Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 260–264, January 1996
Microbiology

Enterococcus faecalis pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation

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Communicated by Dale Kaiser, Stanford Medical Center, Stanford, CA, September 16, 1995

ABSTRACT **Conjugative transfer of the plasmid pCF10 by *Enterococcus faecalis* donor cells occurs in response to a peptide sex pheromone, cCF10, secreted by recipients. The plasmid-encoded cCF10 binding protein, PrgZ, is similar in sequence to binding proteins (OppAs) encoded by oligopeptide permease (*opp*) operons. Mutation of *prgZ* decreased the sensitivity of donor cells to pheromone, whereas inactivation of the chromosomal *E. faecalis opp* operon abolished response at physiological concentrations of pheromone. Affinity chromatography experiments demonstrated the interaction of the pheromone with several putative intracellular regulatory molecules, including an RNA molecule required for positive regulation of conjugation functions. These data suggest that processing of the pheromone signal involves recruitment of a chromosomal Opp system by PrgZ and that signaling occurs by direct interaction of internalized pheromone with intracellular effectors.**

In *Enterococcus faecalis*, conjugative transfer of certain plasmids from donor cells occurs in response to sex pheromones produced by recipients (for reviews, see refs. 1–3). The molecular mechanism by which pheromones induce a mating response is not understood.

The transfer of the tetracycline-resistance plasmid pCF10 from *E. faecalis* donor cells is stimulated by as few as one to five molecules of the peptide pheromone cCF10 (sequence LVTLVFV; see ref. 4). Exposure of the donor cells carrying pCF10 to pheromone cCF10 results in activation of conjugation functions encoded by *prg* genes (pheromone responsive genes). These include *prgB*, encoding the cell surface adhesin Asc10, which mediates donor–recipient attachment (5). Expression of conjugation functions is controlled by negative and positive regulatory circuits (6–8). The pCF10-positive control elements (see Fig. 1) include the protein product of the *prgS* gene and RNA products, Q_S and Q_L, transcribed from *prgQ* and 3′ noncoding sequences (8–10). The region of pCF10 encoding negative control encompasses all plasmid-determined sequences required for signaling (11). PrgZ, the cCF10 binding protein encoded by pCF10, as well as a pheromone binding protein of a second conjugative hemolysin plasmid, pAD1, show significant amino acid sequence similarity to oligopeptide binding proteins (OppAs) from several bacterial genera (12, 13).

Many features of pheromone-inducible conjugation in enterococci resemble signaling processes observed in bacilli. The *spoOK* operon of *Bacillus subtilis* encodes an oligopeptide permease system (Opp), which is required for response to an oligopeptide pheromone, CSF, during competence development (14–17). At present, there are no data in the literature demonstrating direct molecular interaction of CSF with a *SpoOK*-encoded protein, or any other cellular component. *Opp* operons belong to the ABC transporter superfamily (18) and generally encode a periplasmic binding protein (OppA), two transmembrane proteins (OppB and OppC) believed to form a channel for passage of the substrate, and two membrane-associated cytoplasmic ATPases (OppD and OppF). ABC transporters have also been shown to be important in the *Agrobacterium tumefaciens* response to opines (19) and for *Streptococcus pneumoniae* competence (20, 21). No *oppD*, *-B*, *-C*, or *-F* homologs have been identified in the regions of pCF10 and pAD1 required for signaling.

In the experiments described here, we address the mechanism by which cCF10 signals a mating response. Genetic analysis indicated that in wild-type donor cells the plasmid-encoded high-affinity binding protein, PrgZ, recruits the chromosomal OppB, -C, -D, and -F proteins to process the cCF10 signal at physiological pheromone concentrations. To address the question of whether the PrgZ–Opp complex functions as a transporter, as opposed to a signal transducer, we used affinity chromatography to identify putative regulatory molecules that interact with cCF10. The identification of several of these molecules, including a *prgQ*-encoded RNA molecule recently shown to play a role in positive regulation of conjugation functions (10, 22), provides strong evidence for a signaling mechanism involving pheromone internalization and binding to intracellular effectors.

