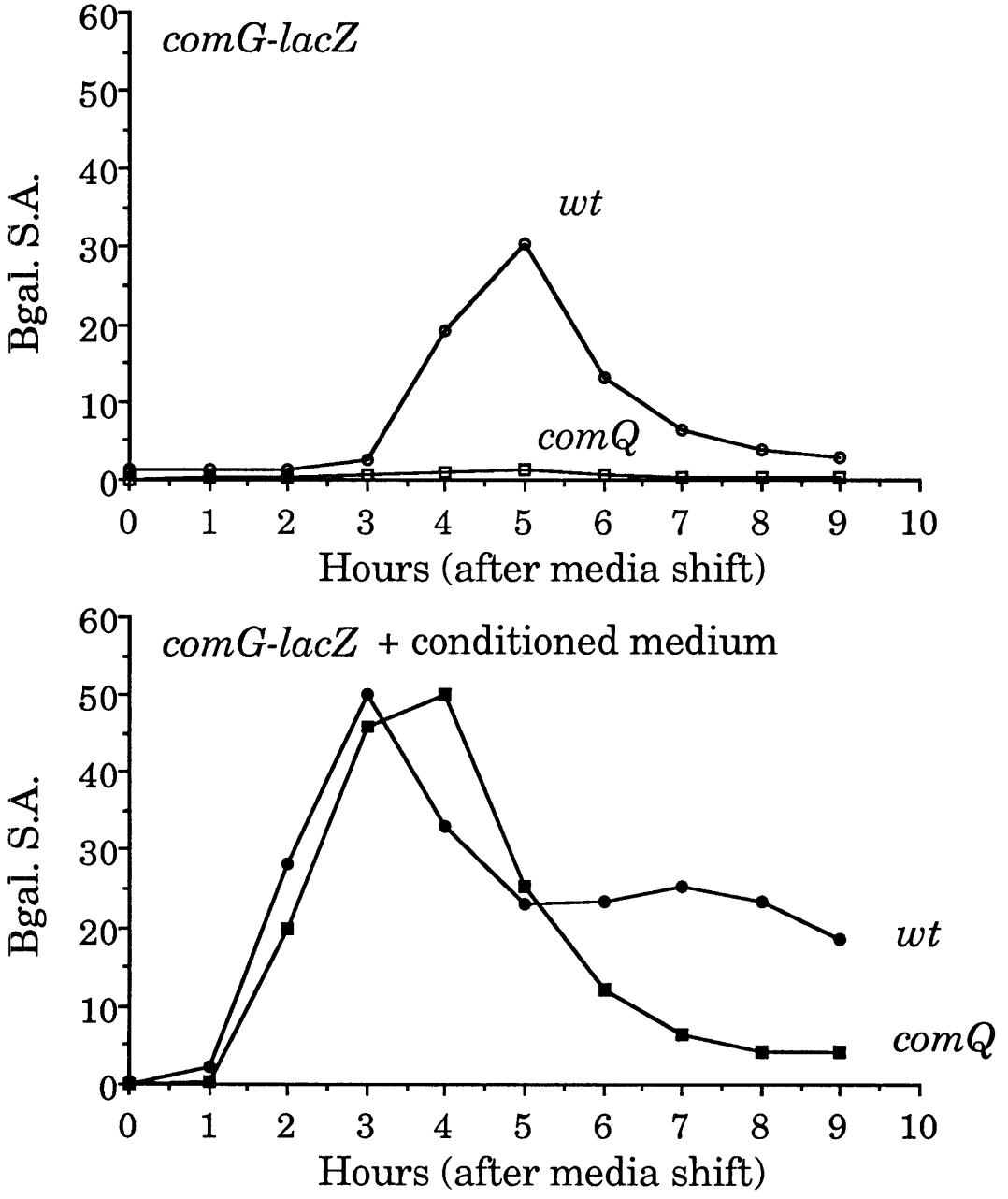

Figure 13. (Top Panal) *comQ* is required for expression of *comG-lacZ*. (Bottom Panel) The defect in expression of *comG-lacZ* in a *comQ* mutant is rescued by the addition of wild-type conditioned medium. *comQ*$^+$ (AG1046, circles) and *comQ*$^-$ (AG1519, squares) cells containing a *comG-lacZ* fusion were grown for three doublings in minimal medium (to 0.080 to 0.120 O.D. at 600 nm) and were then mixed with an equal volume of fresh medium (open symbols, top panel) or conditioned medium (filled symbols, bottom panel). Conditoned medium was prepared from ROM119 (prototroph).

Extracellular Rescue by Cross-streak		**Indicator strain AG1519**
Tester Strains	**Relevant Genotypes**	*comQ::spc amy::(comG-lacZ, neo)*
ROM230	*comX*	-
AG1520	*comQ*	-
ROM100	*wt*	+
ROM140	*comA*	+
KI418	*spo0K*	+
JRL358	*spo0K*	+
AG503	*spo0A*	+
AG132	*spo0A abrB*	+
AG1226	*spo0H*	+
NY120	*kinB kapB*	+
AG169	*abrB*	+
ROM151	*srfA*	+

Table 1. Extracellular Rescue of *comQ* by Cross-streaks. Expression of *comG-lacZ* in the *comQ* indicator strain was rescued by wild-type strains, but not by com*X* or *comQ* strains. Each tester strain was streaked across the indicator strain (AG1519) on SPII Xgal plates, incubated at 37°C for 48 hours and scored for extracellular rescue (Materials and Methods). Rescue (indicated by +) was visually determined by comparing the expression of *comG* near the cross-streak to expression of *comG* distant from the cross-streak. Blue color indicated expression of *comG*.

		Strains and relevant genotypes:			
Effects of Pure pheromone on expression of *comG* and on transformability		AG1046 *comQ*$^+$ *amy::(comG-lacZ, neo)*		AG1519 *comQ::spc* *amy::(comG-lacZ, neo)*	
Additons to Cultures:		**ß-gal. Specific Activity**	Tnf. per ml per O.D. at 600nm	**ß-gal. Specific Activity**	Tnf. per ml per O.D. at 600nm
(Before Additions)		**1.0**	ND	**2.6**	ND
MM		**0.7**	102	**0.4**	28
wild-type CM		**21.9**	3,630	**34.0**	6,670
MM + BSA		**3.7**	3,710	**0.5**	<30
MM + BSA + pheromone		**37.0**	36,500	**29.6**	9,420
MM + pheromone		**6.7**	3,700	**1.2**	133
comQ CM		**29.9**	1,960	**0.8**	42
comQ CM + pheromone		**72.9**	19,600	**63.6**	9,400

Table 2. Effects of Pure Pheromone on Transformability and on Expression of *comG*. Cultures were grown for three doublings in minimal medium to 0.100 to 0.200 O.D. at 600 nm and then additions of equal volume were made to aliquots of each culture, as indicated. Cultures were incubated at 37°C with agitation for 140 minutes. Each culture was then sampled and the density, ß-galactosidase activity and transformability (transformants (Tnf) per ml per O.D. at 600) were determined (Materials and Methods).

Thus, given permissive nutrient conditions, density appears to be the limiting signal for the development of competence.

This result is especially striking since the expression of *comG* and the development of competence is much more heavily regulated than the expression of *srfA*. The expression of late competence genes, such as *comG,* is restricted to the competent fraction of the culture and occurs only in media containing glucose. Expression of *srfA,* in contrast, appears to occur in all cells (Hahn et al., 1994), and is observed in the absence as well as the presence of glucose (Nakano et al., 1988). Thus, *srfA* is an early but imperfect indicator for the development of competence, and although it is required for the development of competence, it is not sufficient for the development of competence. Other signals, such as the presence of glucose, and other regulators, such as *degU, sinR, abrB,* and *comK* are required, in addition to *srfA,* for the expression of late competence genes such as *comG* and for the development of competence (reviewed by Dubnau, 1993). Under these conditions, however, the addition of conditioned medium was sufficient to induce the expression of *comG* (Figure 12) and the development of competence (Table 2). The effects of pheromone and of conditioned medium were most striking in the *comQ* mutant, which has no endogenous production of pheromone.

A role for the competence pheromone in sporulation

At least one extracellular factor is needed for efficient sporulation when cells are induced to sporulate with decoyinine, an inhibitor of GMP synthetase, or by resuspension in nutrient-poor medium (Grossman and Losick, 1988a; Waldburger et al., 1993). To test if the competence pheromone had an important role in sporulation, we tested the effects of a *comQ* null mutation on sporulation in the AG130 strain background. Effects of extracellular sporulation factors (and the *comQ* mutation) are more pronounced and

reproducible in this strain background than others (Grossman and Losick, 1988a). The *comQ* mutation was used instead of the *comX* mutation to avoid potential problems caused by polarity of the *comX* mutation on *comP*, which under some conditions seems to play a role in sporulation (Weinrauch et al., 1990). In nutrient sporulation medium, the *comQ* mutation had little or no effect on sporulation relative to the isogenic wild-type strain. However, when cells were grown in minimal medium and induced to sporulate by addition of decoyinine (Materials and Methods), the sporulation frequency of the *comQ* mutant (ROM261) was approximately 0.1 to 0.5 percent while that of the isogenic wild type (AG130) under similar conditions was typically 10 to 20 percent. Addition of purified competence pheromone to the *comQ* mutant one hour before the initiation of sporulation restored sporulation to levels indistinguishable from wild-type levels. These results indicated that the competence pheromone stimulated sporulation under some conditions.

Homologs of *comQ* and *comX*

In the gram-negative bacterium *Paracoccus denitrificans,* three genes, *moxZ, moxY,* and *moxX* are required for the transcriptional regulation of genes involved in the utilization of methanol. *moxY* and *moxX*, like *comP* and *comA,* are homologous to two-component regulators. The third gene, *moxZ,* shows only a twilight level of similarity to *comQ,* but a striking positional and functional similarity. It will be interesting to know the mechanism by which *moxZ* affects gene regulation *Paracoccus*.

A recent search of the translated DNA database showed two matches, from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, to the deduced sequence of *comQ* (Figure 14). In both cases, *comQ* was homologous to a partial *orf.* These *orfs,* like *comQ,* were located immediatedly downstream

```
B.lic:  1097 MNHFIDLEIPNADLNRTLHAFLDAKDQLHFSELAFYHYQSFGGTDTDAAETLGAGIELLI 1276
             M   ++  I N DL++ L++F+D+K+   F+E +  HY  FGG + D A  LGAGIE+LI
B.sub:   206 MKEIVEQNIFNEDLSQLLYSFIDSKETFSFAESSILHYVVFGGENLDVATRLGAGIEILI 385
             MKEIV   + N  L + L +FI+ K+ FSF   +  HYV F G +    T L AGIE+LI
B.amy:  1377 MKEIVGDKVDNLHLEETLLTFINEKKHFSFGVLAFQHYVAFKGTHSSEITLLAAGIELLI 1556

B.lic:  1277 LAFDIFDDLEDEDSPDEPWMKINRSVAMNAATALYTISI 1393 (truncated)
             L+ DI DDLEDED+    WMKINRS ++NAA +LYT+ +
B.sub:   386 LSSDIMDDLEDEDNHHALWMKINRSESLNAALSLYTVGLTSIYSLNNNPLIFKYVLKYVN 562
             L+ DI DD+EDEDN +  WM+ + + SLNAA SLY++ L +I  L +N
B.amy:  1557 LAFDIFDDIEDEDNFNKAWMQTDHAISLNAATSLYSISLQAICELESN 1700 (truncated)

B.sub:   566 EAMQGQHDDITNKSKTEDESLEVIRLKCGSLIALANVAGVLLATGEYNETVERYSYYKGI 745
B.sub:   746 IAQISGDYYVLLSGNRSDIEKNKHTLIYLYLKRLFNDASEDLLYLISHKDLYYKSLLDKE 925
B.sub:   926 KFQEKLIKAGVTQYISVLLEIYKQKCISAIEQLNLDKEKKELIKECLLSYTKGDTRCKT 1102
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Figure 14. Comparison of *Bacillus subtilis comQ* with homologs in *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. The sequence of ComQ was compared to the translated DNA database using TBLAST (Altschul et al., 1990). ComQ of *B.subtilis* is 49% identical (49 out of 99 residues) to a partial ORF from *Bacillus licheniformis* and 47% identical (51 out of 108 residues) to a partial ORF from *B.amyloliquefaciens*. *comQ* and it homologs are all located immediately downstream of *degQ* (aka *sacQ)* and its homologs. The putative *comQ* homologs are truncated due to the lack of additional sequence. *B.subtilis* sequence is from M7183.Gb_Ba (Weinrauch et al., 1991). *B. licheniformis* sequence is from A02550.Gb_Pat (Amory et al., 1987; Amory et al., 1988). *B. amyloliquefaciens* sequence is from M36595.Gb_Ba (Tomioka et al., 1985).

of *degQ* or its homologs. *degQ* is a small gene whose presence on a multicopy plasmid results in an increase in the production of extracellular proteases (Yang et al., 1986). It should be interesting to see if these homologs of *comQ* regulate competence or sporulation and if they are followed by homologs of *comX, comP* and *comA*.

Discussion

The expression of competence genes (*srfA*, *comG*) and development of genetic competence in *B. subtilis* is controlled by a competence pheromone that accumulates in culture medium as cells grow to high density. This competence pheromone is a peptide of 9 or 10 amino acids with a modification of approximately 206 daltons. The pheromone is produced during growth and the concentration of the pheromone increases with the density of the culture. Possibly, the regulation of competence development by a density dependent pheromone may ensure that cells become competent when they are most likely to encounter homologous DNA. Extracellular DNA is naturally present in competent cultures and thus transformation is taking place even when no exogenous DNA is added (Ephrati-Elizur, 1968). Since pheromone affects sporulation as well as competence, the pheromone may be a general signal for high cell density.

At least two genes are needed for production of the competence pheromone. *comX* is a 55 amino acid open reading frame (Figure 10). The amino acid sequence (disregarding the blank cycle) of purified pheromone matches the C-terminal 10 amino acids of ComX. Mutations in *comX* block production of the pheromone. Non-polar mutations in *comQ*, the gene immediately upstream of *comX*, also block production of the pheromone. It seems likely that the *comQ* gene product is involved in the processing and/or

modification of the pheromone, or in the release of the pheromone from the cell. It may be possible to determine the role of *comQ* once antibodies or other means are availible to detect intermediates in pheromone production. It might also be interesting to further characterize the structure and function of the *comQ* homologs found in other species of *Bacillus*.

Additional genes are likely to be involved in export of the pheromone. It is possible that the pheromone leaves the cell via the major secretion pathway, but ComX does not have a signal sequence or a leader peptidase cleavage site. The competence pheromone might be released by cell lysis, but the production of pheromone was not associated with general cell lysis. Microcins and haemolysins in *E. coli* (reviewed in (Fath and Kolter, 1993)(Kolter and Moreno, 1992), **a**-factor in *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1989), and a competence pheromone in *Streptococcus pneumoniae* (Hui and Morrison, 1991) are but a few of the products that are transported by specific ATP-dependent export systems. This type of transporter might be involved in export of the competence pheromone from *B. subtilis*.

It appears that a larger precurser is modified and processed to yield the mature competence pheromone. Many ribosomally synthesized peptide antibiotics are produced in a similar fashion. The gyrase-inhibitor, microcin B17, and gram-positive lantibiotics such as nisin, epidermin, cinnamycin, and subtilin are modified peptides derived from the C-terminus of a longer precursor peptide (Hansen, 1993; Kolter and Moreno, 1992). Similarly, the α-factor mating pheromone of *Saccharomyces cerevisiae* is a modified peptide derived from a larger precursor (Sprague and Thorner, 1992). The amino-terminal parts these precursor peptides are hypothesized to be important for recognition of the precursor by modification or export proteins. Further work on the competence pheromone may reveal the nature and function of the

modification or may elucidate the pathway for the modification, proteolytic cleavage and export of the competence pheromone.

