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Specificity and Genetic Polymorphism of the *Bacillus* Competence Quorum-Sensing System

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A quorum-sensing mechanism involving the pheromone ComX and the ComP-ComA two-component system controls natural competence in *Bacillus subtilis*. ComX is expressed as a cytoplasmic inactive precursor that is released into the extracellular medium as a cleaved, modified decapeptide. This process requires the product of *comQ*. In the presence of ComX, the membrane-localized ComP histidine kinase activates the response regulator ComA. We compared the sequences of the quorum-sensing genes from four closely related bacilli, and we report extensive genetic polymorphism extending through *comQ*, *comX*, and the 5′ two-thirds of *comP*. This part of ComP encodes the membrane-localized and linker domains of the sensor protein. We also determined the sequences of the *comX* genes of four additional wild-type bacilli and tested the in vivo activities of all eight pheromones on isogenic strains containing four different ComP receptor proteins. A striking pattern of specificity was discovered, providing strong evidence that the pheromone contacts ComP directly. Furthermore, we show that coexpression of *comQ* and *comX* in *Escherichia coli* leads to the production of active pheromone in the medium, demonstrating that *comQ* is the only dedicated protein required for the processing, modification, and release of active competence pheromone. Some of the implications of these findings for the evolution and the mechanism of the quorum-sensing system are discussed.

Under certain growth conditions, *Bacillus subtilis* expresses a set of proteins involved in the uptake and integration of extracellular DNA (reviewed in reference 3). The expression of these proteins in the competent state is tightly controlled and initially regulated by a quorum-sensing (QS) system involving the ComX pheromone (14–17, 30, 31) and the ComP-ComA two-component system (38, 40). The membrane-localized histidine kinase ComP, in the presence of ComX, activates the response regulator ComA, permitting the expression of downstream regulatory genes (4). This signal transduction cascade culminates with the expression of the competence transcription factor ComK (35, 36), resulting in the synthesis of the DNA uptake and recombination proteins. A convergent pathway utilizes a second pheromone, the competence-stimulating factor (CSF) peptide, to modulate this response (16, 31). This second pathway, which exerts a relatively minor effect, probably acts by inhibiting a phosphatase specific for ComA-ComP (22, 23). The two pathways thus appear to converge, both controlling the level of ComA phosphorylation (14, 15, 30).

The ComX pheromone is translated as an inactive propeptide, which is then cleaved and released in the extracellular medium as a modified decapeptide, with the addition of an uncharacterized lipid moiety to a tryptophan residue (17). At least one other gene, *comQ* (39), is required for the production of modified, active ComX (17), but the precise role of ComQ is not understood.

In the laboratory strains of *B. subtilis*, derived from strain 168, the *comQ*, *comX*, *comP*, and *comA* genes are clustered on the chromosome in the order given. Interestingly, a similar genetic organization has been reported for two other bacterial QS systems involving peptide pheromones: the *agrBDCE* and the *comCDE* systems, regulating virulence in *Staphylococcus aureus* and competence in *Streptococcus pneumoniae*, respectively (9, 12, 41). In these cases, substantial genetic polymorphism of the pheromones as well as of the sensor histidine kinases has been reported. This genetic polymorphism is associated with the specificity of each pheromone for its cognate receptor (9, 26), pointing to the presence of distinct pherotypes among staphylococcal and streptococcal species.

A suggestion that such extensive polymorphism may also exist in *Bacillus* has been provided by the recent comparison of the QS loci of *B. subtilis* 168 and *B. natto* NAF4 (34). Since the evolution, ecology, and mechanistic aspects of this polymorphism are of interest, we have extended the initial observations reported in this paper, sequencing all or part of the *comQXPA* loci from a number of closely related bacilli and comparing their pherotype cross-specificities. We report a striking polymorphism within the DNA sequences encoding ComQ, ComX, and the N-terminal two-thirds of ComP. We constructed a set of isogenic *B. subtilis* "producer" strains that release different pheromone molecules into the medium and a set of isogenic "tester" strains expressing different ComP sensors and ComQ proteins. We show that each ComP sensor is specifically activated in vivo by its cognate pheromone and in some cases by a limited set of pheromones from other strains, indicating that ComX-ComP pairs determine different pherotypes in *Bacillus*. In addition, we show that the competence pheromone can be

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
B. subtilis		
OKB125	*his leu met srfA*::Tn*917*Ω OK120 (pTV55) Cm^r	21
BD1658	*his leu met comP*::*cat*	This work
BD2833	*his leu met srfA-lacZ* (*tet*)	This work
BD2834	*his leu met srfA-lacZ* (*tet*) *orf-2*::*kan*	This work
BD2847	*his leu met srfA-lacZ* (*tet*) (*comQ comX comP* replaced by genes from *B. natto* NAF4)	
BD2876	*his leu met srfA-lacZ* (*tet*) *comQ*::Km	This work
BD2877	*his leu met srfA-lacZ* (*tet*) (*comQ*::*phl comX comP* replaced by genes from *B. natto* NAF4)	This work
BD2911	*his leu met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*ery*) (Δ*comQXP*)::Km	This work
BD2913	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*comQ comX comP* replaced by genes from *B. mojavensis* RO-H-1)	This work
BD2914	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*comQ comX comP* replaced by genes from *B. subtilis* RS-B-1)	This work
BD2915	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*comQ comX comP* replaced by genes from *B. natto* NAF4)	This work
BD2935	*his leu met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (Δ*comQXP*)::Km	This work
BD2936	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. mojavensis* RO-H-1)	This work
BD2937	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. mojavensis* RO-C-2)	This work
BD2939	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. subtilis* RO-FF-1)	This work
BD2947	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ comX comP* replaced by genes from *B. subtilis* RO-O-2)	This work
BD2962	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ*::pED345 *comX comP* replaced by genes from *B. mojavensis* RO-H-1)	This work
BD2963	*his met srfA-lacZ* (*tet*) *amyE*::*xylR Pxyl-comK* (*cat*) (*comQ*::pED346 *comX comP* replaced by genes from *B. mojavensis* RO-C-2)	This work
E. coli		
ED318	*KC8 (PCR script with comQ comX* from *B. subtilis* 168 in + orientation)	This work
ED319	*KC8 (PCR script with comQ comX* from *B. subtilis* 168 in − orientation)	This work
KC8	*hsdR leuB600 trpC9830 pyrF*::Tn*5 hisB463 lac*ΔX74 *strA galU galK*	Clontech
Plasmids		
pED302	*PCR script with kan* cassette between *degQ* and *comA*	This work
pED345	*pUS19 with internal comQ* fragment of *B. mojavensis* RO-H-1 in *Eco*RI/*Hin*dIII sites	This work
pED346	*pUS19 with internal comQ* fragment of *B. mojavensis* RO-C-2 in *Eco*RI/*Hin*dIII sites	This work
pUS19	*pUC19 with spc* cassette between the *Nde*I and *Nar*I sites	This work

produced in *Escherichia coli* in the presence of ComQ. Our results show that ComQ and ComX are the only dedicated proteins required for production of the competence pheromone, and that *comQ*, *comX*, and *comP* are the sole determinants of the QS pherotype.