MATERIALS AND METHODS

Plasmids and Strains. The maps of plasmids used in these studies are shown in Fig. 1. All plasmids were expressed in OG1RF (Rif^r, Fus^r) (5). pCF47 and pCF465 represent Tn*917* insertions in *prgW* and *prgZ* genes of pCF10, respectively (6). pMSPS17 is a subclone of pCF10 containing the *prgWZYXQ* region (12). MSP9902 has all the genes necessary for aggregation substance expression and signaling contained on two plasmids, pINY8101 and pMSP6043 (see Fig. 1; ref. 11). MSP9909 contains pINY8101 and a spectinomycin-resistance gene fused in-frame with *prgZ* (pMSP6049, *prgZ::aad9*) (11, 23). No transcription termination sequences exist at the 3′ end of the *aad9* gene and the absence of polar effects has been confirmed by analysis of *prgY* mRNA (B.A.B.L., unpublished results). The complete description of the identification, genetic characterization, and nucleotide sequence of the *E. faecalis opp* operon will be presented in a separate communication (B.A.B.L., G.D. and A.P., unpublished data). Briefly, the operon was identified by using degenerate primers complementary to conserved ATP binding domain sequences of several *oppD* genes (14, 15, 24) to amplify a 400-nucleotide segment of chromosomal DNA via PCR. Sequence analysis of this segment and flanking DNA confirmed its identity as an

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internal region of *oppD*. This fragment was used to create a pG$^+$HOST5 (25) chromosomal Opp insertional mutant (MSP1015) from the signaling strain MSP9902. MSP1023 is a random integrant of pG$^+$HOST5, which served as a control for antibiotic effects on signaling. The *oppD* mutant was cultured in M9YE medium containing chloramphenicol (7.5 μg/ml), erythromycin (2.5 μg/ml), spectinomycin (250 μg/ml), and all 20 amino acids (0.1 mg/ml) except where noted.

Pheromone Induction of β-Galactosidase or Clumping. Overnight cultures (15 hr), grown under appropriate antibiotic selection, were diluted 1:4 in fresh medium containing no antibiotic and were incubated 2 hr at 37°C in a shaking incubator in the presence of synthetic cCF10 (Microchemical Facility, University of Minnesota) at the concentrations indicated in the figure legends. In the case of MSP1013 and MSP1023, erythromycin (1 μg/ml) was used in the overnight culture. β-Galactosidase activity of 0.8 ml of the culture was assayed by the method of Miller with 10-min incubations for color development (26). Induction of *prgB* expression as detected by OG1RF (pCF10) cell clumping was measured by a limiting-dilution microtiter clumping assay (27). The number reported is the inverse of the last dilution in which clumping was observed.

Affinity Chromatography. cCF10Y (LVTLVFVY) was synthesized (Microchemical Facility, University of Minnesota) and activity was confirmed by the microtiter clumping assay (see Table 1). The peptide was covalently coupled to either CNBr-activated Sepharose 4B or epoxy-activated Sepharose 6B according to the manufacturer's instructions (Pharmacia). Any storage of the coupled Sephadex was done at 4°C; however, fresh columns had to be prepared for each experiment. Crude cellular extracts were obtained by lysozyme lysis of 15-hr cultures that had been diluted 1:4 and incubated an additional 2 hr at 37°C with shaking (250 rpm) prior to lysis. The cells were pelleted, washed with phosphate-buffered saline (PBS), resuspended to 1/20th of the culture volume in lysis buffer (1 mM $MgCl_2$/1 mg of lysozyme per ml/500 μg of DNase per ml/250 μg of RNase per ml in phosphate buffer), and incubated for 0.5 hr at 37°C. The crude cellular extract was mixed with cCF10Y-bound Sephadex (1 g of coupled Sephadex per 10 ml of culture volume) and the mixture was incubated 0.5 hr at room temperature. The beads were washed with 3 vol of PBS and bound proteins were eluted with acetonitrile. The eluate was dried under vacuum and the entire sample obtained from 1 g of Sephadex (10-ml culture vol) was analyzed in a single lane for proteins by SDS/PAGE through a nonreducing or reducing (2-mercaptoethanol) 15% polyacrylamide gel followed by silver staining (28). Ethidium bromide staining of the SDS/polyacrylamide gel was accomplished by staining in a 0.5-μg/ml ethidium bromide bath.