Most peptide antibiotics and some peptide pheromones, such as the competence pheromone of *Bacillus subtilis,* contain unusual or modified constituents. In the case of the antibiotics, it seems plausible that the unusual structures might be important for their antimicrobial functions. For cell-cell signaling, this seems somewhat less plausible. The prevalence of modified peptide signaling molecules might be explicable, however, if antibiotics were occassionally recruited to function as cell-cell signals (or *vice versa*). Most antibiotics are small, diffusible, extracellular molecules, and thus, can be good indicators of local cell density. Resistance to antibiotics (such as tetracycline, erythromycin, chloramphenicol and vancomycin) is often induced by the antibiotic itself. In combination, an antibiotic and regulators that sense it, could be used as a device to sense and respond to cell density. Thus, it is possible that the unusual features of some microbial cell-cell signaling molecules might reflect ancestral functions, in microbial warfare, rather than present functions, as cell-cell signals. Thiostrepton, a peptide antibiotic of *Streptomyces*, has both functions. At high concentrations, thiostrepton inhibits protein synthesis and, at lower concentrations, thiostrepton induces a regulon of unknown function (Holmes et al., 1993; Murakami et al., 1989).

Many bacteria, both gram-positive and gram-negative, have pathways for natural transformation, including species of *Streptococcus* (*Pneumococcus*), *Haemophilus*, *Neisseria*, and *Bacillus*. Mechanisms of competence induction differ in these organisms, and only in some cases, such as in *Streptococcus,* is competence known to be induced by an extracellular competence factor (Tomasz and Hotchkiss, 1964; Tomasz and Mosser, 1966). More recent work has identified genes that may be required for the production of (Hui and

Morrison, 1991) and response to (Pearce et al., 1994) the streptococcal competence factor. It will be interesting to see if the pathways for pheromone production and response in species of *Streptococcus* are similar to those in *B. subtilis*.

Chapter 4:
Characterization of Competence Stimulating Factor

Abstract

CSF (competence stimulating factor) is, at least in part, a small extracellular peptide that stimulates the development of competence but that is chromatographically distinct from the competence pheromone. CSF and pheromone both induce expression of *srfA*. The effects of CSF and pheromone on expression of *srfA* are approximately multiplicative.

spo0F, spo0B, spo0A and *spo0H* mutants are defective in the expression of *srfA* and in the production of *srfA*-inducing activity. These mutations partially block production of CSF, but do not block production of pheromone. Conversely, *comX* and *comQ* mutations eliminate production of the competence pheromone without eliminating the production of CSF. Thus, CSF and pheromone are genetically as well as chromatographically distinct.

The defect in expression of *srfA* in a *spo0H* mutant was partially rescued extracellularly, indicating that a significant portion of the competence defect of a *spo0H* mutant was due to the lack of CSF, and that the lack of CSF has appreciable effects on the development of competence.

Competence-inducing signals may be species-specific. Conditioned medium from seven other species of *Bacillus* failed to induce expression of *srfA-lacZ* and in three cases inhibited expression of *srfA-lacZ*. Genetic exchange with other species might be dangerous to the individual bacterium or might erase the distinctions between species.

Introduction

The *spo0* genes are pleiotropic regulatory genes that were first identified because of their involvement in sporulation. Many of these genes are also required for the development of competence. As shown here, the requirement of several of the *spo0* genes for competence is partly explained by the requirement of the *spo0* genes for the production of competence stimulating factor (CSF). Although competence pheromone and CSF are both extracellular *srfA*-inducing factors, they are biochemically, genetically and functionally distinct.

spo0H encodes σ^H (Carter and Moran, 1986; Dubnau et al., 1988), a sigma factor of RNA polymerase that regulates the expression of a large set of genes by determining the promoter specificity of RNA polymerase. *spo0H* is required for the normal expression of *srfA* (Jaacks et al., 1989) and *comG* (Albano et al., 1987) and for the development of competence (Sadaie and Kada, 1983).

Spo0A is a transcription factor of the two-component family (Ferrari et al., 1985; Kudoh et al., 1985). Spo0A is activated by phosphorylation. Spo0F and Spo0B are required to transfer phosphate from histidine protein kinases to Spo0A (Burbulys et al., 1991). *spo0F, spo0B* and *spo0A* are all required for the development of competence (Sadaie and Kada, 1983). Phosphorylated Spo0A activates the transcription of some genes (Satola et al., 1991; Satola et al., 1992; York et al., 1992), but represses the transcription of other genes, such as *abrB* (Perego et al., 1988). AbrB represses the transcription of a variety of genes involved in the production of antibiotics, proteases, and other extracellular enzymes. Thus, phosphorylation of Spo0A leads to the induction of some genes and to the derepression of AbrB-controlled genes. Mutations in

abrB restore antibiotic and protease production in a *spo0A* mutant (Guespin-Michel, 1971a; Guespin-Michel, 1971b; Ito et al., 1971). As shown here, CSF production, like antibiotic production, is reduced in a *spo0A* mutant and is restored by a second mutation in *abrB*.

Results

CSF and pheromone are chromatographically distinct

Pheromone, but not CSF, was extractable with n-butanol. CSF and pheromone were also chromatographically distinct. On a C18 reverse phase column in 0.1% TFA (triflouroacetic acid), CSF eluted at less than 10% acetonitrile, while pheromone eluted at greater than 40% acetonitrile. Thus, pheromone was more hydrophobic than CSF. CSF and pheromone were conveniently separated by their differential hydrophobicity on a single column (Figure 15).

Activities of CSF and pheromone

The maximum response to CSF was markedly lower than the maximum response to pheromone (Figure 16). Response was measured by expression of *srfA*. The difference in the saturation characteristics of CSF and pheromone indicated that the activities of the two factors were intrinsically different. The analysis of the combined effects of CSF and pheromone gave further support to the idea that the activities of CSF and pheromone were different.

In a *comQ* mutant, which has no endogenous source of pheromone, the response to CSF in the presence of pheromone was greater than the response to CSF in the absence of pheromone (Figure 17). Closer inspection of the data shows that although the level of the response to CSF was different in the presence and absence of pheromone (Figure 17), the induction ratio or fold-effect of CSF (relative to fresh medium) was approximately the same in the

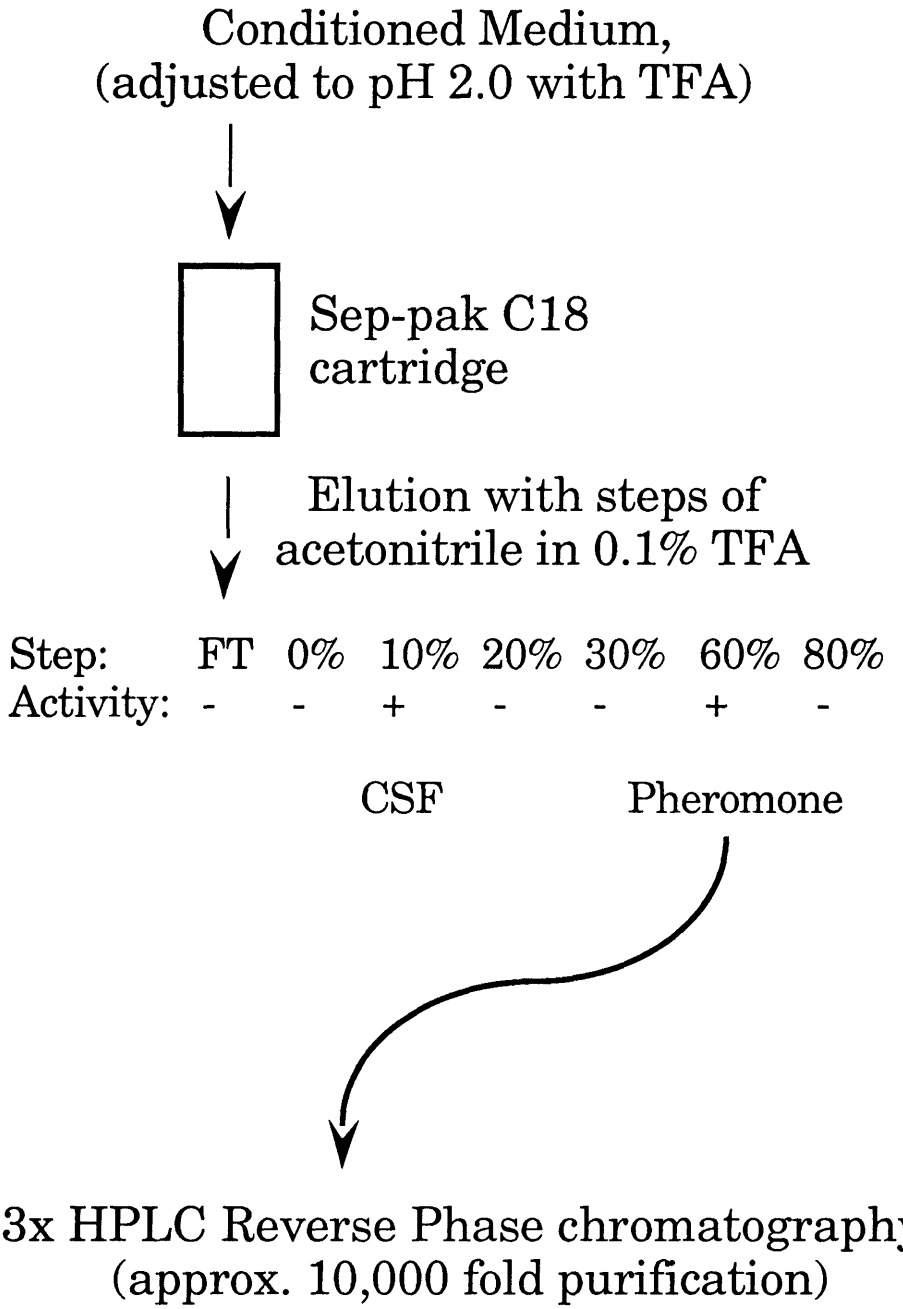


Figure 15. Separation of CSF and Pheromone. 10 to 20 ml of conditioned medium was adjusted to pH 2.0 and applied to a 0.3 gm sep-pak C18 column (Waters). Column was eluted with increasing concentrations of acetonitrile in 5 ml steps. The fractions were dried under vacuum in a speedvac, resuspended in fresh medium, and tested, at various concentrations, for *srfA*-inducing activity. Activity was detected in two distinct fractions, as indicated.

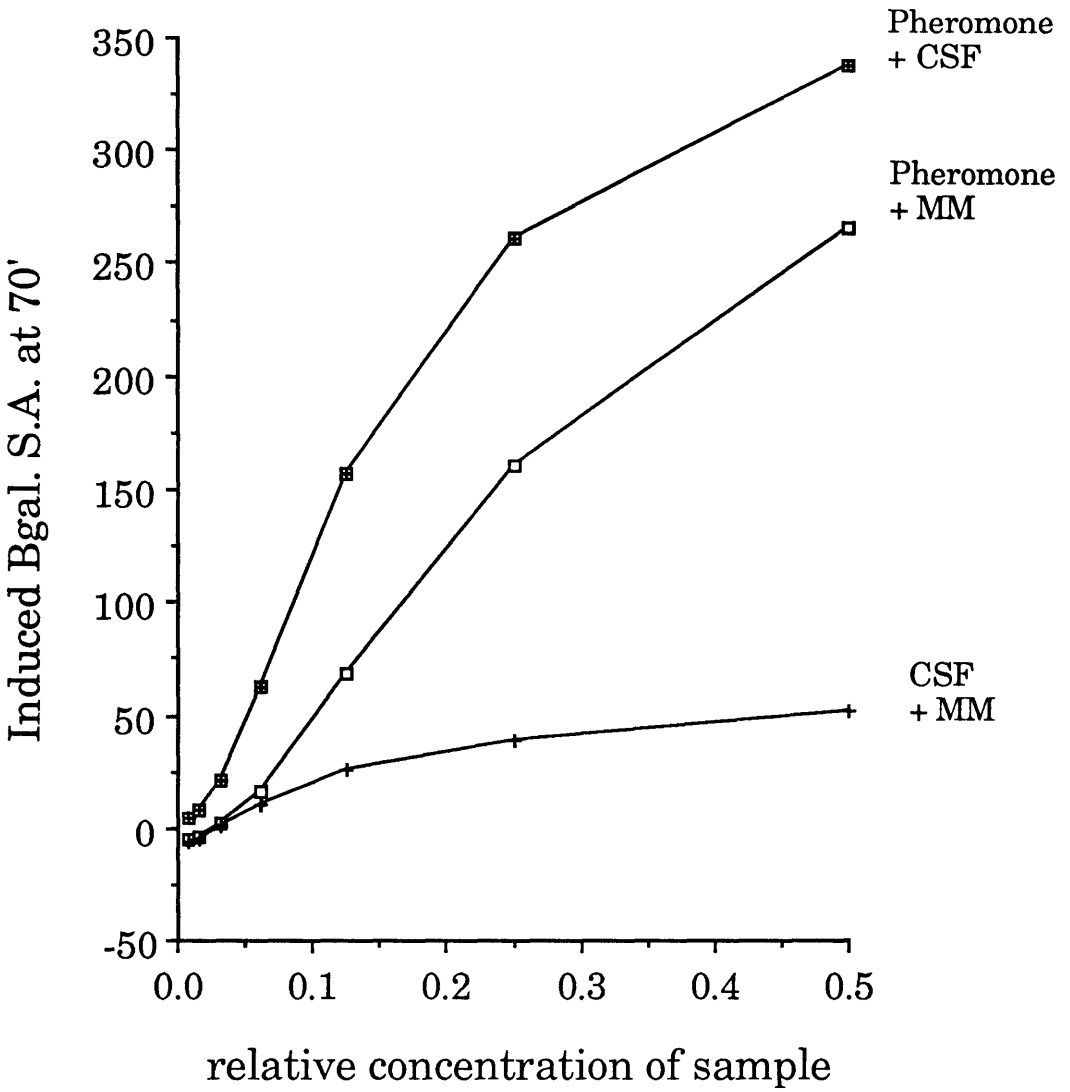


Figure 16. Dose-Response of CSF and Pheromone. CSF gave a lower maximum response than pheromone or crude conditioned medium when tested for *srfA*-inducing activity. CSF and pheromone were separated as described (Figure 15) from conditioned medium prepared from ROM119. Each preparation was mixed with minimal medium (MM) or with the other preparation, and tested for *srfA*-inducing activity in the presence of 50 μg/ml BSA. The indicator strain was JRL293.