MATERIALS AND METHODS

Growth conditions and general methods. *E. coli* KC8 (Clontech Laboratories, Inc.) was used for cloning, and transformants were selected on Luria-Bertani (LB) agar supplemented with ampicillin, (100 μg/ml). *E. coli* KC8 ComX producer strains were grown in liquid competence medium (1) supplemented with histidine, methionine, tryptophan, uracil (20 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG) (2 mM). *B. subtilis* was grown either in liquid competence medium, on minimal medium (2) plates supplemented with glucose and the required amino acids (50 μg/ml), or on Tryptose Blood Agar Base (Difco) plates supplemented with chloramphenicol (5 μg/ml), erythromycin (5 μg/ml), kanamycin (5 μg/ml), phleomycin (1 μg/ml), spectinomycin (100 μg/ml), or tetracycline (20 μg/ml). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a final concentration of 0.004%. *B. subtilis* competent cells were prepared as described previously (1). DNA manipulations, cloning, and standard molecular biological procedures were performed as described previously (29). The strains used in this study are listed in Table 1.

Preparation and assay of conditioned medium. Conditioned medium was prepared by growing producer strains to 2 h past the end of exponential growth. Cells were removed by centrifugation, and the supernatants were sterilized by filtration through 0.2-μm-pore-size filters. Conditioned medium was stored at −20°C. Overnight cultures of tester strains were grown at 32°C in competence medium and then diluted 50-fold into fresh competence medium. An equal volume of prewarmed conditioned medium was added, and aliquots were removed for assay of β-galactosidase at intervals during growth at 37°C. β-Galactosidase assays were carried out as described previously (1) except that the enzyme reactions were carried out in microtiter plates and the course of the reactions were followed using a Tecan Rainbow plate reader. β-Galactosidase activities were calculated from the slopes of the reaction curves.

Sequencing of the RO-H-1 and RS-B-1 *comQXPA* loci. The entire *comQXP* locus from RS-B-1 and RO-H-1 was amplified by PCR with primers RS comB-Pst (CGAATTCCTCGACCTCATAACCGG) and RS comA-Eco (CGGAATT CACGAATCGCTTCCTC) and with primers Q3 (GTGTCCGGATCAAGGA GAAA) and P1 (AAGAACCGAATCGTGGAGATCGGC), respectively. The PCR fragments were then directly sequenced. As sequences were collected, additional primers were synthesized. The sequencing of the *B. natto* NAF4 *comQXP* locus has been described elsewhere (34).

Sequencing of the *comX* genes from RO-O-2, RO-C-2, RO-FF-1, and RO-B-2. PCR products corresponding to the entire *comQ* and *comX* genes and the first 1.7 kb of *comP* were obtained with the primers unicomQ-Xba (GTCTAGAGG AATGGGAGGGGGGAAG) and P1 (see above), located just upstream of *comQ* and within a conserved part of *comP*, respectively. Chromosomal DNA was used as the template. The 3-kb PCR products were purified and cloned into pPCR-Script Amp (SK+) (Stratagene). The resulting inserts were sequenced using the M13 forward and reverse primers and then using primers designed on the basis of the initial sequence obtained.

Plasmid constructions. Plasmid pED302, used to replace the *comQXP* locus from *B. subtilis* BD2833 with a Km^r cassette, was constructed in three steps. A PCR fragment containing the 3′ end of *comP* and the 5′ end of *comA* was obtained by amplification of *B. subtilis* DNA with *Sal*I-′comP (ACTGTCGACA GATGAATGAGCAGATGTC) and comA′-Pst (CCCTGCAGATCCATCTCT ACACCCCCC) primers, and cloned into the *Srf*I site of pPCR-script (Stratagene) to give pED300. A second PCR fragment contained the upstream *degQ* gene and was obtained with the primers Eco-degQ (AGAATTCGGCTGCGG TCAGAATG) and degQ-Xma (AACCCGGGCTGCTCAATAACGACTTCC). This fragment was cloned into pED300, giving rise to pED301. A *Hin*cII kanamycin resistance cassette from pKM1 (11) was then inserted into pED300 at the *Sma*I restriction site, between the two cloned fragments, giving rise to pED302, in which the resistance cassette was flanked by sequences derived from upstream and downstream of the region to be deleted.

The pED318 pheromone production plasmid was derived from the PCR script vector by insertion of PCR-amplified DNA fragments encoding the *B. subtilis* 168 *comQ* and *comX* genes at the *Srf*I restriction site. Primers used for the PCR were uni-comQ-Xba (see above) and 168-comX/Sph (CGCATGCCACCTATTAAT

CACCCC). pED319 is the same as pED318 but with *comQX* cloned in the opposite (unexpressed) orientation.

Construction of *Bacillus* strains. BD2833 was constructed by transforming a strain carrying the Tn*917 lacZ* OK120 insertion in *srfA* (21) with a Cm-to-Tc switching plasmid (32), replacing the Cm^r marker with a Tc^r marker. This insertion was used because it occurred downstream from *comS* and therefore does not interfere with transformation, facilitating further strain construction. The *B. natto* NAF4 QS system was introduced into BD1658 by transformation with pNAF193. This plasmid carries the entire *B. natto comQXPA* locus with an Sp^r cassette inserted in the *Sac*I site of *yuzC*, a small gene of unknown function to the left of *degQ*. BD1658 has a Cm^r cassette inserted in *comP*. Selection for Sp^r was carried out, and transformants were screened for Cm^s. DNA from such a transformant was used to introduce the *B. natto* QS system into BD2834 to create BD2847. BD2834 carries a Km^r marker in an irrelevant open reading frame downstream of *comA* as well as a *srfA-lacZ* reporter construct. Selection for Sp^r was carried out, and transformants were screened for loss of Km^r. The loss of this marker selects for introduction of the entire *B. natto* NAF4 QS locus. A *comX* derivative of BD2847 (BD2877) was made by transformation with a plasmid carrying the QS locus of *B. natto* with a Ph^r cassette inserted in the *Mlu*I site of *comX*. BD2911 was obtained by transforming *B. subtilis* BD2833 with the linearized pED302 plasmid with selection for Km^r. Double recombination leads to a deletion of *comQ*, *comX*, and the first 613 codons of *comP*. BD2911 also contains a copy of *comK* in the *amyE* locus, under *Pxyl* control (6). Since BD2911 was a *comQ*, *comX*, and *comP* mutant, it was made competent by growth in the presence of xylose. To introduce the QS locus of heterologous *Bacillus* strains by congression, BD2911 was transformed with chromosomal DNA from these strains. In these transformations, selection was for the unlinked *leu* marker on X-Gal plates to score for the introduction of the donor QS system. For most of these congression experiments, BD2935, a derivative of BD2911, was used. BD2935 has a Cm^r marker associated with the *Pxyl-comK* construct at the *amyE* locus, instead of Em^r.