Agarose Gel Analysis of Column Extracts. Whole column extracts obtained as described above were analyzed by agarose gel electrophoresis by standard methods (29). RNase treatment of the samples prior to electrophoresis was done by treating samples with ≈400 ng of RNase per ml for 30 min at 37°C followed by direct loading onto either a 0.7% or a 1.5% agarose gel.

Northern Blotting Analysis of Column Extracts. Whole column extracts were separated by electrophoresis through a 1.5% agarose gel and transferred by capillary blotting to Magna Graph membranes (Micron Separations, Westboro, MA). A *prgQ* PCR product corresponding to the *prgQ* promoter through Q_L was used as a probe. The PCRs used to obtain the probe contained 100 nmol of the upstream primer (TTTTTCTAGATGCATACATATTTTAGTTG), 100 nmol of the downstream primer [T(TG)CAC(GT)(GT)CTCTTACGACTAGTTCCAGTAC(AC)(AC)(AC)], 100 ng of pINY4551 (8), 80 μM each dNTP, *Taq* polymerase, and reaction buffer according to the manufacturer's instructions (Boehringer Mannheim). The PCR conditions were 40 cycles of 94°C, 45°C, and 72°C. The resultant product was analyzed by agarose gel electrophoresis and purified by Wizard PCR preparations according to the manufacturer's instructions (Promega). The probe was random prime labeled with ^{32}P (Amersham), and prehybridization, hybridization, and autoradiography were performed as described (29).

RESULTS AND DISCUSSION

PrgZ and the *E. faecalis* Opp System Are Both Involved in cCF10 Signaling. In these studies a recently developed system was used that utilizes β-galactosidase as a measure of pheromone response. The strain harbors one plasmid (pINY8101; Fig. 1) containing the positive control region of pCF10 and a *prgB*::*lacZ* transcriptional fusion and a second, compatible plasmid (pMSP6043) encoding the negative control and signaling genes (11).

To examine the role of *prgZ*, the gene was disrupted by creating pMSP6049 containing an in-frame *prgZ*::*aad9* fusion, which abolished the binding function of PrgZ (30) but did not exert polar effects on downstream genes (ref. 23; see *Materials and Methods*). *E. faecalis* MSP9909, containing this construct, showed a significant induction of β-galactosidase by cCF10 at high pheromone concentrations but was at least 10-fold less sensitive to lower concentrations (Fig. 2, compare responses of MSP9902 and MSP9909 at cCF10 concentrations between 10^{-11} and 10^{-12} M). The residual response of the PrgZ$^-$ strain was likely due to a host-encoded binding protein of lower affinity for cCF10. This is supported by the fact that the response of PrgZ$^+$ cells to cCF10 was not inhibited by a 1000-fold excess of the unrelated pheromone cAD1, whereas 55% inhibition was observed in PrgZ$^-$ cells under the same conditions (data not shown).