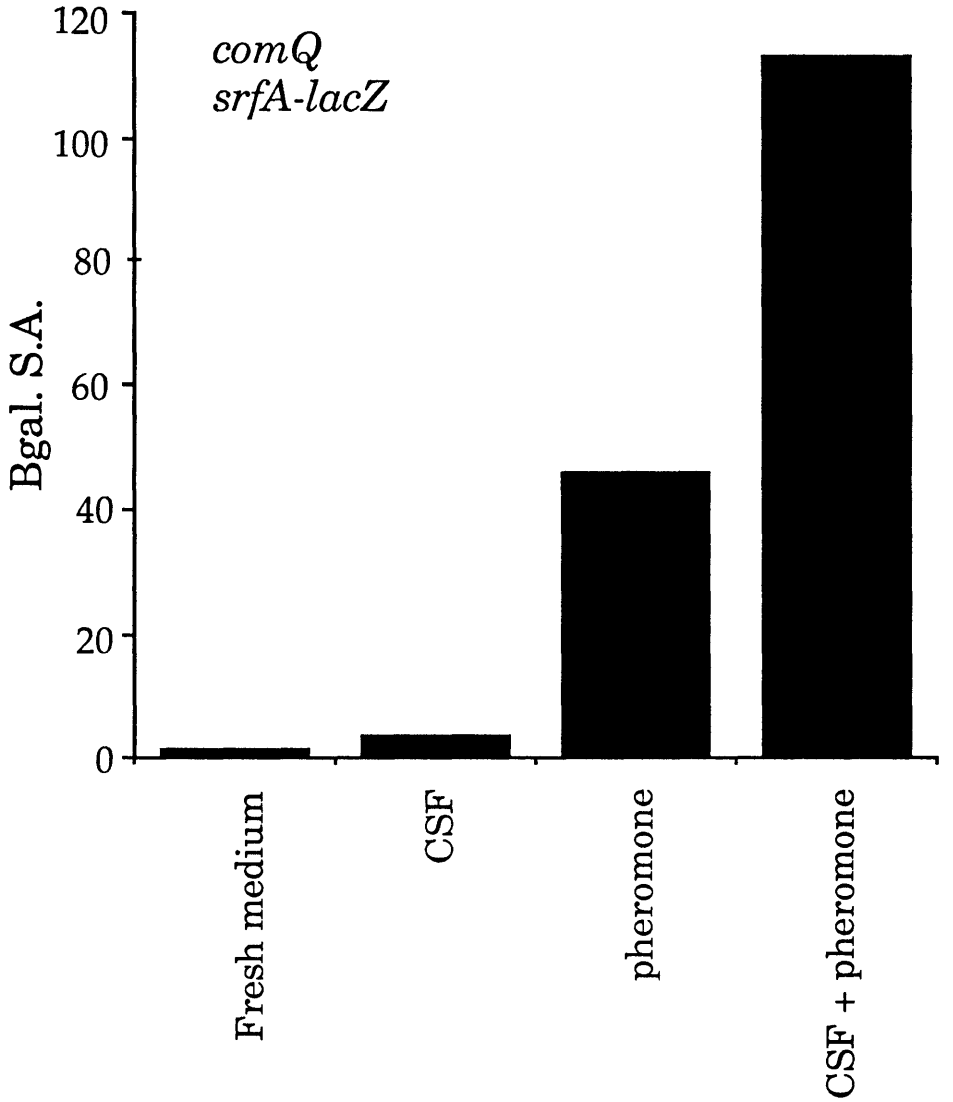


Figure 17.

Combined effects of CSF and Pheromone on expression of *srfA-lacZ*. CSF and pheromone collaborate to induce expression of *srfA*. Partly purified preparations of CSF and pheromone (from ROM119 conditioned medium) were tested separately and together for their ability to induce expression of *srfA-lacZ*. Testing was done in a *comQ* mutant (to eliminate endogenous production of pheromone), at low density (to minimize the effects of any endogenously produced CSF), and in the presence of BSA (to control for any nonspecific effects). The indicator strain was JMS323 (*amyE*::(*srfA-lacZ, cat*), Δ*comQ*::*spc*, *trp, phe*).

presence and in the absence of pheromone (Table 3). The fold-effect of pheromone was similar in the presence and absence of CSF (Table 3).

BSA (Bovine Serum Albumin) (at 50 ug/ml) enhanced the activity of the competence pheromone. As might be expected, this effect was most pronounced with highly purified preparations of pheromone, although effects were also seen with subsaturating quantities of partially purified competence pheromone. In a *comQ* mutant, BSA by itself had no detectable effect on expression of *srfA-lacZ*. BSA had significant effects only when mixed with pheromone. CSF, in contrast to BSA, had a two-fold effect on expression of *srfA-lacZ* (Table 3) in the presence and absence of pheromone, indicating that CSF worked independently of both BSA and pheromone. Thus, it appeared that BSA affected expression of *srfA* by affecting the activity of the competence pheromone, and that CSF affected expression of *srfA* independently of both pheromone and BSA.

Characterization and Purification of CSF

CSF, like pheromone, passes through ultrafiltration membranes with a nominal molecular weight cut-off of 10,000 daltons and is protease sensitive (trypsin and pronase). Therefore, CSF appeared to be, at least in part, a small peptide. CSF was partially purified (approximately 100,000-fold) by cation exchange and reverse phase chromatography. Conditioned medium from a *spo0K* mutant grown to saturation in minimal glucose medium was adjusted to a pH of 2.0 with TFA (triflouroactetic acid) and applied to a Sep-pak C18 cartridge. CSF was eluted with 10% acetonitrile and 0.1% TFA. This material was loaded on an sulfo-propyl sephadex column and eluted (at about 60mM NaCl) with a linear gradient of NaCl in 25 mM sodium acetate, pH 4.0. Material was then HPLC purified twice on a Vydac C18 column using linear gradient of 0.2 % acetonitrile per minute in 0.1% TFA. Material was

Samples, 1x, no BSA	Induction Ratio	Fold-Effect of CSF	Fold-Effect of Pheromone	Fold-Effect of BSA
fresh medium	1.0			
CSF	2.3	2.3		
Pheromone	30		30	
CSF + Pheromone	70	2.5	32	
Samples, 1x, + BSA				
fresh medium	1.0			1.0
CSF	2.3	2.3		1.0
Pheromone	32		32	1.1
CSF + Pheromone	78	2.5	33	1.1
Samples, 1/4x, no BSA				
fresh medium	1.0			
CSF	2.0	2.0		
Pheromone	2.7		2.5	
CSF + Pheromone	6.7	2.5	3.3	
Samples,1/4x, + BSA				
fresh medium	1.0			1.0
CSF	2.0	2.0		1.0
Pheromone	5.7		5.7	2.1
CSF + Pheromone	16	2.9	8.2	2.5

Table 3. Effects of Pheromone, CSF and BSA on expression of *srfA*. CSF, pheromone and BSA were tested, separately and in all combinations, for their effect on expression of *srfA* in 70 minutes. Testing was done in a *comQ* mutant (JMS323), so as to eliminate endogenous production of pheromone, and was done at low density, so as to minimize the effects of any endogenously produced CSF. The induction ratio is the ß-galactosidase activity of cells mixed with sample, divided by the ß-galactosidase activity of cells mixed with fresh medium. The individual, fold-effects of CSF, Pheromone and BSA were calculated by dividing the activity of a particular sample by the activity of a similar sample, lacking the component of interest, but containing all other components. BSA had no effect by itself, but it did have a two-fold effect in the presence of sub-saturating concentrations of pheromone. CSF, in contrast, had a two-fold effect alone, and in the presence of pheromone or BSA or both. Pheromone had approximately a 30-fold effect at high concentrations, and a 3- to 8-fold effect at low concentrations.

repurified a third time with a shallower gradient of 0.04% acetonitrile per minute. CSF eluted at less than 5% acetonitrile under these conditions. After each step in the purification active fractions were concentrated by rotovap or speedvac. Concentrated fractions were resuspended in buffers appropriate for the next step in the purification and the pH was adjusted as necessary.

The most pure preparations of CSF (approximately 100,000-fold purification) showed a complex elution profile (Figure 18). Analysis by mass spectrometry of the active fractions revealed seven species ranging in mass from approximately 520 to 720 daltons. If the mass of CSF is in fact between 520 and 720 daltons, if CSF consists entirely of amino acids, and if the mean residue mass is neither exceptionally large nor exceptionally small, then CSF might be a peptide of 4 to 7 amino acid residues.

Production of *srfA*-inducing activity

spo0A, spo0F, spo0B and *spo0H* mutations caused a defect in the expression of *srfA* (Figure 19), as well as causing defects in sporulation and competence. *spo0A* mutations had a stronger effect on expression of *srfA* than *spo0F, spo0B* and *spo0H.* Since expression of *srfA* was dependent on extracellular factors, it seemed plausible that these *spo0* mutations might affect production of one or both of the extracellular factors.

To test this idea, conditioned medium was prepared from wild-type and *spo0H* cultures that were grown in minimal medium to a density of approximately 4.0 O.D. at 600 nm. These conditioned media were tested for *srfA*-inducing activity (Figure 20). Conditioned medium from a *comQ* strain, which is defective in the production of the competence pheromone, and conditioned medium from a *comQ spo0H* double mutant were also tested for *srfA*-inducing activity. Conditioned media from *comQ* and *spo0H* strains were quantitatively defective relative to wild-type conditioned medium in their

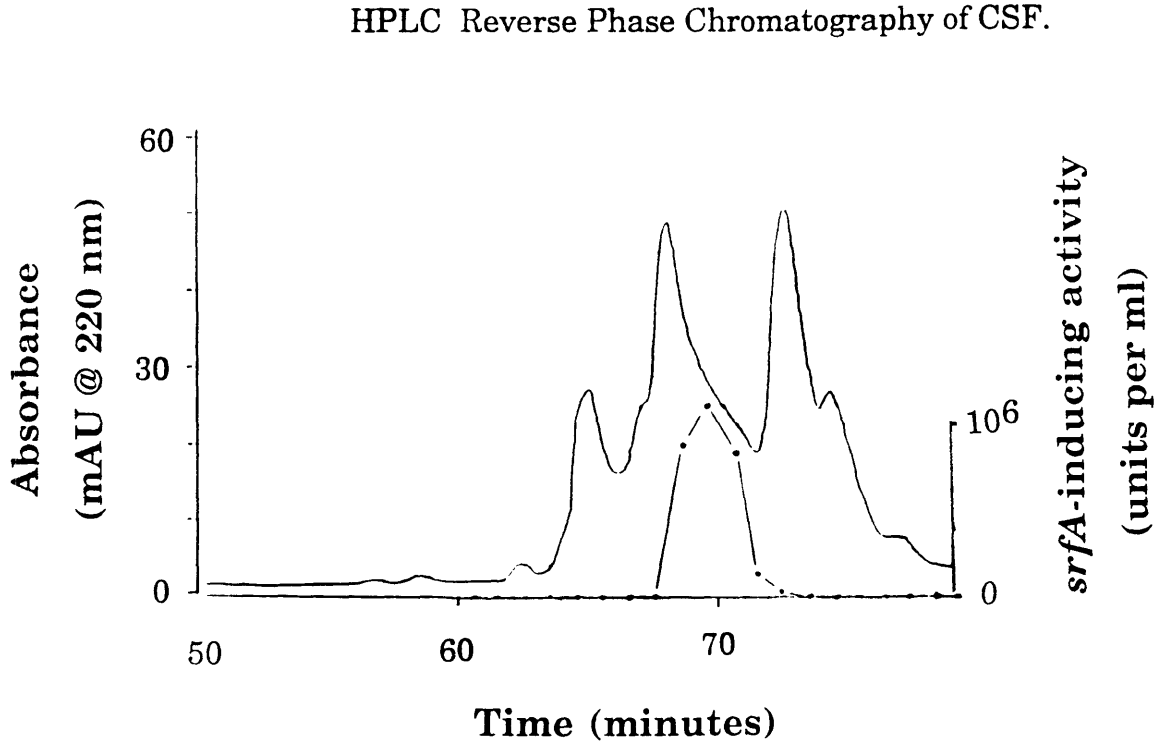


Figure 18. Purification of CSF. The *srfA*-inducing activity (black dots) and the absorbance at 220 nm (smooth curve) are shown for highly purified CSF eluting from an HPLC C18 column in a linear gradient of acetonitrile in 0.1% TFA. The specific activity of this material (activity divided by absorbance at 220 nm) was approximately 100,000-fold greater than the specific activity of conditioned medium. The yield was approximately 6%.

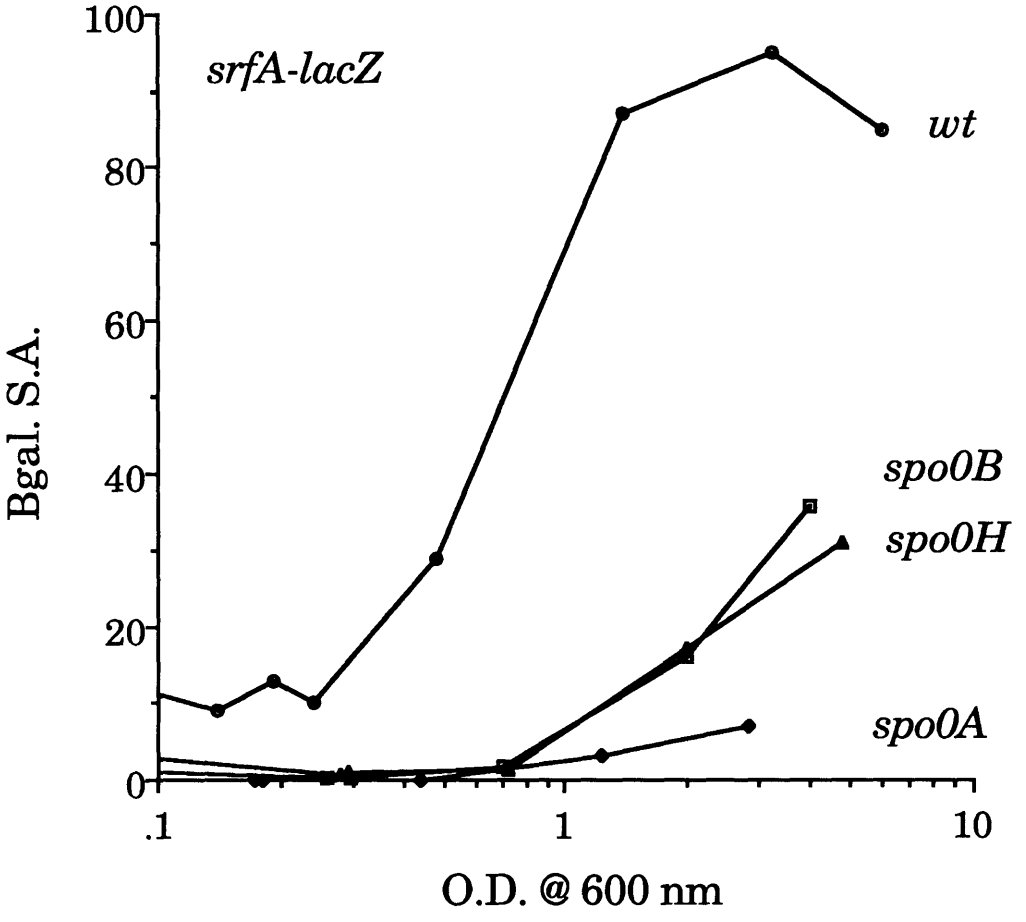


Figure 19. Expression of *srfA-lacZ* is reduced in *spo0B*, *spo0H*, and *spo0A* strains. Strains were grown in minimal medium. Expression of *srfA*, as measured with *csh293*::Tn*917*lac, was lower in *spo0B*, *spo0H* and *spo0A* strains (KJ639, KJ482, ROM050) than in a wild-type background (KJ388).