Tester strains for RO-H-1 (BD2962) and RO-C-2 (BD2963) were obtained by disruption of the *comQ* genes of the respective producer strains (BD2913 and BD2937). An internal fragment of each *comQ* gene (0.45 kb for RO-C-2 and 0.52 kb for RO-H-1) was PCR amplified by PCR using primers with *Eco*RI and *Hin*dIII sites and was cloned into the *Eco*RI and *Hin*dIII restriction sites of pUS19. This vector consists of pUC19 with a Sp^r cassette cloned between the *Nde*I and *Nar*I restriction sites. The resulting plasmids (pED345 for RO-H-1 and pED346 for RO-C-2) were then used to transform the producer strains (BD2913 and BD2937) with selection for Sp^r. This resulted in inactivation of the chromosomal *comQ* genes by Campbell-like recombination.

PCR-RFLP genotyping of the ComX producer strains. Chromosomal DNA was prepared from BD2913, BD2914, and BD2915, and PCR amplification of *comQ*, *comX*, and the 5′ end of *comP* was performed by using primers uni-comQ-Xba and P1, hybridizing upstream from *comQ* and in a conserved region of *comP*, respectively. These PCR fragments were then digested with either *Hin*dIII or *Eco*RV restriction enzyme. This restriction analysis allowed the four QS loci to be distinguished (not shown) and confirmed that BD2913, BD2914, and BD2915 were carrying RO-H-1, RS-B-1, and *B. natto* NAF4 *comQXP* loci, respectively.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences of the *B. mojavensis* RO-H-1 and *B. subtilis* RS-B-1 *comQXPA* loci are AY003901 and AY003900, respectively. The GenBank accession numbers of the *comX* sequences obtained in this study are AY003902 (RO-OO-2), AY003904 (RO-C-2), AY003903 (RO-FF-1), and AY003905 (RO-B-2).

RESULTS

***Bacillus* strains.** A group of *Bacillus* isolates was used for analysis of polymorphism at the QS locus. These are listed and described in Table 2. Most were collected in the Mojave desert by Roberts and Cohan (27), who also described their phylogenetic relationships. Our selected group of strains also includes *B. natto* NAF4 (34) and laboratory strains of *B. subtilis*, derived from strain 168. The *B. subtilis* strains have been assigned to the 168 and W23 subspecies (20, 27) (Table 2).

The *comQ*, *comX*, and *comP* genes are highly polymorphic. The *comQ*, *comX*, *comP*, and *comA* genes from *B. mojavensis* RO-H-1 and *B. subtilis* RS-B-1 were sequenced and compared to those of *B. subtilis* 168 and *B. natto* NAF4 (34). We compared the four *degQ comQXPA* DNA sequences using the PLOTSIMILARITY program (Genetics Computer Group [GCG] Wisconsin package). *degQ* is a flanking gene that is not involved in the QS mechanism. The sequences used for this analysis extended from the *degQ* start codon up to the 63rd codon of *comA*. The resulting plot (Fig. 1) displays the average similarity among the four aligned *degQ comQ comX comP comA* DNA sequences at each position in the alignment, using a moving window of 25 nucleotides. A strong polymorphism is evident, extending from the beginning of the *comQ* open reading frame (starting at the 6th codon) through the first two-thirds of *comP*. In contrast, *degQ*, *comA*, and the C-terminal third of *comP*, corresponding to the histidine kinase domain, are highly conserved among the four strains. The *degQ* and *comA* nucleotide sequences display more than 90% identity. Interestingly, the polymorphism is hypervariable in several regions. One corresponds to the DNA sequences encoding the C-terminal end of ComX extending through the first 20 codons of *comP*. Another hypervariable region is located in the central portion of the DNA that encodes the *comP* linker. We define this linker as extending from the membrane domain of *comP* to the DNA encoding the conserved H box (5) of ComP (position 564 in the *B. subtilis* 168 ComP sequence). In summary, this sequence comparison shows that the DNA sequences are almost identical among the four *Bacillus* strains until the 6th codon of *comQ*. Then a dramatic polymorphism extends roughly up to the ComP histidine kinase domain, where the divergence weakens, so that the *comA* sequences are almost identical in all four strains. A pairwise comparison of the four strains revealed that the entire sequences of *B. mojavensis* RO-H-1 and *B. subtilis* RS-B-1 are quite similar to one another, although distinct from those of *B. natto* NAF4 and *B. subtilis* 168.

In addition to these comparisons, we have sequenced the *comX* genes from the four additional wild-type *Bacillus* isolates listed in Table 2. An alignment of the predicted sequences of all eight ComX proteins is displayed in Fig. 2. The only constraint placed on these comparisons is that we forced alignment of the tryptophan residues, known to be the site of modification in *B. subtilis* 168 (17). Several of the predicted ComX sequences are closely similar to one another (e.g., RO-H-1 and RS-B-1, RO-B-2 and RO-OO-2, and RO-C-2 and 168). The *B. natto* NAF4 sequence is remarkable in that it has both N- and C-terminal extensions. The mature *B. subtilis* 168 ComX molecule is processed to yield an N-terminal alanine (Fig. 2) or aspartate residue (17). It is striking that the most extreme polymorphism occurs downstream of the presumptive process-

TABLE 2. Wild-type *Bacillus* strains

Species	Strain	Group	Reference
B. subtilis	168	168	
B. subtilis	RO-OO-2	168	27
B. subtilis	RO-FF-1	168	27
B. subtilis	RS-B-1	W23	27
B. mojavensis	RO-B-2		27
B. mojavensis	RO-H-1		27
B. mojavensis	RO-C-2		27
B. natto	NAF4		34