Due to the reported homology of PrgZ to OppA (12), the most obvious candidate for the host-encoded pheromone processing system was an Opp. However, an enterococcal *opp*

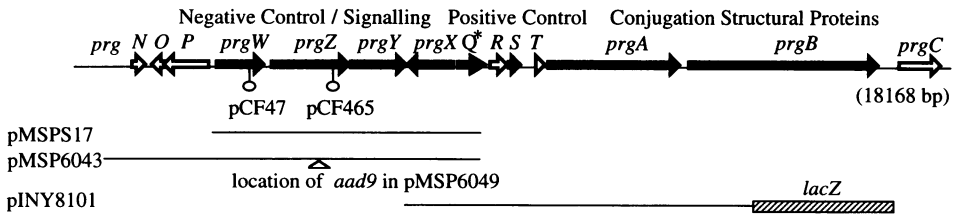

FIG. 1. Genetic organization of the known pheromone response genes. Each gene is designated by an arrow corresponding to the direction of transcription. Solid arrows indicate experimental confirmation of protein products. Region designated *prgQ* (*) actually encodes three products transcribed from the same 5′ end. The first is a peptide (iCF10) encoded in the 5′ end of a 400-bp RNA, Q_S, which represents the second functional product from this region. The third product from this region is a long mRNA molecule (Q_L; 530 bp), which represents a 3′ extension of Q_S and is produced under cCF10-induced conditions. The general function attributed to each gene is denoted above the genes; circles indicate position of Tn*917* insertions in pCF10. Lower rows indicate subcloned regions of pCF10 used in these studies; triangle shows position of the *aad9* fusion in *prgZ* as discussed in the text.

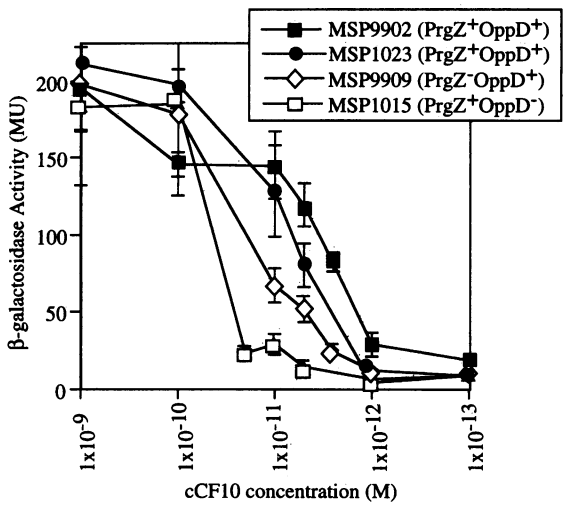

FIG. 2. Pheromone response of the *prgZ* and *oppD* mutants. Freshly diluted overnight cultures were induced with synthetic cCF10 at the indicated concentrations for 2 hr. Signaling efficiency was measured by assaying the amount of β-galactosidase activity expressed from a *prgB::lacZ* fusion. Uninduced levels of activity were <5 MU (Miller units) for all strains.

operon had not been identified prior to these studies. Using degenerate PCR primers complementary to conserved sequences in the ATP binding domains of *oppD* genes in several bacteria, a 400-bp fragment was amplified whose sequence indicated that it represented an internal fragment of *oppD*. Further cloning and sequencing of the flanking chromosomal DNA revealed the existence of a five-gene operon with the gene order (*oppA*, *-B*, *-C*, *-D*, and *-F*) identical to those of several other operons in this family, including *spoOK* (16). The complete sequence and genetic characterization of this operon will be reported in a separate communication. The PCR product was cloned into pG$^+$HOST5 and used for insertional inactivation to generate an Opp$^-$ derivative of MSP9902 called MSP1015, which allowed for examination of the role of the Opp in pheromone signaling. The failure of the insertional mutant to obtain essential amino acids for growth from peptides confirmed that it was an Opp mutant (data not shown).

As shown in Fig. 2, disruption of the *opp* operon had a deleterious effect on cCF10 signaling in MSP1015, even in the presence of a wild-type *prgZ* gene. Although a pheromone response was seen in this strain at >0.1 nM cCF10, the observed response could be due to a low level of diffusion of the hydrophobic peptide into the cell, a subpopulation of revertants, or to the action of another transport system that processes the signal inefficiently. In these experiments, the use of MSP1023, a PrgZ$^+$, Opp$^+$ strain containing randomly integrated pG$^+$HOST5 as a control, ruled out significant effects of the vector or antibiotics on signaling (Fig. 2).