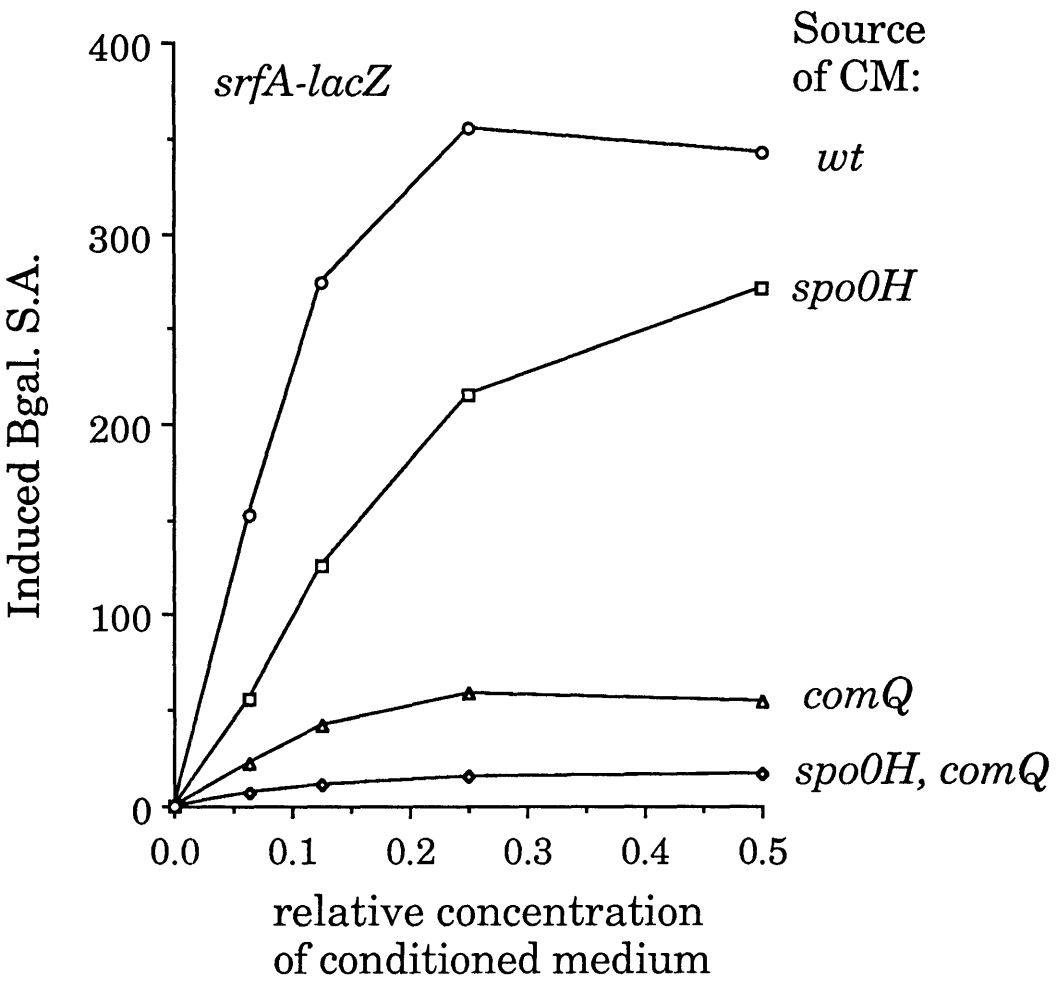


Figure 20. *srfA*-inducing activity of conditioned media. Conditioned medium from the *comQ spo0H* double mutant was more defective than conditioned medium from either single mutant. A similar result was observed with *spo0A comQ* double mutants. *wt, spo0H, comQ* and *spo0H comQ* conditioned media were harvested from minimal cultures of, respectively, ROM100, AG665, ROM297, and ROM302 at a density of approximately 3.0 O.D. at 600 nm. The conditioned media were tested for *srfA*-inducing activity on JMS374 (*amy::(srfA-lacZ, neo), trp, phe*) in the presence of 50 μg/ml BSA.

ability to induce expression of *srfA-lacZ*. Interestingly, conditioned medium from the *spo0H comQ* double mutant had a stronger defect in *srfA*-inducing activity than the defect of either single mutant. Furthermore, the maximum response to *comQ* and *spo0H* conditioned media was lower than the maximum response to *spo0H* conditioned medium (Figure 20), just as the maximum response to CSF was lower than the maximum response to pheromone (Figure 16).

Conditioned media prepared from wild-type, *spo0F, spo0B* and *spo0A* strains were also tested for *srfA*-inducing activity (Figure 21). The results indicated that these mutants, like *spo0H,* were quantitatively defective in the production of *srfA*-inducing activity. Mutations in *spo0A* had the largest defect in production of *srfA*-inducing activity and the largest defect in expression of *srfA*. Production of *srfA*-inducing activity in a *spo0A* mutant was restored by a second mutation in *abrB* (Figure 21).

Fractionation of Conditioned Media

In order to further characterize the defects in production of *srfA*-inducing activity, conditioned media from various mutants were fractionated on sep-pak columns and the fractions were tested for activity (Table 4). As expected, *comX* and *comQ* mutations abolished the production of pheromone. The residual activity in these strains was due to CSF. Conversely, *spo0A* mutations caused a strong defect in the production of CSF, but did not affect the production of pheromone. Mutations in *spo0F, spo0B* and *spo0H* also caused a similar but smaller defect in the production of CSF, but did not affect the production of pheromone. Thus, CSF and pheromone appeared to be genetically as well as chromatographically separable.

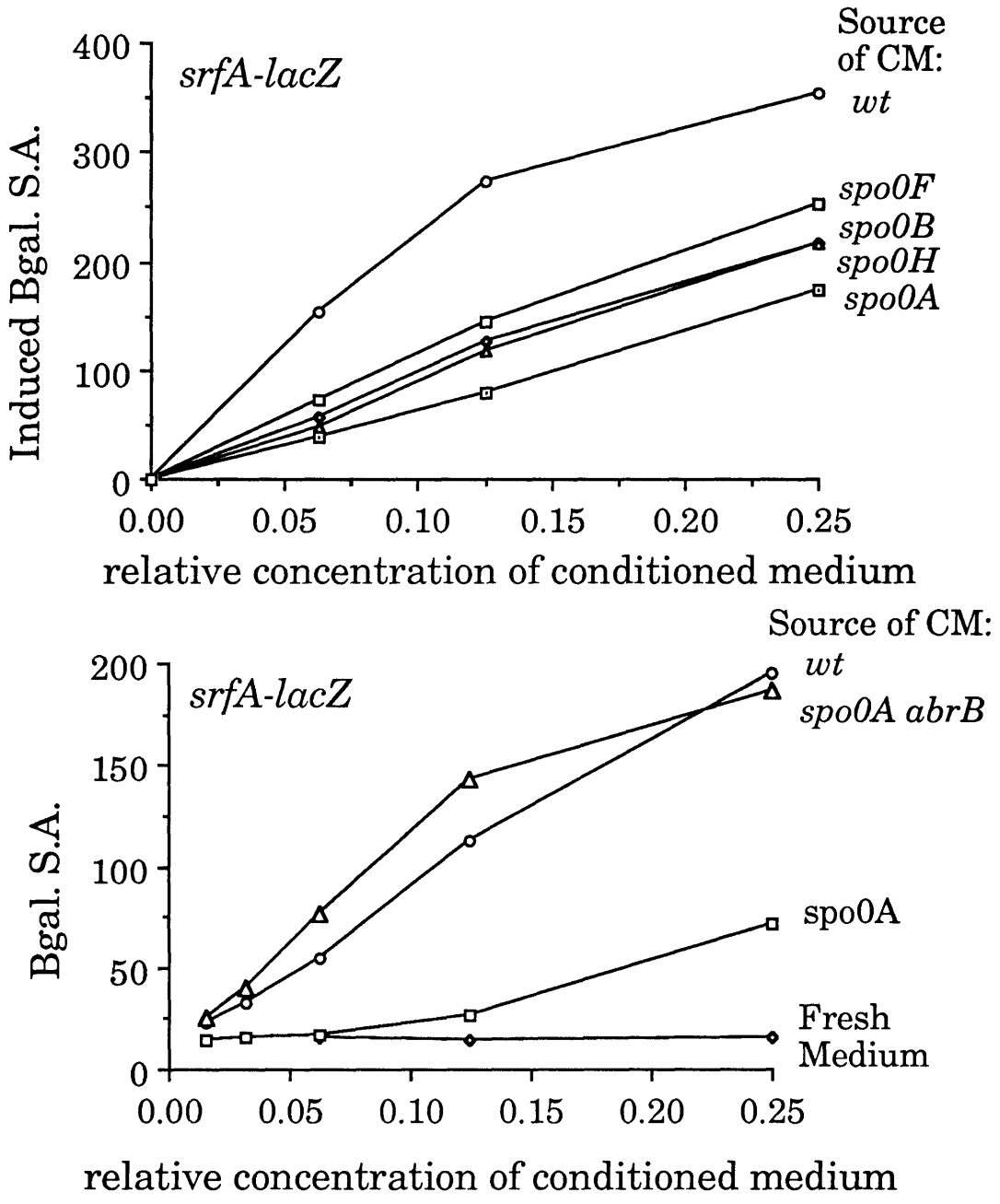


Figure 21. *srfA*-inducing activity of conditioned media from various strains. Conditioned media (CM) for each experiment were prepared from cultures of comparable cell density. **Top Panel:** Conditioned media from *spo0F, spo0B, spo0H* and *spo0A* (AG144, AG141, AG665 and AG665) showed a mild defect in the production of *srfA*-inducing activity relative to wild-type conditioned medium (ROM100). JMS374 (*amyE::(srfA-lacZ, neo), trp, phe*) was used as the indicator strain. **Bottom Panel:** Production of *srfA*-inducing activity in a *spo0A* mutant was restored by a second mutation in *abrB*. *wt, spo0A* and *spo0A abrB* conditioned media were prepared from ROM100, AG503, and AG132, respectively. JRL293 (*amyE::(srfA-lacZ, cat), trp, phe*) was used as an indicator strain.

Strain	Relevant Genotype	Crude Activity (%)	CSF Activity (%)	Pher. Activity (%)
ROM100	*wt*	100	100	100
AG132	*spo0A abrB*	186	123	166
JRL358	*spo0K*	275	191	428
AG1032	*comP*	86	82	107
ROM230	*comX*	61	74	**1**
ROM230	*comX*	10	5	**<2**
AG1520	*comQ*	11	6	**<2**
ROM297	*comQ*	31	22	**6**
ROM301	*srfA comQ*	19	24	**4**
NY120	*kinB kapB*	77	**20**	107
AG144	*spo0F*	20	**19**	110
AG141	*spo0B*	20	**13**	110
AG1226	*spo0H*	18	**8**	78
AG665	*spo0H*	16	**9**	68
AG503	*spo0A*	11	**4**	40
AG503	*spo0A*	42	**3**	129
ROM298	*comQ spo0A*	**3**	**1**	**8**
ROM302	*comQ spo0H*	**3**	**1**	**1**

Table 4. Fractionation and analysis of conditioned media. Conditioned medium from each strain was fractionated on small sep-pak columns (Figure 15). Original and fractionated conditioned media were tested for *srfA*-inducing activity on JRL293 in the presence of BSA. The activity of crude conditioned medium; the activity of the CSF fraction; and the activity of the pheromone fraction were normalized to the activities of similar samples from wild-type conditioned medium, prepared, fractionated, and assayed at the same time as a given mutant conditioned medium.

Partial Rescue of *spo0H*

Since *spo0F, spo0B, spo0A* and *spo0H* were defective in expression of *srfA* and were defective in the production of CSF, it seemed possible that the defect in expression of *srfA* in these mutants could be rescued extracellularly. To test this hypothesis, we tried to rescue the competence defect of a *spo0H* mutant by adding conditioned medium from a wild-type strain. We found that the addition of conditioned medium rescued the *spo0H* defect in expression of *srfA-lacZ*. That is, the expression of *srfA-lacZ* in *spo0H* cells was delayed or reduced relative to the expression of *srfA-lacZ* in *spo0H*$^+$ cells, but in the presence of exogenously added wild-type conditioned medium, expression of *srfA-lacZ* in *spo0H* cells was virtually indistinguishable from expression of *srfA-lacZ* in *spo0H*$^+$ cells (Figure 22). *spo0F, spo0B* and *spo0A* showed some response to conditioned medium, but were only partially rescued, indicating that these genes may affect expression of *srfA-lacZ* by an intracellular as well as by an extracellular route. The interpretation of conditioned medium shifts in *spo0F, spo0B* and *spo0A* strains was complicated by cell lysis and/or the cessation of cell growth in these strains upon addition of conditioned medium. Wild type and *spo0H* strains were not adversely affected by the addition of conditioned medium.

Species specificity of *srfA*-inducing activity

Genetic exchange is observed in test mixtures of *Bacillus subtilis* in liquid culture (Ephrati-Elizur, 1968) and in soil (Graham and Istock, 1979) and appears to be frequent and efficient in the wild (Istock et al., 1992). Genetic exchange within a species should be beneficial (Felsenstein, 1985), but efficient

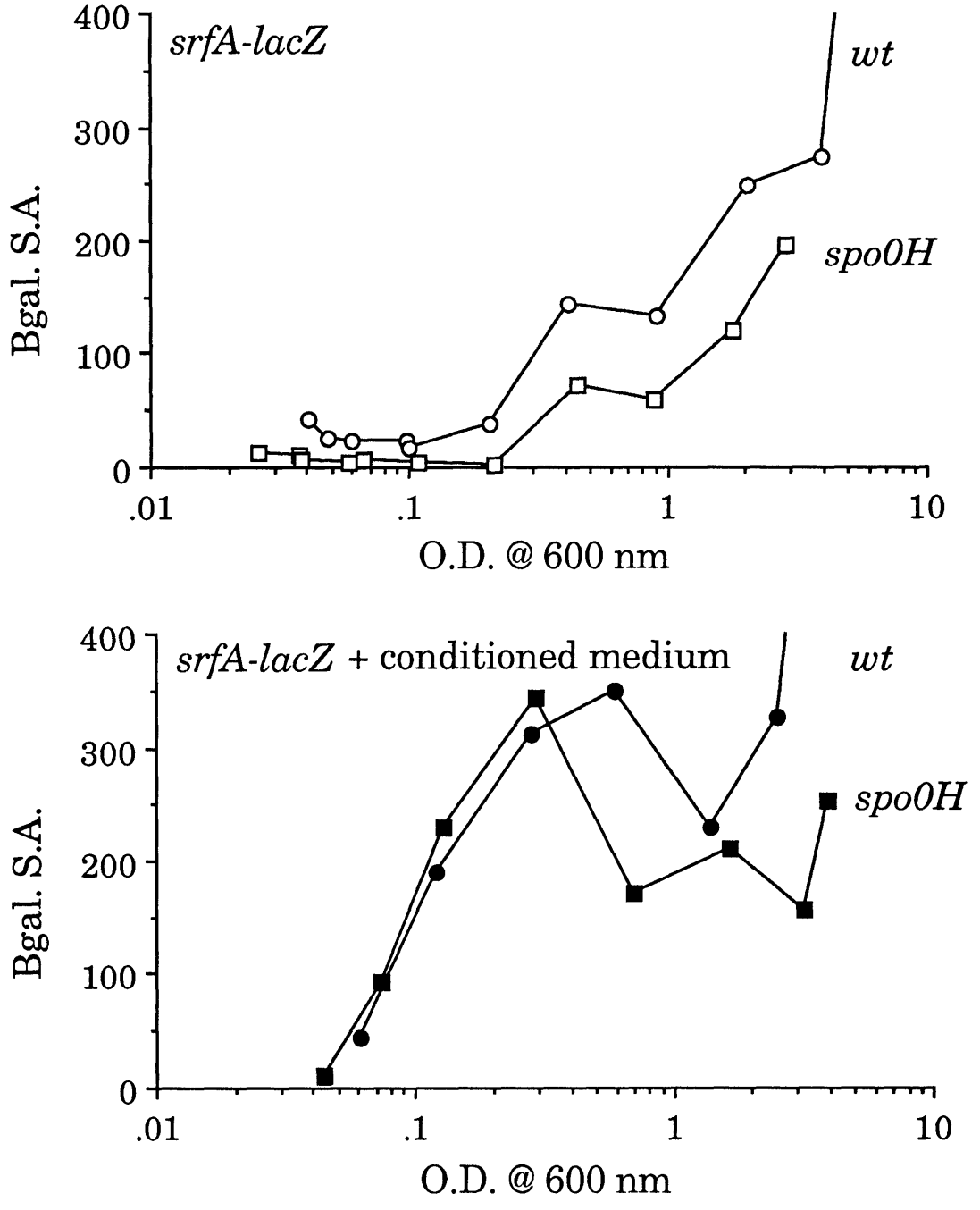


Figure 22. The defect in expression of *srfA-lacZ* in a *spo0H* mutant (top panel) is rescued by conditioned medium (bottom panel). Cells were grown for three doublings in minimal medium before the start of the experiment. Expression of *srfA-lacZ* in wild-type (JRL293, circles) and in *spo0H* (JMS139, squares) backgrounds was measured following the addition of fresh medium (open symbols) and conditioned medium (filled symbols).

genetic exchange between species could be dangerous or could erase species distinctions. Soil is a rich and diverse microbial habitat, containing high concentrations of many microorganisms. Recombinational barriers offer partial, but imperfect, protection against heterologous transformation (Duncan et al., 1989). Thus, "mate selection" or sexual selectivity might be important in the wild. Mechanisms that enhance sexual selectivity may minimize the dangers of sex while preserving the benefits and may also be important in the establishment and maintenance of bacterial species. It was hypothesized that CSF and pheromone might help to determine the sexual selectivity of *Bacillis subtilis.* If the competence-inducing factors were species specific, then *Bacillus subtilis* would become competent only when surrounded by *Bacillus subtilis*, and thus, might be preferentially transformed by *Bacillus subtilis* DNA.