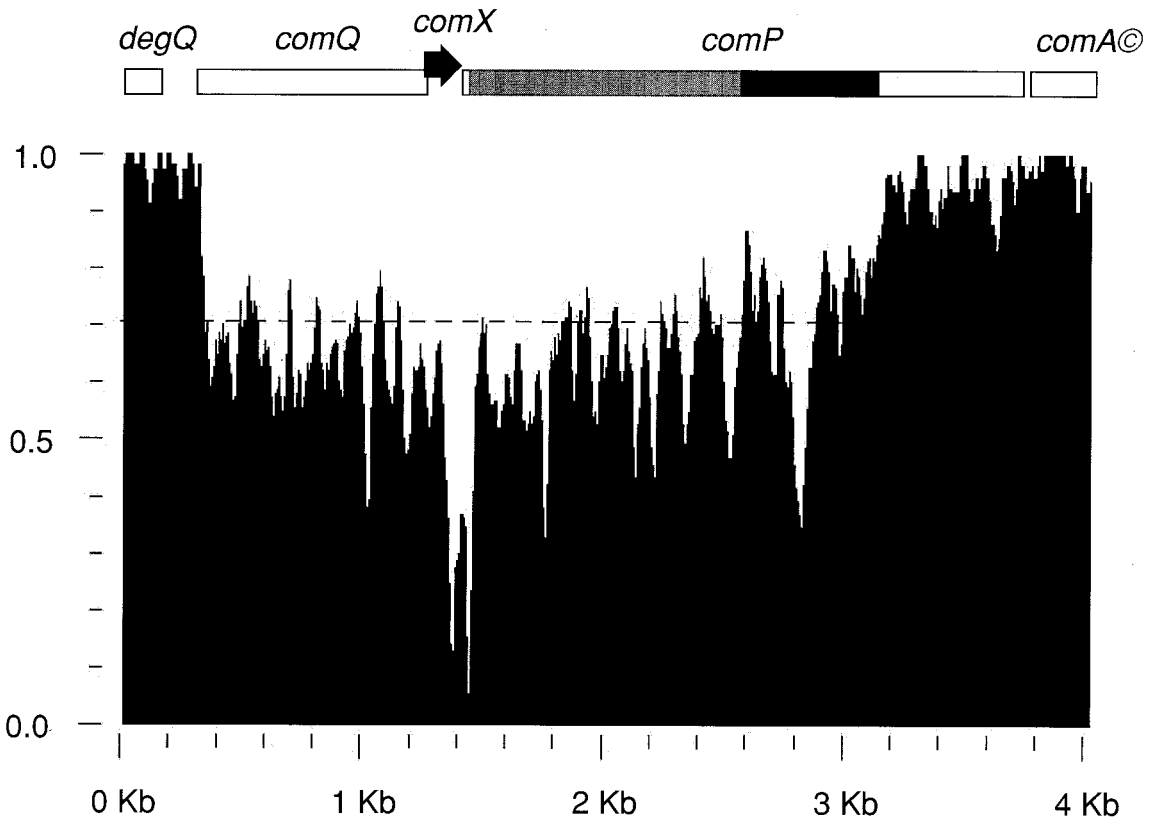

FIG. 1. Polymorphism at the *Bacillus* QS locus. DNA sequences of *B. subtilis* 168 and RS-B-1, and of *B. mojavensis* RO-H-1 and of *B. natto* NAF4, extending from the *degQ* start codon to codon 63 of *comA*, were aligned using the PILEUP alignment program (GCG Wisconsin package). This alignment was used as an input sequence for the PLOTSIMILARITY program. A 25-nucleotide window of comparison was moved one position at a time along the sequence, and the average similarity within the window is plotted at the middle position of the window. The overall average similarity is plotted as a dotted line. A genetic map is also shown above the plot. The membrane and linker domains of ComP are shown as shaded and solid bars, respectively. The linker domain is defined as extending from the C-terminal end of the 8th putative membrane-spanning segment to the 1st residue of the ComP H box.

ing sites, with the sole exception of the tryptophan residue that bears the uncharacterized posttranslational modification.

Replacement of *comQXP* genes from *B. subtilis* 168 with their orthologs from other bacilli. In order to compare the specificities of these QS systems, it was necessary to construct isogenic strains that produce pheromones (producer strains) and can respond to them (tester strains). This requirement for isogenicity is more than just good genetic practice. The addition of conditioned media from heterologous strains generally resulted in pronounced growth inhibition (not shown), most likely due to the presence of defective phages (8), bacteriocins (42), antibiotics, or other toxic substances (not shown). Two genetic methods were employed for the construction of these strains, and the results obtained with them were the same. We first constructed strains BD2911 and BD2935. These *B. subtilis* 168 strains carry a *srfA-lacZ* reporter for QS activity, and a replacement of the *comQXP* genes by a Km^r marker. At the *amyE* locus is a copy of *comK* under the control of a xylose-inducible promoter. BD2911 and BD2935 differ only in the antibiotic resistance markers associated with the *Pxyl-comK* constructs at the *amyE* locus. When they are grown in competence medium, the addition of 2% xylose enables transforma-

```
Consensus      #~.##~~##.~P~##..#....#~L#~#~..~...I#.^%.~.    .......W..
RS-B-1      ---MQEMVGYLIKYPNVLREVMEGNACLLGVDKDQSECIINGFK---GLEIYSMMDWHY---------------
RO-H-1      ---MQEMVGYLIKYPNVLREVMEGNACLLGVDKDQSECIINGFK---GLEIYSMLDWKY---------------
RO-B-2      ---MQEIVGYLVKNPEVLDEVMKGRASLLNIDKDQLKSIVDAFG---GLQIYTNGNWVPS--------------
RO-OO-2     ---MQEIVGYLVKNPEVLDEVMEGRASLLNIDKDQLKSIVDAFR---GMQIYTNGNWVPS--------------
B. natto    MKHIDKIISHLVNNPEAFDQFKNGNLTLLNINEKEKKAILYAFEQ---GEVPRTSKWPPIEAISNFFEDDKRKS
RO-C-2      ---MQDLINYFLSYPEVLKKLKNREACLIGFSSNETETIIKAYNDYHL-SSPTTREWDG----------------
168         ---MQDLINYFLNYPEALKKLKNKEACLIGFDVQETETIIKAYNDYYL-ADPITRQWGD----------------
RO-FF-1     ---MQELISYLLKYPEVLKKLKSNEASLIGFSSDETQLIIEGFEGIEEVKRGNAGKWGPE--------------
```

FIG. 2. Alignment of ComX amino acid sequences. The conserved W residue is marked in boldface and was used to align the sequences with the ZEGA program (http://molsoft.com/services/help/intro.htm). The following symbols indicate similar residues: #, hydrophobic residues; ~, polar residue; ^, small residue; %, aromatic residue. Nonconserved residues are indicated by dots. The N-terminal alanine in *B. subtilis* 168 ComX is underlined.