These data support the model shown in Fig. 3 (*Upper*), where the PrgZ protein binds pheromone with high specificity and recruits the chromosomal OppBCDF complex to allow for signaling. In the absence of PrgZ, OppA can carry out the same process if the pheromone concentration is sufficiently high ($\geq 5 \times 10^{-11}$ M). Because the level of pheromone produced by recipient cells is $\approx 2 \times 10^{-11}$ M (4, 31) and there could be a number of competing peptides in the environment, it is likely that *prgZ*$^-$ donor cells would not be induced effectively in normal mating mixtures and Opp$^-$ cells would show no pheromone response. In the pAD1 system, it has been observed that phenotypic differences in cAD1 pheromone receptor mutants related to the host strain (13, 32); our results suggest that these observations could be due to differences in chromosomal Opp systems in different *E. faecalis* strains.

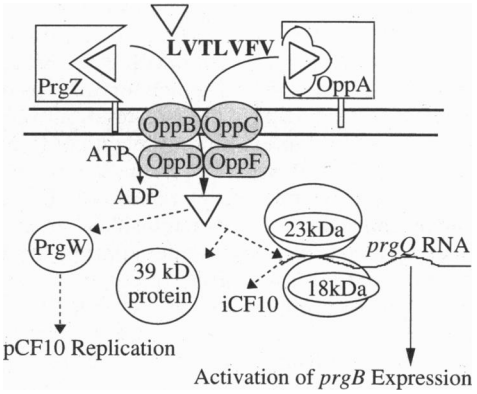

FIG. 3. Model of cCF10 signaling during pCF10 conjugation. Recipient-secreted pheromone (cCF10) interacts with either the plasmid-encoded specific binding protein PrgZ or the lower affinity chromosomal binding protein OppA on the donor cell. cCF10 is then transported into the cell via the chromosomally encoded oligopeptide permease system. Once inside the cell, the pheromone interacts with intracellular effector molecules, including possibly displacing iCF10 from a ribonucleoprotein complex, leading to activation of *prgB* (aggregation substance) and the subsequent conjugative transfer of the plasmid to the recipients.

Signaling Can Be Inhibited by Tethering cCF10 to Sephadex Beads. The fact that oligopeptide permease systems, including SpoOK and the *E. faecalis* Opp, are required for pheromone signaling and can mediate uptake of peptides used as sources of essential amino acids for growth could be construed as *a priori* evidence for pheromone internalization. However, it has been shown in studies of phosphate regulation in *Escherichia coli* that the ABC transporter Pst can function either in substrate transport or in signal transduction (33). Thus, more direct biochemical experiments were required to distinguish between these possibilities. The effect on signaling of tethering cCF10 to a bulky group that could not be transported by the Opp system was determined. Synthetic cCF10 was tethered to Sephadex beads by either the C or the N terminus. To supply a reactive group on the C terminus, a derivative cCF10 was synthesized with a tyrosine group at the C terminus (LVTLVFVY). This modified form of cCF10 (cCF10Y) retained full activity, as determined by standard microtiter plate clumping induction assays (Table 1). When cCF10Y was tethered by the N terminus to Sephadex 4B, it was found to no longer bind PrgZ or to signal at estimated cCF10 concentrations ≈1000 times higher than the minimal concentration necessary for signaling (ref. 4; Table 1). However, if cCF10Y was tethered by the C terminus, it retained the ability to bind the PrgZ as judged by affinity chromatography (see below), but it was unable to act as a signal (Table 1).

Interaction of cCF10 with Putative Effector Proteins. To determine whether cCF10 could bind to possible cellular effector molecules, affinity chromatography was carried out with an affinity matrix consisting of cCF10Y coupled by either the N terminus to CNBr-activated Sepharose 4B or by the C terminus to epoxy-activated Sepharose 6B (see *Materials and*

Table 1. Induction of clumping

Pheromone modification	Titer* Exp. 1	Titer* Exp. 2	PrgZ binding†
cCF10	256	64	
Tyrosinated cCF10	256	256	
Sepharose 4B-tyrosinated cCF10	0	0	No
Sepharose 6B-tyrosinated cCF10	0	0	Yes

*Response of cells to various forms of pheromone as determined by limiting-dilution microtiter plate clumping assays.
†Receptor binding was determined by affinity chromatography.