Conditioned media made from various strains of *Bacillus* were tested for their ability to induce expression of *srfA-lacZ* at low density in *Bacillus subtilis* (Table 5). This test can detect either CSF or pheromone activity. Only conditioned medium from *Bacillus subtilis* 168 induced expression of *srfA-lacZ.* Conditioned medium from three species (*Bacillus cereus*, *Bacillus globigii* and the divergent, W23 isolate of "*Bacillus subtilis*") failed to induce *srfA-lacZ* and also inhibited the induction of *srfA-lacZ* by conditioned medium from *Bacillus subtilis* 168. Such inhibitory activity might be due to the action of proteases, antibiotics, phage or competitive inhibitors. Conditioned medium from four other species (*Bacillus licheniformis, Bacillus megaterium, Bacillus pumilis* and most isolates of *Bacillus thuringiensis*) neither induced expression of *srfA-lacZ* nor interfered with the induction of *srfA-lacZ* by conditioned medium from *Bacillis subtilis*.

Strain	BGSC code	Species	Activity of test CM:	Activity of a mixture of test and control CMs:	Net Activity of test CM:
ROM264	1A1	*Bacillus subtilis*	+	+	+
ROM265	2A2	*Bacillus subtilis (W23)*	O	O	-
ROM266	3A1	*Bacillus subtilis*	+	+	+
ROM267	4A1	*Bacillus thuringiensis serot-1*	O	+	O
ROM268	4B1	*Bacillus thuringiensis serot-2*	O	+	O
ROM269	4C1	*Bacillus thuringiensis serot-3a*	O	O	-
ROM270	5A2	*Bacillus licheniformis*	O	+	O
ROM271	5A24	*Bacillus licheniformis* transformable	O	+	O
ROM272	6A1	*Bacillus cereus*	O	O	-
ROM273	7A1	*Bacillus megaterium*	O	+	O
ROM275	7A13	*Bacillus pumilis*	O	+	O
ROM279	11A1	*Bacillus globigii*	O	O	-

Table 5. Species specificity of *srfA*-inducing activity. Conditioned media were prepared from various species of *Bacillus* grown to saturation in minimal medium at 30°C. These conditioned media were tested alone and in combination with active conditioned medium from *Bacillus subtilis* for *srfA*-inducing activity. JRL293 was used as the indicator strain and BSA (50 μg/ml) was included in all sample. **O**, no activity; **+**, *srfA*-inducing activity; **-** , inhibitory activity. Strains were obtained from the Bacillus Genetic Stock Center (BGSC).

At least one of these species, *Bacillus licheniformis,* has a homolog of *comQ*, is transformable itself, is often found in the same habitat as *Bacillus subtilis,* and has DNA that can transform *Bacillus subtilis*, albeit poorly (Duncan et al., 1989).

The inability of conditioned media from *Bacillus licheniformis* and other species to induce expression of *srfA-lacZ* in *Bacillus subtilis* is consistent with the hypothesis that extracellular competetence factors might exist in order to improve the sexual selectivity of *Bacillus subtilis*. Recombinational barriers and other extracellular factors, such as antibiotics and proteases, might also increase sexual selectivity.

Discussion

Mutations in *spo0A* reduce the production of CSF and of other extracellular products such as antibiotics and proteases. These effects are alleviated by a second mutation in *abrB*. Spo0F and Spo0B transfer phosphate groups from kinases to Spo0A. Since *spo0F* and *spo0B* mutations also reduce the production of CSF, it appears that phosphorylation of Spo0A has a positive effect on the production of CSF. The defect in expression of *srfA-lacZ* in a *spo0H* mutant, could be largely rescued extracellularly, indicating that a large portion of the *spo0H* defect in expression of *srfA* can be ascribed to the defect in production of CSF and that a defect in the production of CSF produces a significant defect in the expression of *srfA*. Mutations in *spo0F*, *spo0B* and *spo0A* also caused a defect in expression of *srfA-lacZ*, but these defects, unlike the defect in a *spo0H* mutant, were only partially rescued by wild-type conditioned medium. Thus, it appears that these genes may have an intracellular effect, as well as an extracellular effect, on expression of *srfA*.

Pheromone and CSF were found to be genetically as well as chromatographically distinct. That is, mutations that abolished the production of pheromone did not eliminate the production of CSF, and mutations that reduced the production of CSF did not reduce the production of pheromone. Experiments with partially purified preparations of CSF and pheromone indicated that the two factors had multiplicative effects on expression of *srfA-lacZ* in a *comQ* mutant, indicating that the two factors were functionally as well as biochemically distinct. Cross streak experiments (Chapter 5, Table 6) showed that strains lacking one factor were rescued by strains lacking the other factor and thus confirmed that the two factors were functionally as well as biochemically distinct.

Conditioned medium from seven other *Bacillus* species did not induce *srfA*, and in some cases inhibited the induction of *srfA*. The apparent absence of the competence-inducing signals in other species may help ensure that *Bacillus subtilis* becomes competent only in the presence of *Bacillus subtilis* and thus, is preferentially transformed by *Bacillus subtilis* DNA. The existence of two competence-inducing signals might enhance this sexual selectively and thus might help *Bacillus subtilis* to distinguish itself from other, closely related species. In the absence of such sexual selectivity, genetic exchange might be dangerous for *Bacillus subtilis* or might erase the distinctions between *Bacillus subtilis* and related, cohabiting bacterial species.

The idea that sexual selectivity might be generally desirable is supported by the fact that some species of transformable bacteria have a completely different and more direct way of exercising sexual selectivity. *Haemophilus influenzae* takes up DNA containing a certain 11 bp sequence that is common in its own DNA but is uncommon in the DNA of other, unrelated species (Danner et al., 1980). A similar mechanism is observed in the constitutively transformable gram negative species *Neisseria gonorrhea* (Goodman and Scocca, 1988). Thus, while *Bacillus subtilis* chooses the company in which it becomes competent, *Neisseria gonorrhea* and *Haemophilus influenzae* choose what particular pieces of DNA to take up. Both strategies enhance sexual selectivity.

In *Bacillus subtilis,* it is possible, as discussed above, that the existence of multiple competence-inducing extracellular signals is important in maintaining or establishing the species specific induction of competence. It is also possible that two signals may also simply increase the general specificity and magnitude of the response. Alternatively, having two signals may provide a way to integrate additional information. For example if the production of one

signal requires glucose, and the production of the other signal requires minimal media (no amino acids), then two nutritional conditions will have been added to the original density condition. The effect of nutrients on the production of CSF and pheromone has not been examined.

Extracellular effects on competence are observed in a variety of gram positive bacteria, including *Streptococcus pneumoniae* (Tomasz and Hotchkiss, 1964) , *Streptococcus sanguis* (Pakula and Walczak, 1963), *Bacillus stearothermophilus* (Streips and Young, 1971), and *Bacillus cereus* (Felkner and Wyss, 1964). Extracellular competence signals may be a fairly common way of regulating the development of competence. It will be interesting to see if multiple signaling molecules are involved in competence development in organisms other than *Bacillus subtilis*.

Several other examples of dual signaling molecules have been described in bacteria. In *Agrobacterium tumefaciens*, two classes of compounds commonly released by plant wounds, namely sugars (such as glucose) and phenolic compounds (such as acetosyringone), act synergistically through the *virA/virG* two component regulatory system to induce expression of other *vir* genes that are required for the transfer of the Ti plasmid from *Agrobacterium tumefaciens* to host plant cells. The requirement for two plant derived signals may increase the specificity and the sensitivity of the bacterial response. Also in *Agrobacterium tumefaciens*, opines (produced by Ti infected plant cells) and conjugal factors (homoserine lactone compounds produced by the *Agrobacterium tumefaciens*), act cooperatively to induce the conjugal transfer of the Ti plasmids from one bacterium to another. In this case, the two signals are produced by different agents, and thus provide different information. Temporal and spatial information may be provided by the multiple extracellular signals that regulate the formation of fruiting bodies in

Myxococcus xanthus. It appears that dual signaling may not be uncommon among microorganisms, and that dual signals may be used to increase specificity, to integrate signals, or coordinate developmental events.

Chapter 5:
Analysis of the Response to Pheromone and Competence Stimulating Factor

Abstract

Cross-streak experiments revealed the existence of two extracellular complementation groups, corresponding to defects in CSF and pheromone. The finding that members of one class could rescue members of the other class in a cross-streak assay indicated that CSF and pheromone were functionally, as well as biochemically, distinct.

comP, comA and *spo0K* strains are defective in the expression of *srfA*. However, these strains rescued *spo0A* and *comQ* mutants in cross-streak assays, indicating that these strains make both CSF and pheromone. Furthermore, preparation and fractionation of conditioned medium from *spo0K* and *comP* strains showed that these strains produced relatively normal levels of CSF, of pheromone, and of total *srfA*-inducing activity. *comP, comA* and *spo0K* mutants were not rescued extracellularly, either in cross-streak or in liquid culture. Thus, these three loci are required for the response to, rather than the production of, the two extracellular density signals.

A preliminary analysis of the residual expression of *srfA-lacZ* in single and double mutant combinations of *comQ, spo0A, comP* and *spo0K*, performed in collaboration with Jonathan Solomon, indicated that pheromone was sensed by ComP and that CSF was sensed by Spo0K. Further work by Jonathan Solomon has confirmed and extended these preliminary findings.

Introduction

Two biochemically and genetically distinct extracellular factors, CSF and pheromone, collaborate to induce the expression of *srfA*, and thus, to initiate the development of competence in *Bacillus subtilis*. Mutations in certain genes, notably *spo0A* and *spo0H,* reduced the production of CSF but not the production of pheromone. Conversely, mutations in *comX* and *comQ* abolished the production of pheromone without abolishing the production of CSF (Chapter 4). This chapter addresses the question of how these two extracellular factors are sensed. It is naively expected that the loss of a sensing component might cause a defect in expression of *srfA,* without causing a defect in the production of CSF or pheromone. Furthermore, this defect ought not be rescued by the addition of exogenous conditioned medium.

Experiments presented here show that the histidine protein kinase, ComP, the cognate response regulator, ComA, and the Spo0K oligopeptide permease are required for the response to, but not for the production of, the two extracellular factors. Furthermore, preliminary experiments performed in collaboration with Jonathan Solomon (and since extended by Jonathan), indicated that the sensing of the two extracellular factors, like the production of the two factors, occured by two genetically distinct pathways.

ComA, a transcription factor belonging to the two-component family of response regulators (Nakano and Zuber, 1989; Weinrauch et al., 1989), is required for the expression of *srfA* (Nakano and Zuber, 1989), and has been shown to bind to the promoter of *srfA* (Roggiani and Dubnau, 1993). Furthermore, phosphorylated ComA binds the *srfA* promoter better than nonphosphorylated ComA (Roggiani and Dubnau, 1993), and alteration of the ComA binding sites alters expression of *srfA* (Nakano and Zuber, 1993).

ComP is homologous to the two-component family of histidine protein kinases (Weinrauch et al., 1990), and is required for the expression of *srfA* (Nakano et al., 1991). The N-terminal portion of ComP has eight proposed membrane-spanning segments. Thus, ComP may be well situated to interact with an extracellular ligand.

spo0K, a five gene operon encoding an ATP-dependent transport system, is required for the uptake of oligopeptides (Perego et al., 1991; Rudner et al., 1991), for sporulation, for competence (Sadaie and Kada, 1983), and for expression of *srfA* (Grossman et al., 1991; Hahn and Dubnau, 1991; Magnuson et al., 1994; Nakano and Zuber, 1991). Spo0K belongs to a large, evolutionarily conserved family of transport systems, the ABC (ATP-Binding Cassette) transporters, that couple ATP hydrolysis to the transport of a variety of compounds (Higgins, 1992). ABC transporters are found in prokaryotes and eukaryotes, can be involved in import or export, and include such well-known proteins as the multi-drug resistance protein (MDR) and the cystic fibrosis transmembrane regulator (CFTR). Spo0K and the homologous oligopeptide permeases (Opp) from *E. coli* and *S. typhimurium* are not known to transport peptides larger than 5 amino acids. The transport of oligopeptides of up to five amino acids appears to be nonspecific (Hiles et al., 1987). A similar oligopeptide permease from *Lactococcus lactis* appears to transport some oligopeptides containing between 5 and 8 amino acids (Tynkkynen et al., 1993).

Results

Two extracellular complementation groups

comQ and *comX* mutants were defective in the production of pheromone. Similarly *spo0A* and certain other *spo0* mutants were specifically defective in the production of CSF and not in the production of pheromone (Chapter 4, Table 4). Thus, CSF and pheromone were not only biochemically different, but also appeared to be produced by genetically distinct pathways. Since the activities of CSF and pheromone were different (Figure 16, Figure 17), it seemed possible that their functions and mechanisms of action might also be different.

Cross-streak experiments provided positive evidence that CSF and pheromone were functionally distinct. In a cross-streak assay on SpII Xgal plates, *comQ* cells, lacking pheromone, were rescued by *spo0A* cells, and, conversely, *spo0A* cells, lacking CSF, were rescued by *comQ* cells (Table 6). If *spo0A* and *comQ* mutants were missing functionally similar factors, then one, less defective strain, might rescue the other, more defective strain, however, the two strains would not rescue each other. The reciprocity of the observed rescue indicated that *spo0A* and *comQ* were defective in functionally distinct extracellular *srfA*-inducing factors. *comQ* was defective in the *comQ* rescuing activity but not in *spo0A* rescuing activity. Conversely, *spo0A* and *spo0H* were defective in *spo0A* rescuing activity, but produced the *comQ* rescuing activity. Thus, two extracellular complementation groups, corresponding to the two extracellular factors, were defined. Wild-type strains produced both the *spo0A* and the *comQ* rescuing activities (Table 6).

Extracellular Rescue by Cross-streak:	**Indicator Strains:**	ROM291	ROM286	ROM292
Tester Strains:	**Relevant Genotype:**	*srfA-lacZ* *comQ*	*srfA-lacZ* *spo0A*	*srfA-lacZ* *spo0A* *comQ*
ROM297	*comQ*	-	+	-
AG503	*spo0A*	+	-	+/-
ROM298	*spo0A comQ*	-	-	-
AG665	*spo0H*	+	-	+/-
ROM302	*spo0H comQ*	-	-	-
ROM100	*wt*	+	+	+
JMS122	*spo0K*	+	+	+
AG1082	*comP*	+	+	+
ROM140	*comA*	+	+	+

Table 6. Cross-streaks show two extracellular complementation groups. The *comQ* indicator strain was rescued by wild-type and by *spo0A* strains, but not by a *comQ* strain. Similarly, The *spo0A* indicator strain was rescued by wild-type and *comQ* strains, but not by a *spo0A* strain. Thus, *comQ* and *spo0A* are defective in functionally distinct extracellular factors. *spo0K, comP,* and *comA* tester strains rescued both *comQ* and *spo0A* indicators. Furthermore, *spo0K, comP,* and *comA* indicator strains were not rescued by a wild-type cross-streak (not shown). Thus, *spo0K, comP,* and *comA* were required for the response to, but not the production of, extracellular signals. Each tester strain was cross-struck against each indicator strain on SPII Xgal plates, incubated at 37°C for 48 hours and scored for extracellular rescue (Materials and Methods). Rescue (indicated by +) was visually determined by comparing the expression of *srfA* near the cross-streak to expression of *srfA* distant from the cross-streak.

Like the wild-type strain, *comP, comA* and *spo0K* strains also produced both the *spo0A* and the *comQ* rescuing activities (Table 6), suggesting that they were defective in the production of neither CSF nor pheromone. As expected, conditioned media from *spo0K* and *comP* strains had no defect in total *srfA*-inducing activity, and fractionation of *spo0K* and *comP* conditioned media revealed normal levels of CSF and of pheromone (Chapter 4, Table 4).