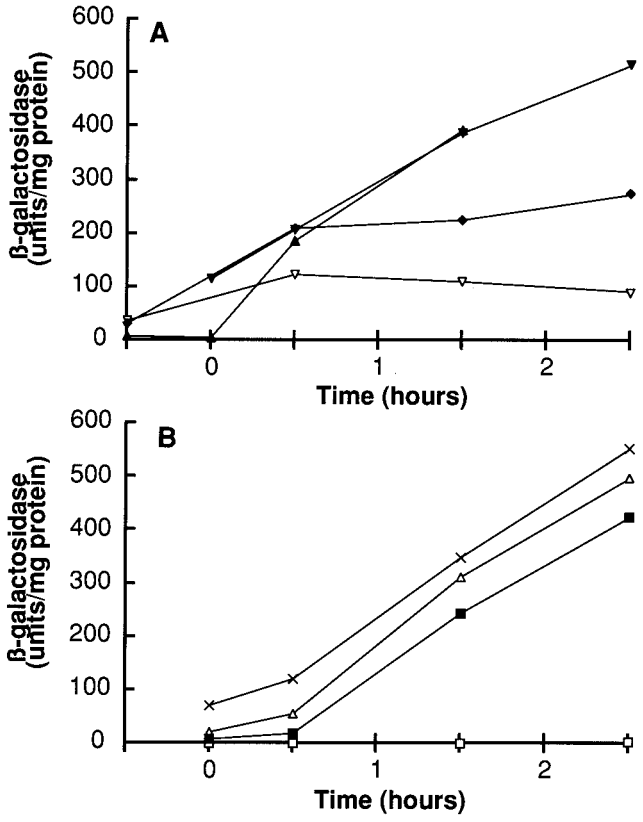

FIG. 3. Expression of *srfA-lacZ* by producer strains. β-Galactosidase activities were measured in a set of isogenic *B. subtilis* strains expressing heterologous *comQXP* genes. Time is in hours before or after the time of transition from exponential to stationary phase. The specificities of the producer strains in the two panels were as follows. (A) ▲, 168 (BD2833); ▼, RO-H-1 (BD2913); ▽, RS-B-1 (BD2914); ◆, *B. natto* NAF4 (BD2915). (B) ×, RO-FF-1 (BD2939); △, RO-B-2 (BD2936); ■, RO-C-2 (BD2937); □, RO-OO-2 (BD2947).

tion to occur in the *comQXP* deletion strains. BD2911 or BD2935 were transformed with chromosomal DNA prepared from a given heterologous strain, with selection for leucine prototrophy on plates containing X-Gal. Those Leu transformants that had incorporated foreign *comQXP* genes by congression were visualized as blue colonies due to the expression of *sfrA-lacZ*. These strains were checked for Km^s, which results from the replacement of the Km^r marker by the foreign *comQXP* genes, and were further characterized by PCR genotyping (see Materials and Methods). In addition to strain 168 itself, seven such additional producer strains were constructed from RO-OO-2, RO-FF-1, RS-B-1, RO-B-2, RO-H-1, RO-C-2, and *B. natto* NAF4. As shown in Fig. 3, all of these strains except RO-OO-2 expressed significant levels of *srfA*, although the amounts of β-galactosidase expressed by the various strains varied somewhat. For instance, the RS-B-1 producer strain consistently exhibited lower β-galactosidase activity than the others, and the *B. natto* NAF4 producer exhibited an intermediate level. To exclude the possibility that a relevant unsuspected gene was integrated into *B. subtilis* by congression during the construction of these producer strains, we employed a second strategy to construct producer and tester strains carrying the *B. natto* NAF4 system as described in Materials and Methods. A plasmid carrying only the QS locus and a few flanking genes from *B. natto* was used to replace the QS locus of *B. subtilis* 168 as described in Materials and Methods. A tester strain was derived from this producer by inactivation of *comX*. This producer strain expressed a level of β-galactosidase comparable to that shown in Fig. 3 (data not shown) and produced a conditioned medium that could activate the tester strain. Taken together, these results demonstrate that the QS systems from other bacilli can replace that of *B. subtilis* 168, strongly suggesting that all of the strains, with the possible exception of RO-OO-2, possess active ComX-mediated QS systems.

Specificity of pheromone recognition. The striking polymorphism noted above suggests that there is specificity of interaction between a given pheromone, its receptor protein ComP, and the processing protein ComQ. We have investigated this predicted specificity with regard to ComX and ComP. For this, the isogenic producer strains were grown in competence medium to prepare conditioned medium. Four tester strains were constructed by inactivating the *comQ* or *comX* genes of the 168, RO-H-1, RO-C-2, and *B. natto* NAF4 producer strains, as described in Materials and Methods. The tester strains cannot produce active pheromone but are expected to express ComP sensor proteins. Each conditioned medium was mixed with an equivalent volume of fresh competence medium and used to grow each of the isogenic tester strains. β-Galactosidase activities were measured at different times for each of the producer-tester combinations, and representative experiments are reported in Fig. 4. A summary of the pattern of responses is reported in Table 3, which includes results for RO-FF-1 that are not shown in Fig. 4. Since the growth of the tester strains in diluted conditioned medium was slightly less than in fresh medium, in some experiments we used conditioned medium from the tester strain itself as a control, instead of fresh medium. This seemed to give a slightly higher "baseline" response, probably due to the presence of CSF (31), but the qualitative pattern was the same when either control was used. In all cases, although conditioned medium was added at the onset of the experiment, the specific activity of β-galactosidase increased during growth. This was expected, since additional control mechanisms for *srfA* are known to exist (4). Conditioned medium from the RO-OO-2 "producer" strain failed to induce expression of *srfA-lacZ* from any of the tester strains, consistent with the absence of activity of this strain when tested by itself (Fig. 3). Several examples of specificity are evident from the results in Table 3 and Fig. 4. For example, the *B. natto* NAF4 pheromone induces only the cognate tester, and conversely, the *B. natto* NAF4 tester is induced only by its cognate pheromone. Some cross talk is also evident. For instance, the 168 pheromone induces the RO-C-2 tester, and the RO-H-1, RS-B-1, and the RO-B-2 pheromones induce the RO-H-1 tester. It should be noted that this is not a complete response matrix, and the full pattern of specificity is therefore not evident.