Methods). Proteins apparently bound to the N terminus of cCF10Y were 61, 43, and 36 kDa, while the column containing the free C-terminal cCF10Y bound proteins of 43, 39, and 15 kDa (Fig. 4, lanes 1 and 2). It should be noted that results essentially identical to those reported here were obtained when cCF10 (10^{-6} M in buffer) rather than acetonitrile was used to elute bound proteins (data not shown).

Further analysis of the products was carried out by analyzing pheromone-bound proteins from extracts of *E. faecalis* strain OG1RF carrying either cloned fragments of pCF10 or pCF10::Tn*917* derivatives (6). Tn*917* insertion into *prgZ* in the context of pCF10 resulted in loss of the 61-kDa protein corresponding to the molecular mass of PrgZ and the appearance of a 36-kDa species (Fig. 4, lane 5). The 36-kDa protein was eliminated by an insertion in *prgW* and corresponds to the predicted molecular mass of PrgW. Insertional inactivation of *prgW* also resulted in loss of the 61-kDa PrgZ-related band; this likely results from polar effects of the *prgW* mutation on *prgZ* expression (12). The 43-kDa proteins from both columns (lanes 1 and 2) are probably chromosomally encoded since similar-sized proteins were retained when extracts from plasmid-free OG1RF cells were used (data not shown). However, the 43-kDa protein species interacting with N-linked pheromone columns are probably different from the similar-sized proteins retained by the C-linked pheromone columns, since the species retained on the column with the free C terminus separated into 23- and 18-kDa molecules when run on reducing gels (lanes 6 and 7), while the 43-kDa protein interacting with the free N terminus of cCF10 did not (data not shown). In some experiments, these 23- and 18-kDa proteins each comprised a single band, while in other experiments clustered triplet bands were observed in these size ranges. The 39-kDa protein interacting with the C terminus of cCF10 (lanes 2, 6, and 7) was also host encoded, based on its presence in extracts from all strains. A small amount of a 15-kDa band was sometimes detectable in extracts from plasmid-free cells, but much more material in this size range was retained in strains carrying pCF10 DNA (lanes 2 and 6). The differences observed in cellular constituents bound to the two types of pheromone affinity columns suggest that there is specificity in the protein interactions with the column.

The deduced sequence of PrgW strongly suggests a cytoplasmic location (12). The PrgW protein was previously implicated as either a positive regulator or a structural component of the signaling machinery that includes PrgZ (12); this protein also shares significant sequence similarity with the RepA protein shown by Weaver *et al.* (34) to be required for replication of pAD1. The region of pCF10 conferring negative control and signaling has recently been shown to function as an autonomous replicon (11), suggesting that PrgW could have more than one function and that pheromones could regulate plasmid replication as well as conjugation.

The 15-kDa Species Bound to Pheromone Affinity Columns Contains *prgQ* mRNA. Interestingly, the plasmid-specific 15-kDa band did not appear to correspond to any pCF10-encoded protein, since extracts from a strain containing only the *prgW*, *-Z*, *-Y*, *-X*, and *-Q* genes (MSPS17) still had the product but would not be expected to encode a protein with a molecular weight between 1000 and 30,000. Further analysis of the 15-kDa band indicated that it was not protein but a fragment of *prgQ* mRNA. The band stained with ethidium bromide, was RNase sensitive, and hybridized with a *prgQ*-specific probe in Northern blots of the affinity column extracts (Fig. 5, lanes 2 and 4). Since no hybridizing species were detected in extracts