These results indicated that *spo0K, comP* and *comA* were required for the response to, rather than the production of, density signals. If this is true, then extracellular rescue of these strains should not be possible. In fact, cross-streaking with a wild-type strain did not induce expression of *srfA* in these mutants. Similarly, the addition of wild-type conditioned medium did not restore expression of *srfA-lacZ* in *comP*, *comA*, or *spo0K* mutants (Figure 23, Figure 24, and Figure 25, respectively).

The background expression of *srfA-lacZ* was lower in the *spo0A comQ* double mutant than in the single mutants, consistent with the finding that these mutants were defective in different factors (Figure 26). Similarly, expression of *srfA* was lower in the *comP spo0K* double mutant than in the single mutants. These results suggested that two functionally distinct extracellular factors, CSF and pheromone, might be sensed by two different and independent sensors, *spo0K* and *comP*. It seemed that it would be possible, by analyzing the residual expression in various single and double mutants, to deduce which factor was sensed by which sensor. In a collaboration, Jonathan Solomon and I constructed the requisite double mutants, tested them on SPII Xgal plates and found the pattern of gene expression indicated in Figure 26.

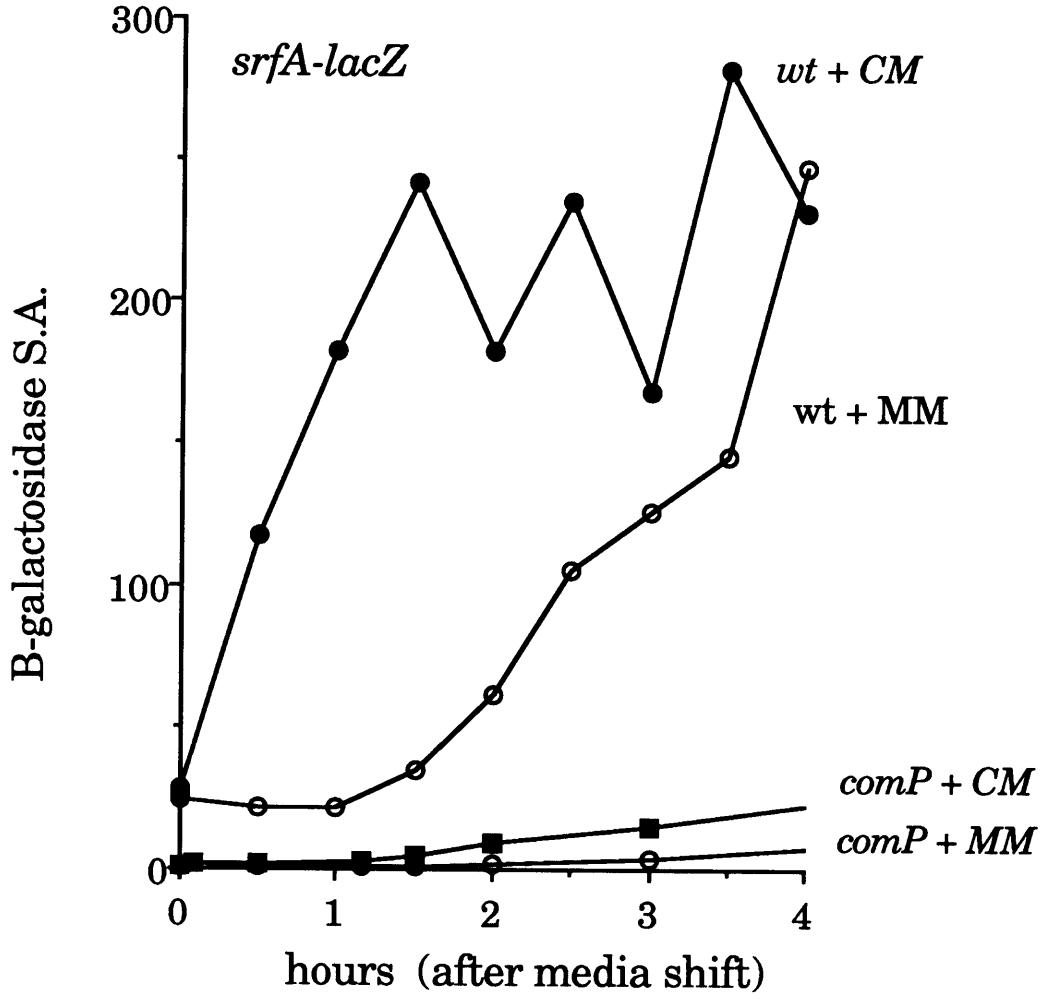


Figure 23. Expression of *srfA* in a *comP* mutant was not rescued by the addition of conditioned medium. Strains were grown in minimal medium for three doublings (to approximately 0.1 O.D. at 600 nm) and were then mixed with minimal medium (MM) or conditioned medium (CM). The optical density and ß-galactosidase activity of samples was determined hourly as the cultures continued to grow at 37°C with agitation. The expression of *srfA-lacZ* was monitored in an otherwise wild-type background (circles, JRL293) and in a *comP* mutant (squares, JRL359) after the addition of minimal medium (open symbols) or conditioned medium (closed symbols).

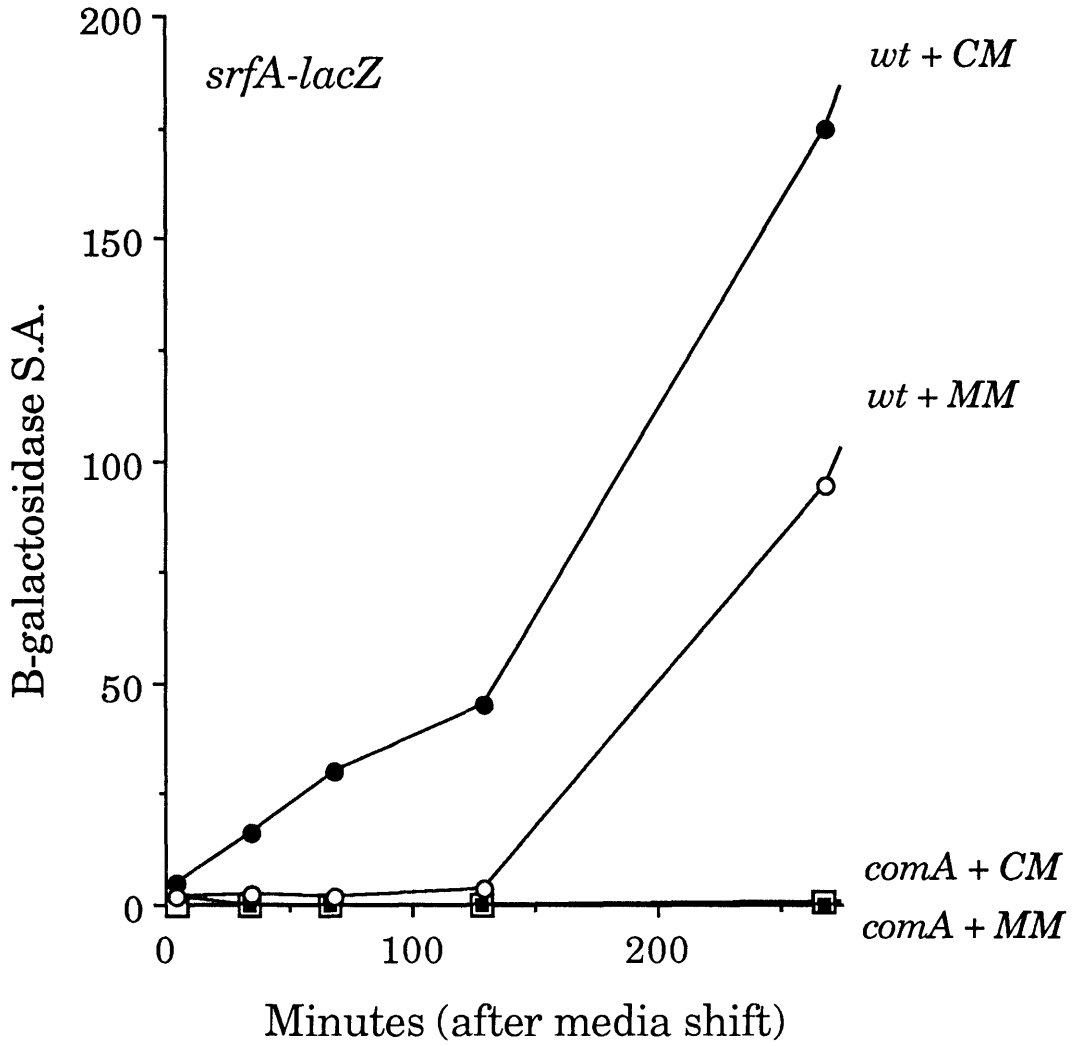


Figure 24. Expression of *srfA-lacZ* in *comA* strain was not rescued by the addition of wild-type conditioned medium. Strains were grown in minimal medium for three doublings (to approximately 0.1 O.D. at 600 nm) and then mixed with an equal volume of minimal medium (MM) or conditioned medium (CM). The optical density and the ß-galactosidase activity of samples was determined at intervals as the cultures continued to grow at 37°C with agitation. The expression of *srfA* was monitored with the *csh293::*Tn*917lac* fusion in an otherwise wild-type background (circles, KJ388) and in a *comA* mutant (squares, ROM053); after the addition of minimal medium (open symbols) or conditioned medium (closed symbols).

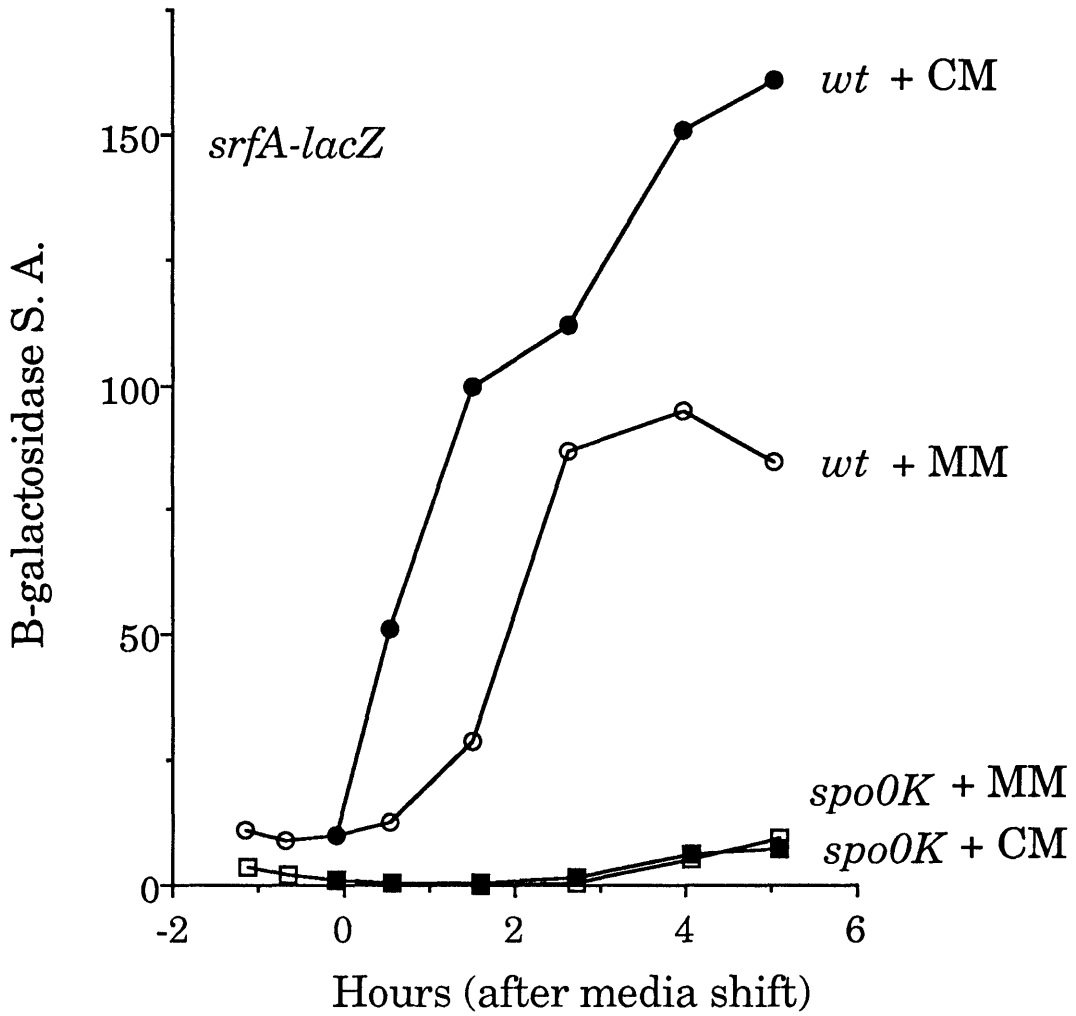
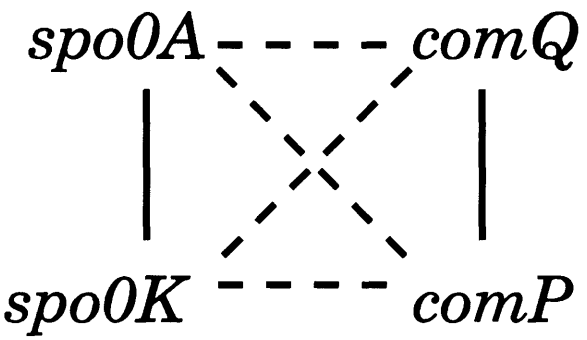


Figure 25. Expression of *srfA-lacZ* in *spo0K* strain was not rescued by the addition of wild-type conditioned medium. Strains were grown in minimal medium for three doublings (to approximately 0.1 O.D. at 600 nm) and were then mixed with an equal volume of minimal medium (MM) or conditioned medium (CM). The optical density and the ß-galactosidase activity of samples was determined hourly as the cultures continued to grow at 37°C with agitation. The expression of *srfA* was monitored with the *csh293::*Tn*917lac* fusion in an otherwise wild-type background (circles, KJ388) and in a *spo0K* mutant (squares, KJ590) after the addition of minimal medium (open symbols) or conditioned medium (closed symbols).



Strain:	Genotype: *amyE::(srfA-lacZ, neo), trp, phe* in:	Expression of *srfA-lacZ* on SPII Xgal
JMS374	*wt*	+++
ROM306	*comA::cat*	-
ROM286	*spo0A::erm*	+
JMS384	*Δspo0K::erm*	+
ROM291	*ΔcomQ::spc*	+
ROM305	*comP::spc*	+
ROM303	*spo0A::cat, spo0K::erm*	+/-
ROM294	*ΔcomQ::spc, comP::cat*	+
ROM292	*spo0A::cat, ΔcomQ::spc*	-
ROM304	*spo0A::cat, comP::spc*	-
JMS426	*Δspo0K::erm, ΔcomQ::spc*	-
JMS425	*Δspo0K::erm, comP::spc*	-

Figure 26. Summary of the residual expression of *srfA* in single and double mutants. Double mutants that expressed *srfA-lacZ* at or near the level of the constituent single mutants are indicated by a solid line. Double mutants that expressed *srfA-lacZ* at much lower level than the constituent single mutants are indicated by a dashed line. Expression of *srfA-lacZ* was much better in a wild-type background than in *spo0A, spo0K, comQ* and *comP* strains. Similarly, expression of *srfA-lacZ* was much better in *spo0A, spo0K, comQ* or *comP* single mutants than in certain double mutants, indicated in the figure by a dashed line, or in a *comA* single mutant. Similar results were observed using *spo0H* instead of *spo0A,* and in liquid as well as solid medium (Solomon et al., 1995).

Expression of *srfA-lacZ* in the *spo0A spo0K* double mutant was found to be almost as high as expression in the single mutants, indicating that *spo0A* and *spo0K* affected the same process or pathway. Similarly, expression of *srfA-lacZ* in the *comQ comP* double mutant was found to be no worse than expression in either single mutant, indicating that *comQ* and *comP* affected the same process or pathway. Expression of *srfA-lacZ* in all other combinations of double mutants, indicated by dashed lines, was much worse than expression in the constituent single mutants (Figure 26). These results indicated that *spo0A* and *spo0K* affected expression of *srfA* in one way, and that *comQ* and *comP* affected expression of *srfA* in a second and independent manner. More explicitly, these results suggested that ComP sensed pheromone and that Spo0K sensed CSF.