Production of active ComX pheromone from *E. coli*. The experiments reported above, as well as unpublished work described below, demonstrate that *comX*, *comQ*, and *comP* are sufficient to specify the pherotype. We next determined whether *comQ* and *comX* are sufficient for production of active ComX. For this, the *B. subtilis comQ* and *comX* genes were

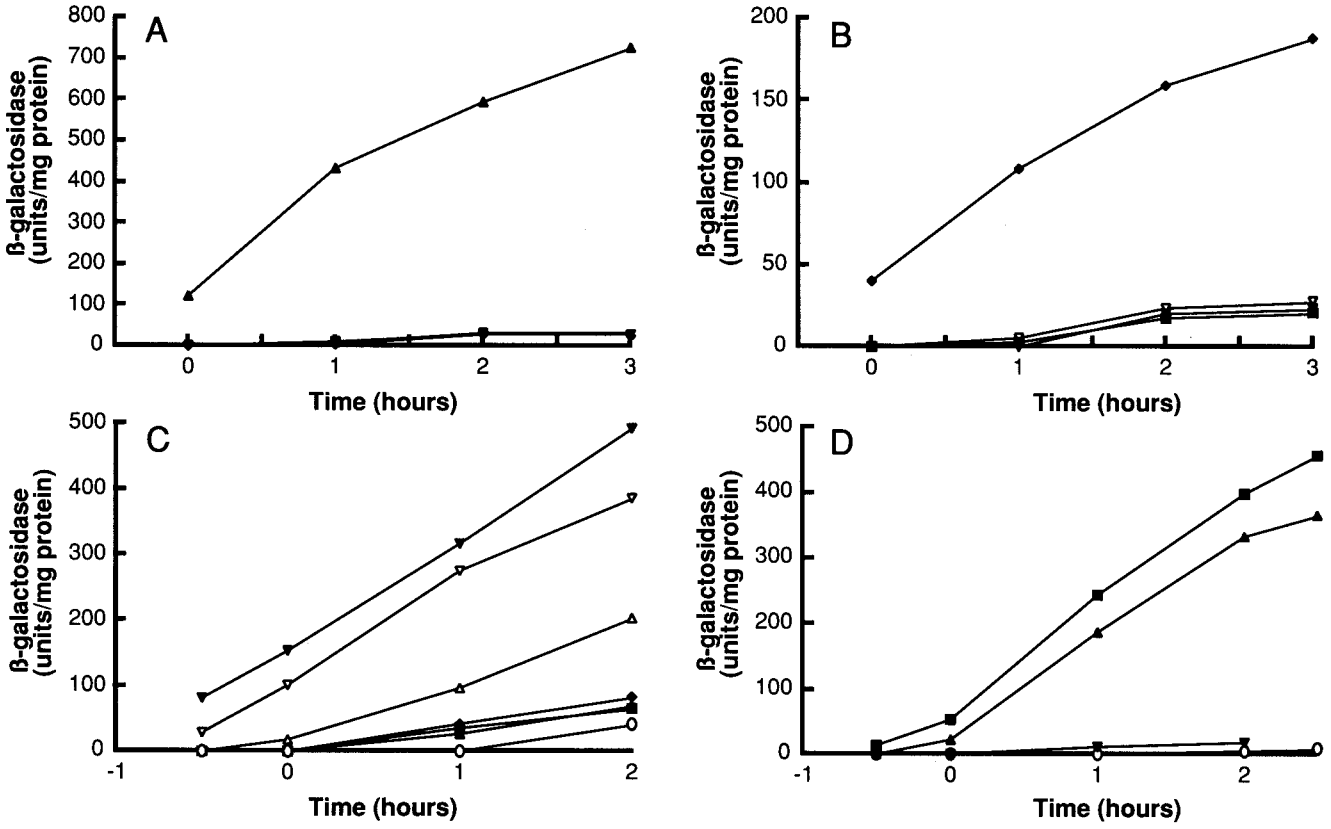

FIG. 4. Specificity of the QS response. Isogenic *B. subtilis* tester strains that do not produce ComX were grown in the presence of conditioned media prepared from isogenic producer strains. β-Galactosidase expressed from a *srfA-lacZ* reporter construct was assayed during growth. The following tester strains were used: BD2876 (derived from *B. subtilis* 168) (A), BD2877 (derived from *B. natto* NAF4) (B), BD2962 (derived from *B. mojavensis* RO-H-1) (C), and BD2963 (derived from *B. mojavensis* RO-C-2) (D). Conditioned media were from strain 168 (BD2833) (▲), RO-H-1 (BD2913) (▼), RS-B-1 (BD2914) (▽), *B. natto* NAF4 (BD2915) (◆), RO-B-2 (BD2936) (△), and RO-C-2 (BD2937) (■). The open circles in panels C and D show the effect of adding fresh medium to the tester (panel D) or conditioned medium from the tester itself (panel C). Time is in hours before or after the time of transition from exponential to stationary phase. Note that the ordinate scales differ in the four panels.

cloned into an *E. coli* plasmid under the control of the *lac* promoter. A second plasmid containing the same cloned fragment inserted in the opposite (unexpressed) direction was used as a control. *E. coli* strains carrying these plasmids were grown for 5 h in liquid competence medium supplemented with IPTG in order to induce *comQX* expression. Cells were harvested by centrifugation, and the supernatants were collected and filter sterilized. These supernatants were diluted into fresh competence medium, and *B. subtilis* BD2876 was used as a tester strain. As shown in Fig. 5, *srfA-lacZ* expression was induced to a level comparable to that of the BD2833 reference strain. The activity detected was dose dependent. The supernatant prepared from the reversed-orientation control did not lead to detectable *sfrA* expression. These data demonstrate that the ComX pheromone can be produced and secreted in *E. coli*, and that no accessory gene from *B. subtilis*, other than *comQ*, is required for this production. In other experiments we have shown that the active pheromone produced by *E. coli* does not appear to accumulate in the periplasm (not shown).

DISCUSSION

The QS system that governs *srfA* and competence gene expression in *B. subtilis* joins the Agr system of *S. aureus* (9, 10, 18) and competence regulation in *S. pneumoniae* (7, 24, 26, 41) in exhibiting striking polymorphism at the relevant loci and a corresponding variability in pherotype specificity. *Bacillus* and *Staphylococcus* QS systems represent a class of gram-positive mechanisms that involve peptide pheromones and membrane-localized histidine kinase sensors with complex topologies (12).

TABLE 3. Summary of QS specificities

Producer specificity	Response of the indicated tester strain to conditioned medium from the producer strain[a]			
	168	RO-C-2	RO-H-1	NAF4
B. subtilis 168	++	++	−	−
B. mojavensis RO-C-2	+/−[b]	++	−	−
B. subtilis RO-OO-2	−	−	−	−
B. subtilis RS-B-1	−	−	++	−
B. mojavensis RO-B-2	−	−	+	−
B. mojavensis RO-H-1	−	−	++	−
B. natto NAF4	−	−	−	++
B. subtilis RO-FF-1	−	−	−	−

[a] Determined as described in the text. The approximate magnitude of each response is indicated.

[b] This response is very weak but was noted in several experiments. It is not evident in Fig. 4.