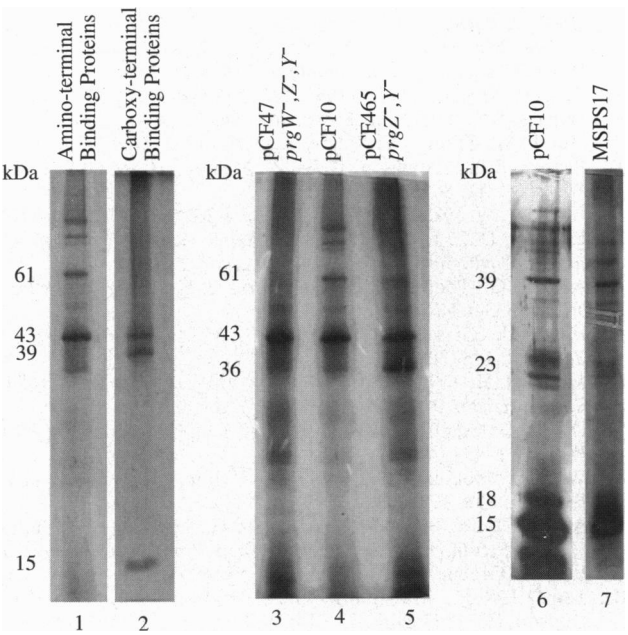

FIG. 4. cCF10 binding proteins. Modified cCF10 containing a Y at the C terminus was attached to Sephadex beads by either the N or C terminus. Cells were treated with lysozyme, RNase, and DNase, and the crude cell lysates were passed over the columns. Proteins were extracted by acetonitrile and analyzed by nonreducing SDS/PAGE and silver staining. Proteins extracted from columns with cCF10 attached by the N terminus leaving a free C terminus are designated carboxy-terminal binding proteins, while those binding to columns with cCF10 tethered by the C-terminus leaving a free N terminus are designated amino-terminal binding proteins. Lane 1, all of the N terminal binding proteins identified in extracts from pCF10-containing cells, whereas C-terminal binding proteins are shown in lane 2. Lanes 3–5, N-terminal binding proteins from cells carrying pCF10 (lane 4), pCF47 (lane 3), or pCF465 (lane 5). Lanes 6 and 7, reducing SDS/PAGE of the C-terminal binding molecules from OG1RF carrying either pCF10 (lane 6) or the subclone pMSPS17 (lane 7).

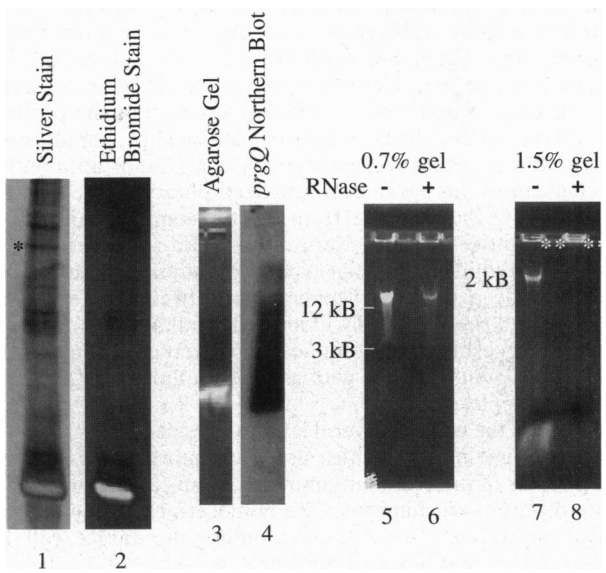

FIG. 5. Coisolation of *prgQ* RNA with C-terminal binding proteins. Extracts of C-terminal binding proteins from MSPS17 were analyzed by SDS/PAGE followed by silver staining (lane 1). *, Protein band that was inconsistently isolated during affinity chromatography experiments. Lane 2, same gel staining with ethidium bromide and viewed under UV light. Whole C-terminal binding molecules eluted from the pheromone affinity column were run into a 1.5% agarose gel and ethidium bromide stained (lane 3), and the autoradiogram represents the blotted RNA probed with ^{32}P-labeled *prgQ* DNA (lane 4). Column extracts were pretreated with or without RNase and then analyzed on either a 0.7% agarose gel (lanes 5 and 6) or a 1.5% agarose gel (lanes 7 and 8). **, Presence of a significant amount of ethidium bromide-stained material in the wells of the 1.5% agarose gel.