Discussion

Mutations in *comP, comA* and *spo0K* reduced expression of *srfA*, yet had no effect on the production of *srfA*-inducing factors and were not rescued extracellularly by wild-type conditioned medium. It appears then that these genes are required for the response to, and not the production of, *srfA*-inducing factors.

Since expression of *srfA* was not significantly worse in a *spo0K spo0A* or a *comQ comP* double mutant than in the constituent single mutants; and since all other combinations of these mutations were profoundly defective in expression of *srfA,* it appeared that *spo0K* and *spo0A* acted on one pathway to induce expression of *srfA,* while *comQ* and *comP* affected expression of *srfA* by a different and complementary pathway. Similar results were obtained using *spo0H* instead of *spo0A,* and Jonathan Solomon has since shown that these results hold in liquid as well as on solid media. Furthermore, by analyzing the

residual response of single mutants to purified factors, Jonathan has shown that *spo0K* was required for the response to CSF, but not pheromone, and similarly, that *comP* was required for the response to pheromone, but not CSF. (Solomon et al., 1995). Thus, pheromone and CSF appeared to act independently, through ComP and Spo0K, respectively, to induce expression of *srfA-lacZ* and thereby initiate the development of competence.

The *comP* and *comA* gene products are members of the large family of two-component regulatory systems found in prokaryotes (Bourret et al., 1991) and recently in eukaryotes (Alex and Simon, 1994; Swanson and Simon, 1994). ComP is homologous to histidine protein kinases (Weinrauch et al., 1990) that autophosphorylate on a histidine residue. ComA is homologous to response regulators (Nakano and Zuber, 1989; Weinrauch et al., 1989) and is phosphorylated, probably on an aspartate in the conserved N-terminal domain. ComA binds, *in vitro*, to the *srfA* promoter; phosphorylated ComA binds better than unphosphorylated ComA (Roggiani and Dubnau, 1993). Thus, it is suggested that pheromone binds to ComP and stimulates the kinase activity of ComP; that ComP autophosphorylates and transfers phosphate groups to ComA; and that phosphorylated ComA binds to the *srfA* promoter and stimulates transcription.

The mechanism by which SpoOK might affect transcription of *srfA* is less clear. Spo0K is an oligopeptide permease (Perego et al., 1991; Rudner et al., 1991), thus Spo0K might simply act to transport CSF into the cell or alternatively, upon interacting with CSF, Spo0K might send a transmembrane signal. In either case, it is not clear how these events might lead to the induction of *srfA*. The two signaling pathways, defined by *spo0K* and *comP,* might converge upon ComA or upon the *srfA* promoter. *comA* mutants, like *comP spo0K* and other double mutants that eliminate both pathways, have

almost no detectable expression of *srfA* (Solomon et al., 1995). One attractive and very speculative hypothesis is that CSF might inhibit a phosphatase, thereby simultaneously decreasing the rate at which ComA is dephosphorylated and increasing the steady state level of phosphorylated ComA.

Chapter 6:
Discussion of Competence, Speciation and Cell Density Signals

Under permissive nutrient conditions, competence in *Bacillus subtilis* is induced by two extracellular peptides. The effects of the two peptides on expression of *srfA* are approximately independent and multiplicative. One of the peptides, competence pheromone, is a modified 9 or 10 amino acid peptide derived from the C-terminus of the deduced 55 amino acid precursor encoded by *comX*. A second, slightly overlapping gene, *comQ,* is also required for the production of pheromone and may be involved in the modification or proteolytic processing of the deduced pheromone precursor.

comP and *comA,* located immediately downstream of *comX,* are required for the response to pheromone and encode, respectively, a histidine protein kinase and a response regulator of the two-component family. The ComP histidine protein kinase has eight predicted transmembrane segments (Weinrauch et al., 1990). Thus, pheromone may not need to be imported to stimulate ComP. *In vitro*, ComA binds to the promoter of *srfA* and phosphorylated ComA binds better that unphosphorylated ComA (Roggiani and Dubnau, 1993). It is therefore proposed that the binding of pheromone to ComP on the exterior of the cell might induce a conformational change to activate the cytoplasmic kinase activity of ComP. Activated ComP may then autophosphorylate on a histidine residue and transfer the phosphate group to an aspartate residue of ComA. Finally, phosphorylated ComA may bind the promoter of *srfA* and stimulate transcription of *srfA* (Figure 27). A biochemical demonstration of an interaction between pheromone and ComP, or portions of ComP, would do much to support this model.

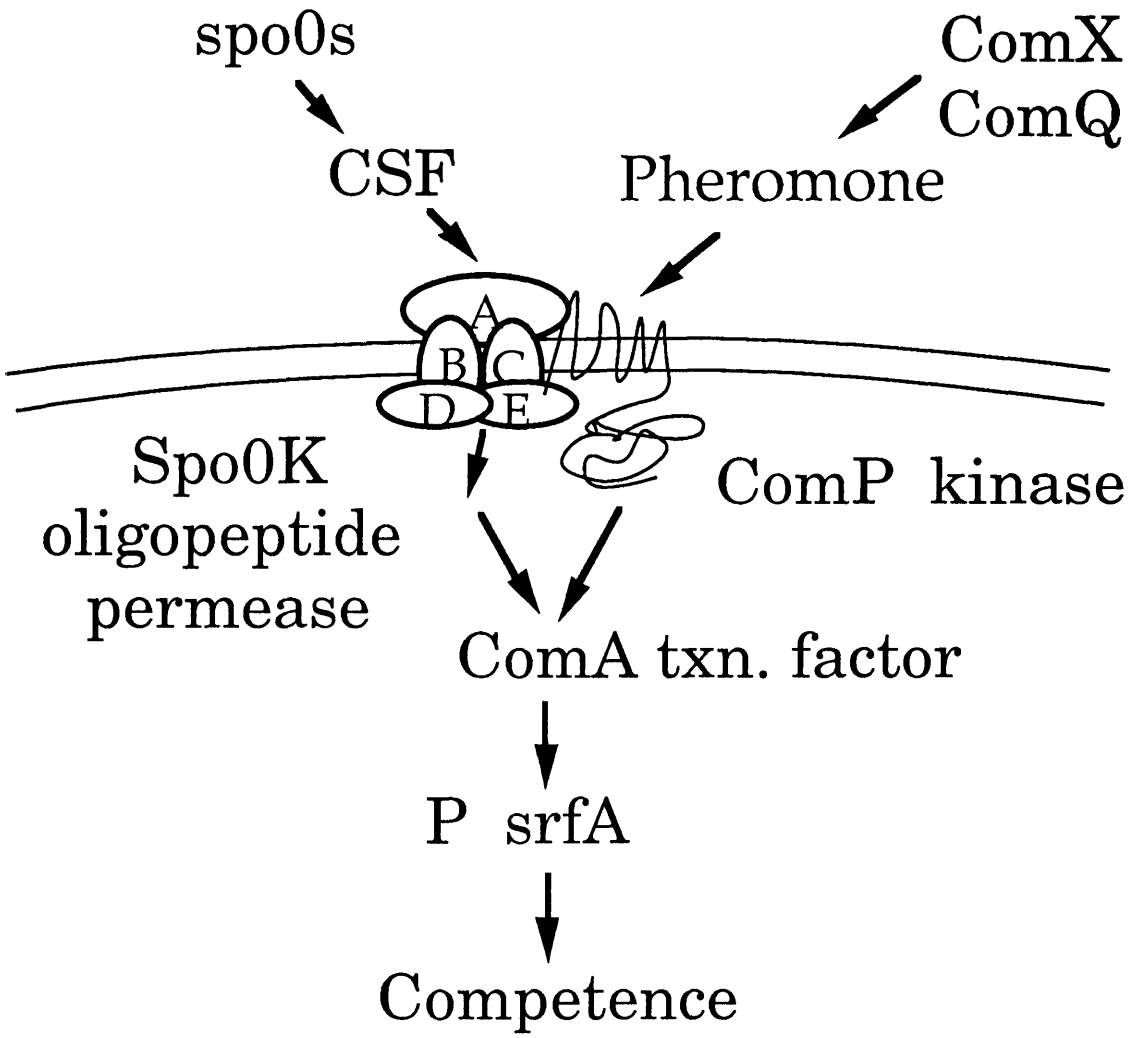


Figure 27. Model for the induction of *srfA* by two extracellular peptide density signals. *comQ* and *comX* are required for the production of pheromone, a 9 or 10 amino acid modified peptide derived from the C-terminus of ComX. Pheromone is sensed by ComP, which phosphorylates ComA, a transcription factor that binds to the promoter of *srfA* and stimulates transcription. Spo0A is required for the (full) production of CSF, a second, probably smaller extracellular peptide. The response to CSF requires the Spo0K oligopeptide permease. Pheromone and CSF appear to act by independent and complementary mechanisms that converge upon ComA or upon the *srfA* promoter.

A second extracellular peptide, CSF, appears to regulate *srfA*, independently of pheromone and ComP, via the Spo0K oligopeptide permease. The precise functions of CSF and of Spo0K are not known. Spo0K may simply transport CSF into the cell or, alternatively, Spo0K might function as a receptor for CSF. Ultimately, CSF and Spo0K might exert their effect on the ComA transcription factor or upon the *srfA* promoter (or upon RNA polymerase). In principle, the activity of the *srfA* promoter might be influenced by removing a repressor or, in a DNA looping model, by inducing a DNA bend between the two ComA bindings sites (Nakano and Zuber, 1993). The level of phosporylated ComA might be increased by activating a kinase, by inhibiting a phosphatase, or by increasing the general expression or stabililty of ComA. Further experimentation may clarify the mechanism by which CSF and Spo0K regulate expression of *srfA*.

In *Bacillus subtilis,* cell-cell signaling and signal integration are involved in the initiation of sporulation (Grossman and Losick, 1988a; Waldburger et al., 1993) as well as in the development of genetic competence. Both competence and sporulation are most efficient at high cell density. Although density signals appear to be sufficient to initiate the development of competence (as defined by expression of *srfA*), nutrient deprivation and DNA related signals are required, in addition to cell density signals, to initiate sporulation (Grossman, 1991; Grossman and Losick, 1988a; Ireton and Grossman, 1992; Ireton and Grossman, 1994; Ireton et al., 1993; Waldburger et al., 1993). Since *comQ* mutants sporulate poorly under some conditions, but are rescued by the addition of purified pheromone, it is clear that pheromone can contribute to the extracellular regulation of sporulation. In addition to pheromone, a *spo0A*-dependent signal, possibly including CSF, is required for the extracellular

stimulation of sporulation. In preliminary experiments, the addition of CSF did not restore the sporulation stimulating activity of *spo0A* conditioned medium. Therefore, as previously indicated (Grossman and Losick, 1988b), there may be a *spo0A*-dependent sporulation stimulating activity that is not due to CSF. The Spo0K oligopeptide permease, which is needed for sporulation as well as for competence, may be required for the response to one or more extracellular sporulation factors (Rudner et al., 1991).

Many two-component systems (Nixon et al., 1986) are involved in sensing environmental conditions. It now also appears that it may not be uncommon for ABC transporters to be involved in sensing environmental signals. ABC transporters (or proteins with homology to components of ABC transporters) are implicated in the export and import of the competence factor of *Streptococcus pneumoniae* (Hui and Morrison, 1991; Pearce et al., 1994) Proteins homologous to the ligand binding protein of *spo0K* are required for the binding and sensing of peptide pheromones that induce the conjugation of plasmids in *Enterococcus faecalis* (Ruhfel et al., 1993; Tanimoto et al., 1993). One of the loci involved in the response to the extracellular A-signal of *Myxococcus xanthus* appears to be an ABC transporter similar to *spo0K* (H. Kaplan, personal communication).

ABC transporters are also involved in the conjugation of the Ti plasmid of *Agrobacterium tumefaciens.* In this case, two extracellular signals, an opine, produced by the Ti plasmid transformed plant cells of a crown gall tumor, and a density dependent homoserine lactone autoinducer, act synergistically to induce conjugation (Greene and Zambryski, 1993). The opine is transported into the cells by an ABC transporter (Valdivia et al., 1991; Zanker et al., 1992). Once inside the cells, the opine interacts with a transcriptional regulator to induce genes required for the synthesis and sensing of the second signal, the

Agrobacterium (homserine lactone) autoinducer (AAI, formerly called conjugation factor or CF) (Fuqua et al., 1994). Thus one signal, from the plant, is required for the synthesis and sensing of a second, autogenously produced density signal. Thus, two signals, reflecting two independent environmental conditions, are integrated by a simple mechanism. In *Bacillus subtilis,* it is possible that nutrient conditions might regulate the production of CSF or pheromone or both. Thus, some nutritional signals could be integrated into the final cell density signal.

There is a second example of dual signaling molecules in *Agrobacterium tumefaciens.* Two synergistic signals, sugars and phenolic compounds, act via the VirA/VirG two-component system to induce the transfer of the Ti plasmid from *Agrobacterium tumefaciens* to plant cells. One signal, the phenolic compound, appears to act directly on the cytoplasmic or transmembrane domain of the VirA histidine protein kinase. The second signal, the sugars, appear to interact with ChvE, the ligand binding protein of an ABC family sugar transporter; and ChvE then interacts with the extra-cytoplasmic domain of VirA. The two signals, sugars and phenolic compounds, are both produced by wounded plants, which are more susceptible to infection by *Agrobacterium tumefaciens.* By sensing and integrating two signals stemming from the same source, a plant wound, the bacterium may be able to respond with greater sensitivity or specificity (Winans, 1992).

Similarly, the combination of CSF and pheromone may regulate competence with greater sensitivity and specificity than could be obtained with a single signal. The specificity of the regulation of competence might be especially important in preserving the distinction between *Bacillus subtilis* and related species. CSF and pheromone appear to be species-specific factors, as conditioned medium from seven other *Bacillus* species did not induce *srfA*, and

in several cases inhibited the induction of *srfA*. Inhibitory activity could be due to the action of proteases, antibiotics or competitive inhibitors. The species specificity of competence-inducing (and competence-inhibiting) signals could help ensure that *Bacillus subtilis* becomes competent only in the presence of *Bacillus subtilis* and thus, that it is preferentially transformed only by *Bacillus subtilis* DNA.

Extracellular effects on competence are observed in other gram-positive bacteria, including *Streptococcus pneumoniae* (Tomasz and Hotchkiss, 1964; Tomasz and Mosser, 1966), *Streptococcus sanguis* (Pakula and Walczak, 1963), *Bacillus stearothermophilus* (Streips and Young, 1971), and *Bacillus cereus* (Felkner and Wyss, 1964). It will be interesting to see if these extracellular effects are mediated by peptides similar to those of *Bacillus subtilis*. Hints of similarities are already apparent. Peptide permeases similar to Spo0K are required for the development of competence in *Streptococcus pneumoniae* (Pearce et al., 1994). The observation that *Bacillus amyloliquefaciens* and *Bacillus licheniformis* have homologs of *comQ* suggests that they might also make and sense pheromones.

Although the regulation of competence by extracellular signals may be common, some species of transformable bacteria have a more direct way of exercising sexual selectivity. *Haemophilus influenzae* takes up DNA containing a certain 11 bp sequence that is common in its own DNA but is uncommon in the DNA of other, unrelated species (Danner et al., 1980). A similar mechanism is observed in the constitutively transformable gram negative species *Neisseria gonorrhea* (Goodman and Scocca, 1988). The sexual specificity of these bacteria may be fairly fixed, since a change in the specificity of the receptor would have to accompanied by many different compensatory changes to restore self-specificity. In contrast, the sexual

specificity of *Bacillus subtilis* may be relatively flexible, since a change in the specificity of the receptor requires only a single compensatory change in the pheromone to re-establish a working system.