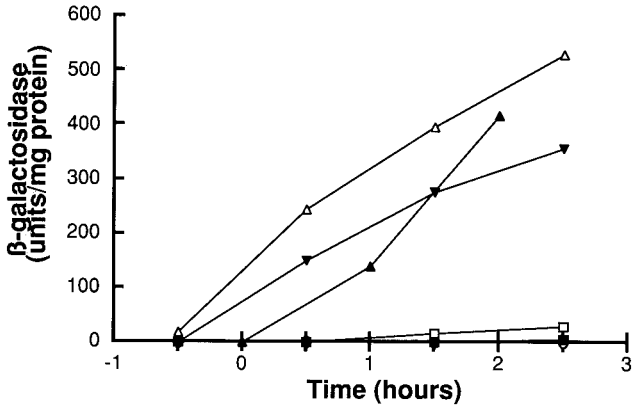

FIG. 5. Competence pheromone produced in *E. coli*. The *B. subtilis* 168 tester strain (BD2876) was grown in the presence of supernatant prepared from *E. coli* (ED318) carrying a plasmid with the *B. subtilis* 168 *comQ* and *comX* genes. β-Galactosidase activities produced from a *srfA-lacZ* reporter construct were assayed during growth. BD2876 was grown in the presence of 10-fold (△), 20-fold (▼), and 100-fold (□) dilutions of the *E. coli* supernatant. The activities of the *B. subtilis* producer (BD2833) (▲) and tester (○) strains grown in fresh medium are included for comparison. (The open circles are obscured by the solid squares). Conditioned medium from an isogenic *E. coli* strain (ED319) carrying the *comQ* and *comX* genes in reversed (nonexpressing) orientation (■) exhibited no activity.

It is likely that other QS systems in this group will also exhibit polymorphism at the relevant loci.

In the *Bacillus* system it appears that the *comQ*, *comX*, and *comP* genes are sufficient to determine specificity and that the strain 168 ComA can interact well with any of the ComP molecules. This was expected from the pattern of polymorphism in these genes (Fig. 1), since both ComA and the C-terminal domain of ComP with which it interacts are highly conserved. It has been confirmed by unpublished experiments (M. Ansaldi and D. Dubnau) in which chimeras containing all or part of the N-terminal domain of a given ComP respond to the cognate pheromone even if the *comA* gene is from strain 168.

The mechanism of transmembrane signaling in these systems is not understood, nor is the role of the unusual polytopic histidine kinase membrane domains characteristic of this group of sensor proteins. ComP for instance, has six to eight membrane-spanning segments and two large extracellular loops (25). Sequence comparisons may assist in making testable predictions concerning the interacting residues in ComX and ComP, as well as those in ComX and ComQ. For instance, it is interesting that hypervariable regions seem to exist in ComQ, in the C terminus of ComX, in the center of the linker region of ComP, and in about five additional segments in the N-terminal domain of ComP (Fig. 1). The C-terminal ComX variations presumably determine the observed variations in pheromone specificity, since they encode the mature pheromones. This is supported by the correspondence between the predicted amino acid sequences of the mature ComX molecules and the pherotype specificities (see below). Hypervariability in the N-terminal region of ComP suggests that some or all of these segments may be involved in determining response specificity. We have reported that deletion of one of the extracellular loops and two of the membrane-spanning segments of ComP confers complete ComX independence on *srfA* transcription (25). Clearly, the ComX-ComP interaction and the mechanism of transmembrane signaling are complex. The apparent conservation of the tryptophan residue in the eight ComX sequences, together with the many conserved residues in the four available ComQ sequences, strongly suggest that the uncharacterized modification of ComX is the same in all the strains. The conserved residues upstream of the presumptive cleavage site in ComX probably reflect common features in ComQ recognition.

In the staphylococcal Agr system, the peptide pheromone produced by one strain inhibits the response of another (9). We have tested the *B. subtilis* 168 tester strain for evidence of such interference (not shown). The homologous (168 specificity) conditioned media were mixed with equal amounts of each of the producer-conditioned media, and the *srfA-lacZ* expression was compared to that obtained by diluting the 168 conditioned medium with an equal volume of tester conditioned medium. In contrast to the staphylococcal results, we observed no dramatic interference. Dilution of the 168 conditioned medium with tester strain conditioned medium produced a reduction in *srfA-lacZ* activity. Dilution with the heterologous conditioned media gave approximately the same response. At most, an inhibition of about 25% was noted when the *B. mojavensis* RO-H-1 conditioned medium was used. However, since we do not know the concentrations of the various pheromones in the conditioned media, the absence of a strong effect makes this experiment somewhat inconclusive. Tran et al. (34) have also failed to detect interference between *B. natto* NAF4 and *B. subtilis* 168.

Several additional features of the pherotype specificity pattern deserve discussion. First, the RO-OO-2 producer strain exhibits no β-galactosidase activity, indicating that the system fails to induce *srfA* transcription, and the conditioned medium from this strain fails to induce any of the testers we have used. This has been observed with independently made RO-OO-2 producer constructs. We have no explanation for this failure, except to suggest that the original *B. subtilis* RO-OO-2 strain may carry an inactivating mutation in the QS locus. We have sequenced the *comX* gene from this strain, and it is evident from Fig. 2 that it is nearly identical to that of the fully active *comX* from *B. mojavensis* RO-B-2. There are however, three differences in the predicted amino acid sequences of these two precursor ComX molecules. These differences, or a mutation in *comQ*, may be responsible for the failure of the RO-OO-2 producer strain to synthesize detectable pheromone.

Second, the RO-FF-1 producer strain exhibits robust expression of *srfA-lacZ*, while conditioned medium from this strain does not activate any of the tester strains. Inspection of the C-terminal sequence of the RO-FF-1 pheromone confirms that its predicted mature pheromone differs from the others at every position except the conserved tryptophan residue (Fig. 2).

A third point concerns *B. mojavensis* RO-H-1 and *B. subtilis* RS-B-1. The entire QS loci of these two strains are quite similar (not shown), and conditioned media from both the RO-H-1 and RS-B-1 producer strains activate the RO-H-1 tester equally well. The RS-B-1 producer, however, exhibits low activity (Fig. 3). We have constructed an RS-B-1 tester strain and have failed to demonstrate a response to condi-

tioned medium from either the RS-B-1 or RO-H-1 producers (not shown). We have no explanation for this anomaly except that insertion of an inactivating cassette in the *comQ* gene of RS-B-1 may exert a polar effect on *comP* expression in this strain if the locations of promoter sequences in RS-B-1 differ from those of the other strains.

Fourth, an asymmetry is apparent in the responses of *B. subtilis* 168 and *B. mojavensis* RO-C-2. The RO-C-2 tester is activated by conditioned medium from both producer strains, whereas the 168 tester is activated by the homologous conditioned medium but very poorly by the RO-C-2 pheromone (not shown). This is likely due to the nature of the presumed ComP binding sites for pheromone. The mature pheromones of RO-C-2 and 168 are similar, sharing 4 out of 10 identities (Fig. 2). It is therefore apparent that the patterns of pheromone and receptor activities need not be identical.