from plasmid-free strains, the faint 15-kDa band identified in some extracts from plasmid-free cells is not the same material (data not shown). Because the cells are treated with RNase during the lysis procedure prior to affinity chromatography, the hybridizing material is believed to be a protected fragment of *prgQ* RNA associated with one or more cCF10 binding proteins. Indeed, when column eluates were analyzed by agarose gel electrophoresis, two bands were detected. A larger RNase-resistant species (>12 kb; lanes 5 and 6) apparently contained RNA bound to protein, which could be resolved on a 0.7% gel and did not migrate into a 1.5% agarose gel. A more rapidly migrating RNase-sensitive band, presumably representing free RNA, was seen on a 1.5% agarose gel (lanes 7 and 8). In addition, when *in vitro* synthesized *prgQ* RNA was run over the column in the absence of the other material in the *E. faecalis* extracts, it was not retained (data not shown). Thus, binding of *prgQ* RNA to the pheromone affinity column is probably effected by one or more of the other proteins that simultaneously bind cCF10 and the RNA. This conclusion is supported by experimental results involving analysis of the binding of *E. faecalis* cellular components to *prgQ* RNA (B. A. Bensing and G.M.D., unpublished results), which suggest an interaction of this positive regulatory RNA with two putative ribosomal proteins similar in size to the 18- and 23-kDa C-terminal binding proteins identified in the present studies (lane 6).

The Intracellular Stage of Pheromone Signaling. The data described above suggest that ribonucleoprotein complexes containing pheromone, *prgQ* RNA, and some of the cCF10 binding proteins identified in our affinity chromatography studies may play a role in the regulation of *prgB* expression. One possible model to account for the existence of this type of complex is that the intracellular destination of the imported pheromone is a ribosome translating a polycistronic mRNA whose synthesis initiates at the *prgQ* promoter. The inhibitor molecule iCF10, which is the peptide product of *prgQ* (31), could have cis effects on ribosomes translating the Q RNAs by affecting mRNA stability or the translation of downstream positive control gene messages or both. Translational regulation by cis-acting peptides has been reported in other bacterial systems (35), and analysis of the transcription of the positive control region (ref. 22; B. A. Bensing and G.M.D., unpublished data) indicates that message stability is an important control mechanism in this system. Internalized pheromone could act by displacing the inhibitor from the ribosome and thus alleviating negative control. Confirmation or rejection of this model will require additional experimentation, but the present data provide a direct molecular link between the signaling pathway and the previously identified regulatory circuits and strongly suggest that pheromone internalization is required to allow for its interaction with an intracellular *prgQ* RNA–protein complex.

Based on the work in several laboratories, it is now clear that bacterial signaling molecules used for quorum sensing and other forms of intercellular communication can act by at least three different mechanisms. The homoserine lactone autoinducer of marine *Vibrio fischeri* enters the target cell by diffusion (36) and binds to the LuxR regulator (37), whereas the ComX pheromone of *B. subtilis* is believed to act via a two-component signal transduction mechanism (15). The enterococcal sex pheromones and probably the *B. subtilis* CSF competence pheromone (15) use peptide transport systems to reach intracellular target molecules.

The authors would like to acknowledge Barbara Bensing for providing *prgQ* RNA and Dr. Patrick Cleary, Barbara Bensing, and David Mills for helpful discussion. This work was supported by Public Health Service Grants 1RO1 GM49530 to G.M.D. and 1F32 AI08742 to B.A.B.L. and by Deutsche Forschungsgemeinschaft Grant 3914/3-2 to A.P.

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