Mechanisms that enhance sexual selectivity may minimize the dangers of genetic exchange while preserving the benefits offered by increased genetic variation. In the absence of such sexual selectivity, competence might be dangerous to the individual bacterium or might erase the distinctions between *Bacillus subtilis* and related, cohabitating bacterial species. Once two related species have sufficiently diverged, recombinational barriers may reinforce any sexual barriers. Thus sexual selectivity or other means of sexual isolation might be especially important during speciation, when recombinational barriers are low.

Speciation and the development of sexual selectivity may be causally connected events. Speciation occuring between two geographically isolated populations is described as "allopatric." Speciation occuring without geographical isolation is described as "sympatric." It is commonly believed, but still hotly contested, that allopatric speciation may be much more common than sympatric speciation (Smith, 1989). According to this view, sexual selectivity must evolve after the (allopatric) speciation of geographically isolated bacteria; either by genetic drift, or by selection at the geographical frontier between two sexually compatible but genetically incompatible species.

Alternatively, it may be argued that changes in sexual selectivity might drive sympatric speciation. If sexual specificity can be as simple as a single ligand-receptor interaction, then sympatric speciation events might be common. It is expected, however, that only a small proportion of such proto-species would manage to develop a profitable ecological niche and thus, to

proliferate into a common and stable species. It will be interesting to learn more about the mechanisms of speciation in sexual microorganisms.

There are many examples of cell-cell signals regulating microbial development. Cell-cell signals are required for sporulation of *Myxococcus xanthus*, antibiotic production, sporulation, and morphogenesis of *Streptomyces coelicolor*, pattern formation in the cyanobacterium *Anabaena*, and plasmid conjugation in *Enterococcus faecalis* (reviewed by Kaiser and Losick, 1993). Furthermore, a family of structurally related, density dependent homserine lactone autoinducers regulate virulence factors in *Pseudomonas aeruginosa*, antibiotic and exoenzyme production in *Erwinia caratovora*, plasmid conjugation in *Agrobacterium tumefaciens,* and luminescence in *Vibrio harveyi* and *Vibrio fisheri* (reviewed by Fuqua et al., 1994). Sexual processes, and synthesis of extracellular products such as antibiotics and degradative enzymes, are inherently social strategies that are most successfully pursued at high cell density. The regulation of such inherently social strategies by cell density signals makes good biological sense. In some cases, density signals may also be predictive of impending nutrient limitation. By sensing and responding to such density signals, cells may gain more time in which to escape from or adapt to the upcoming nutrient limitation. Thus cell density may be an important and advantageous regulator of aspects of metabolism, development, sexuality, antibiotic production and exoenzyme production.

Chapter 7:
Materials and Methods

Strains

Escherichia coli strains used for cloning have been described previously (Ireton et al., 1993). Except for strains from the Bacillus Genetic Stock center, all strains were derived from AG174 (JH642). The translational *srfA-lacZ* fusion (*amyE*::(*srfA-lacZ, cat*)), located in single copy in the chromosome at the non-essential *amyE* locus, was provided by J. Hahn and D. Dubnau. A transcriptional *srfA-lacZ* fusion (*amy::(srfA-lacZ, neo*)), used in all the strains listed in Figure 26, was constructed by Jonathan Solomon (Solomon et al., 1995). The *comG-lacZ* transcriptional fusion was constructed by K. Jaacks Siranosian, as described (Magnuson et al., 1994). The *comA::cat, comP::cat,* and *comP::spc* alleles were constructed in the lab of D. Dubnau. The Δ*spo0K-erm* mutation was made by J.R. LeDeaux. All strains were constructed according to standard techniques (Harwood and Cutting, 1990). Various species of *Bacillus* were obtained from the Bacillus Genetic Stock Center (BGSC). Experiments involving these strains were performed at 30°C rather than 37°C, due to the growth characteristics of some of these strains.

Strain	genotype
AG130	*prototroph* (Grossman and Losick, 1988b)
AG132	*Δspo0A abrB703, trp, phe*
AG141	*spo0B136, trp,*
AG144	*spo0F221, trp, phe*
AG169	*abrB703, trp, phe*
AG174	*trp, phe (JH642)*
AG503	*spo0A::cat, trp, phe*
AG665	*spo0H::cat, trp, phe*
AG1046	*amyE::(comG-lacZ, neo), trp, phe*
AG1082	*comP::cat* (Weinrauch et al., 1990), *trp, phe*
AG1226	*spo0H::cat, trp, phe*
AG1489	*amyE::(comG-lacZ, neo), comP::pRO12, trp, phe*
AG1519	*amyE::(comG-lacZ, neo), ΔcomQ::spc, trp, phe*
AG1520	*ΔcomQ::spc, trp, phe* (Magnuson et al., 1994)
KI418	*spo0K::Tn917lac, trp, phe* (Rudner et al., 1991)
KJ388	*csh293::*Tn*917lac, trp, phe* (Jaacks et al., 1989)
KJ482	*spo0H::cat,csh293::*Tn*917lac, trp, phe*
KJ639	*spo0B, csh293::*Tn*917lac, trp, phe*
KJ590	*csh293::*Tn*917lac, spo0K141, trp (PB2 background)*
JMS139	*amyE::(srfA-lacZ, cat::neo), spo0H::cat*
JMS323	*amyE::(srfA-lacZ, cat), ΔcomQ::spc, trp, phe*
JMS374	*amy::(srfA-lacZ, neo), trp, phe*
JMS384	*amyE::(srfA-lacZ, neo), Δspo0K::erm, trp, phe*
JMS426	*amyE::(srfA-lacZ, neo), Δspo0K::erm, ΔcomQ::spc, trp, phe*
JMS425	*amyE::(srfA-lacZ, neo), Δspo0K::erm, comP::spc, trp, phe*
JRL293	*amyE::(srfA-lacZ, cat), trp, phe*
JRL358	*Δspo0K-erm, trp, phe* (J. R. LeDeaux)
JRL359	*amyE::(srfA-lacZ, cat), comP::cat, trp, phe*
NY120	*kinB kapB, trp, phe* (LeDeaux et al., 1995)
ROM050	*spo0A::cat, csh293::*Tn*917lac, trp, phe*
ROM100	AG174
ROM102	KJ388
ROM053	*comA::cat, csh293::*Tn*917lac, trp, phe*
R0M119	ROM100 converted to prototrophy (by *his leu met* strain)
ROM140	*comA::cat* (Weinrauch et al., 1989), *trp, phe*
ROM051	*srfA::*Tn*917-cat, trp, phe*
ROM183	*spo0A::erm, abrB::cat,* prototroph (derived from ROM119)
ROM230	*comX::spc, trp, phe*
ROM261	AG130 *ΔcomQ::spec*
ROM264	*Bacillus subtilis* BGSC 1A1

ROM265	*Bacillus subtilis (W23)* BGSC 2A2
ROM266	*Bacillus subtilis* BGSC 3A1
ROM267	*Bacillus thuringiensis serot-1* BGSC 4A1
ROM268	*Bacillus thuringiensis serot-2* BGSC 4B1
ROM269	*Bacillus thuringiensis serot-3a* BGSC 4C1
ROM270	*Bacillus licheniformis* BGSC 5A2
ROM271	*Bacillus licheniformis* transformable BGSC 5A24
ROM272	*Bacillus cereus* BGSC 6A1
ROM273	*Bacillus megaterium* BGSC 7A1
ROM275	*Bacillus pumilis* BGSC 7A13
ROM279	*Bacillus globigii* BGSC 11A1
ROM286	*amyE::(srfA-lacZ, neo), spo0A::cat, trp, phe*
ROM291	*amyE::(srfA-lacZ, neo), ΔcomQ::spc, trp, phe*
ROM292	*amyE::(srfA-lacZ, neo), ΔcomQ::spc, spo0A::cat, trp, phe*
ROM294	*amyE::(srfA-lacZ, neo), ΔcomQ::spc, comP::cat, trp, phe*
ROM297	*ΔcomQ::spc, trp, phe* (AG1520)
ROM298	*ΔcomQ::spc, spo0A::cat, trp, phe*
ROM301	*srfA::pRO101, ΔcomQ::spc, trp, phe*
ROM302	*ΔcomQ::spc, spo0H, trp, phe*
ROM303	*amyE::(srfA-lacZ, neo), spo0A::cat, Δspo0K::erm, trp, phe*
ROM304	*amyE::(srfA-lacZ, neo), spo0A::cat, comP::spc, trp, phe*
ROM305	*amyE::(srfA-lacZ, neo), comP::spc, trp, phe*
ROM306	*amyE::(srfA-lacZ, neo), comA::cat, trp, phe*

Table 7. Strains.

Media

LB medium (Davis et al., 1980) was used for routine growth of *E. coli* and *B. subtilis*. DS medium (Schaeffer et al., 1965) was used for some spore assays. Minimal medium for competence assays, for sporulation assays with decoyinine, and for the small scale preparation of conditioned media contained S7 minimal salts (Vasantha and Freese, 1980) except that MOPS (morpholinepropanesulfonic acid) was used at 50 rather than 100 mM (Jaacks et al., 1989). Minimal medium was supplemented with 1% glucose, 0.1% glutamate, and required amino acids (40 μg/ml) as needed. Small batches of conditioned medium were prepared by growing strains (without *lacZ* fusions)

in minimal medium at 37°C with agitation to approximately 4.0 O.D. at 600 nm. The cultures were then clarified by centrifugation, and the supernatants were sterile filtered. Conditioned medium was stored at 4°C or -20°C. In experiments involving other species of *Bacillus,* conditioned medium was harvested from cultures grown to saturation at 30°C. Conditioned medium made in a 10 liter fermentor contained Spizizen minimal salts (Spizizen, 1958) supplemented with trace metals instead of S7 salts. Glucose was fed into the fermentor to meet the demands of growth. pH in the fermentor was controlled by the automatic addition of a 1 M solution of ammonium hydroxide. Fermentor cultures reached 20 to 40 O.D. at 600 nm. Cross streak assays were performed on SpII media (Dubnau and Davidoff-Abelson, 1971), containing agar at 15 gm per liter and 5-Bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (Xgal) at 120 μg/ml.

Assays

Assay of ß-galactosidase activity

ß-galactosidase specific activity was measured essentially as described (Jaacks et al., 1989; Miller, 1972) and is defined as (ΔA_{420} per minute per ml of culture per optical density unit at 600 nm) x 1,000.

Assay *srfA*-inducing activity

The concentration of *srfA*-inducing activity was defined by the ability to induce ß-galactosidase activity in a low density culture containing the *srfA-lacZ* fusion (usually JRL293). *srfA*-inducing activity was defined as the response (induced ß-galactosidase specific activity) in the linear range times the dilution factor of the sample giving that response. Samples were tested for activity over a range of concentrations. Two-fold dilutions were preferred, although on occassion three-fold, four-fold, five-fold and even 10-fold dilutions

were used. Crude conditioned medium gave a fairly linear response over a 10-fold range of concentrations. The response reached a plateau approximately 10- to 20-fold above the background levels of ß-galactosidase activity. Pheromone behaved similarly. The response to CSF was less robust, as the maximum response obtained by CSF was notably lower than the maximum response obtained by pheromone or crude conditioned medium. Activity of CSF was therefore calcuated from samples that produced, approximately, a doubling of the background ß-galactosidase activity. In the standard assay, 0.25 ml of cells (containing the *srfA-lacZ* fusion) at an O.D. at 600 nm of 0.05 to 0.1 was added to 0.25 ml of the sample to be tested. Bovine serum albumin (BSA) was included at a final concentration of 50 μg/ml as indicated to prevent non-specific loss of pheromone activity. The samples (mixed with cells) were incubated at 37°C for 70 minutes and then assayed for ß-galactosidase specific activity.

Assay of competence

Cells were grown in minimal medium and the transformation frequency determined at different times. Typically, cells were mixed with *B. subtilis* chromosomal DNA (~1 μg/ml) containing a selectable marker (e.g. *cat*, chloramphenicol resistance) incubated at 37°C for 20 minutes and then put on selective plates. The transformation frequency is the total number of transformants per viable cell. Viable cells were determined in parallel, except that nonselective plates were used. In some cases, O.D. @ 600 nm was used as an alternative measure of viable cells. Typical frequencies at the peak of competence for the strain background used here ranged from 5×10^{-5} to 8×10^{-4} transformants per viable cell.

Assay of sporulation

Sporulation frequencies were determined as the number of heat resistant (80°C for 20 min) colony forming units as a fraction of total colony forming units on LB plates. For experiments in minimal medium, cells were grown to an O.D.at 600 nm of 0.5 to 0.6 and decoyinine (U-7984; Upjohn Co.), an inhibitor of GMP synthetase, was added to a final concentration of 500-1000 μg/ml to initiate sporulation, essentially as described (Grossman and Losick, 1988a; Mitani et al., 1977).

Cross-streak assays

Expression of *lacZ* fusions on SpII Xgal plates was indicated by blue color. Mutations affecting expression of *srfA* were tested in three different cross-streak assays on SpII Xgal plates. Pheromone producing strains were identified by their ability to induce expression of *srfA-lacZ* in a *comQ* indicator strain. Similarly, CSF producing strains were identified by their ability to induce expression of *srfA-lacZ* in a *spo0A* indicator strain. Finally, mutant strains containing a *srfA-lacZ* fusion were tested for their ability to express *srfA* in response to a wild-type cross-streak. Positive and negative controls were included in each experiment. Cells for cross-streak assays were taken from colonies grown overnight on LB plates at 30°C. Cross streak plates were incubated at 37°C for approximately 48 hours and then scored. Distinctions were often sharper after an additional day at room temperature.

Molecular biology

Construction of *srfA* clones

An *E. coli* origin was introduced into the *csh293* transposon (Jaacks et al., 1989) by transformation with pTV21Δ2. Chromosomal DNA was then isolated, digested with *Sph*I, and ligated to generate a covalently closed, circular plasmid containing a 4 kb fragment of DNA upstream of the transposon insertion, by established techniques (Youngman, 1993; Youngman et al., 1984). This resulting plasmid was transformed into *E. coli,* propagated, purified and manipulated by standard techniques (Ausubel et al., 1990; Sambrook et al., 1989). The 4kb fragment was subcloned into pGem-*cat,* a vector suitible for single strand DNA sequencing or for reintegration (insertion-duplication) into the *Bacillus subtilis* chromosome (Youngman et al., 1989). A restriction map was generated. Fragments between convenient restriction fragments were deleted in order to generate plasmids with different segments of DNA abutting the vector. Single stranded DNA was prepared from these plasmids and sequenced. Sequencing reactions, using sequenase (USBC), were initiated with the universal primer (which was complementary to a portion of the vector), labeled with ^{35}S-dATP and terminated with dideoxynucleotides (Sanger et al., 1977).

Construction of *comQ* mutants

comQ was cloned, based on published sequence, and *comQ* mutants were constructed by N. Gunther and A. D. Grossman as described (Magnuson et al., 1994).

Complementation of *comQ*

The effect of the *comQ* mutation on pheromone production and competence was not due to polarity on expression of *comX*. When the *comQ*$^+$ allele (without *comX*) was provided in trans, pheromone production (measured by a cross-streak assay) and competence (measured by expression of *comG-lacZ)* were restored. When this strain was propagated nonselectively, the strain was occassionally cured of the plasmid. Pheromone production and competence were also defective in these cured isolates, indicating that the plasmid had truly complemented, rather than repaired, the *comQ* defect.

Construction of *comX* mutants

comX was cloned by PCR using oligonucleotide primers based on published sequence (Weinrauch et al., 1991; Weinrauch et al., 1990). The PCR product, containing DNA from 91 bp upstream of the *comX* start codon to 362 bp downstream of the *comX* stop codon, was cloned into pJH101 (Ferrari et al., 1983) between the EcoRI and BamHI sites, to yield pRO17. A DNA cassette conferring resistance to spectinomycin was cloned into the unique HindIII site of *comX* to produce pRO19. pRO19 was recombined into the chromosome by double crossover to disrupt *comX* (strain ROM230).

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