Our analysis demonstrates the existence of at least four pherotype specificities in these *Bacillus* strains. One group consists of *B. subtilis* 168 and *B. mojavensis* RO-C-2, although these strains exhibit asymmetric responses, as just noted. A second group consists of *B. subtilis* RS-B-1 and the *B. mojavensis* isolates RO-H-1 and RO-B-2. The predicted mature pheromone sequences of RO-H-1 and RS-B-1 are nearly identical, exhibiting only two conservative replacements. The pheromone of RO-B-2 is predicted to share 5 out of 10 identical amino acid residues and 1 similar residue with the two other members of this group. The third group contains a single member, *B. subtilis* RO-FF-1, that exhibits little similarity to the others in the predicted sequence of its mature pheromone. The fourth group consists only of *B. natto* NAF4, with its unique predicted mature pheromone sequence. The long C-terminal extension on the *B. natto* NAF4 ComX precursor protein is notable. Tran et al. (34) have also tested the responses of *B. natto* NAF4 and *B. subtilis* 168 to one another, using transformability as a downstream reporter, and detected a low degree of cross-activation (~15%), in contrast to the present results (Fig. 4). This discrepancy might be due to the higher sensitivity of the transformation assay. Additionally, they introduced the heterologous genes on multicopy plasmids, which may have increased the sensitivity of the system by overexpression of ComP. Finally, detection of low-level cross-activation will depend on the concentration of active pheromone in conditioned medium in a given experiment, over which we have little control. In general, the predicted sequences of the mature pheromones are consistent with the observed pattern of responses. Clearly the variations in ComP sequence must determine response specificity, and our data provide very strong evidence that ComX must directly contact ComP. We have not shown that ComQ variation establishes specificity with respect to processing of ComX, although this is likely to be true, and has been demonstrated for *B. natto* NAF4 and *B. subtilis* 168 (34).

Our data raise questions concerning the evolution of the QS system and also provide some insights. For instance it is likely that the polymorphic regions of the QS loci have been acquired recently by the host organisms. The average G+C contents of the sequenced portions of the *comQ*, *comX*, *comP*, and *comA* genes regions are lower than the value of 43.5% reported for the entire *B. subtilis* genome (13). The G+C averages for the QS genes are 34.0% for *B. subtilis* 168, *B. subtilis* RS-B-1, and *B. mojavensis* RO-H-1 and 35.5% for *B. natto* NAF4.

How was QS polymorphism generated? It is formally possible that QS polymorphism reflects a series of independent recent horizontal-transfer events from distinct donors. This appears unlikely and would merely displace discussion of the evolutionary forces driving polymorphism to other organisms. It seems more reasonable to assume that a single transfer event took place in an ancestor of the contemporary strains. Any model for the evolution of divergent pherotypes should be general. It should be adaptable not only to the transformation systems of *B. subtilis* and *S. pneumoniae*, but also to the Agr system of *S. aureus*. We assume that each bacterial type tends to evolve a QS system that senses population density, resulting in the activation of downstream genes so as to increase its own fitness. The activation of these target genes will then occur optimally only in response to the needs of the given strain and not when another, possibly competing organism releases pheromone. Given this assumption, if another such strain is in the environment and produces a pheromone with cross-specificity, mutations that minimize this heterologous response without eliminating the homologous response would be advantageous. If the heterologous pheromone can actually interfere with response to the homologous molecule, as reported for the Agr system (9), then the selective pressure for change would be even greater. This situation would favor evolution toward greater specificity in the presence of foreign pheromones that possess partial or complete cross-activating activity. The result would be the evolution of polymorphism at the QS locus. A first step in this evolution might be an alteration in the sensor protein that reduces activation or interference by the foreign pheromone without eliminating homologous activation. Such a situation may obtain in the case of *B. subtilis* 168 and *B. mojavensis* RO-C-2, since the RO-C-2 pheromone activates the 168 tester very weakly (not shown), whereas in the reverse situation, robust activation occurs (Fig. 4D). The 168 *comP* may carry mutations that mitigate its response to the heterologous pheromone. Such restrictive mutations may sometimes decrease the extent of the homologous response, creating a situation in which selective pressure exists for compensating mutations in *comX* and then in *comQ*. This scenario predicts the coevolution of the three genes. Since the C-terminal domain of ComP that presumably interacts with ComA does not determine pherotype, there would be no pressure for divergence of this domain or of *comA*, as observed.

This model has the virtue of generality but is not concrete. For each system we must further consider the specific forces that select for function. In the case of the Agr system it has been postulated that there is competition for colonization of a new host, and each strain evolves to minimize the chances that a competing strain will gain a foothold (9). In our case, it would be premature to argue that transformability, whatever its adaptive role (33), provides the sole driving force for the evolution of the QS system, because it is clear that the *Bacillus* QS system does more than regulate competence. Although the QS system was first identified on the basis of its function in competence, it is certainly involved in regulating many genes, among them at least several that are involved with survival-enhancing stationary-phase adaptations. ComA-PO_4 activates the transcription of *srfA* (encoding surfactin synthesis). In addition *degQ*,

rapA, and *rapC* are ComA activated (19), and these genes are known to regulate the synthesis of degradative enzymes, sporulation, and competence. In *B. natto* NAF4, *comA* also regulates the synthesis of capsular γ-polyglutamate, postulated to provide protection against bacteriophages (34). A computer analysis revealed more than 400 potential ComA-binding DNA sequences in the *B. subtilis* genome (15). We can explain the selective pressure driving the evolution of polymorphism by supposing that it would be disadvantageous for the ensemble of genes activated by ComA-PO_4 (including competence genes) to be expressed inappropriately in response to foreign QS signals.

It is striking that the pherotype and phylogenetic classifications are not congruent. In fact, individual members within the first and second pherotype groups described above have been assigned to different species based on restriction site differences at three housekeeping loci, fatty acid composition differences, genetic transformation between the species, and DNA hybridization experiments (27, 28). The lack of congruence between the QS specificities and the phylogenetic relationships among the *Bacillus* strains suggests that the QS loci have passed horizontally among these strains, most likely by transformation, and that this horizontal transmission must have occurred more frequently than for most other genes. Alternatively, as noted above, they may have entered this group of bacilli from another source in a number of independent transmission events, again more frequently than transfer of other genes. Why the QS genes would spread so readily is mysterious. Perhaps pherotype switching transiently enhances fitness. This would be a Red Queen situation (37), defined as an evolutionary mechanism in which there is selection for change, usually in response to alterations in the biological environment, but without any long-term increase in fitness. Another, more gene-centered view is that the QS locus behaves like selfish DNA and that a special pheromone-activated mechanism exists for the transfer of this locus. Possibly an answer will present itself when the complete catalog of genes activated and repressed in response to ComX are identified by genomic approaches.

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