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A Membrane Protein with Similarity to *N*-Methylphenylalanine Pilins Is Essential for DNA Binding by Competent *Bacillus subtilis*

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In a cloned copy of *comG* open reading frame 3 (ORF3), an in-frame deletion was generated by site-directed in vitro mutagenesis, removing the coding sequence for 15 amino acids from the central portion of this pilin-related protein. The mutagenized ORF3 was incorporated into the *Bacillus subtilis* chromosome, replacing the wild-type ORF3. The presence of the deleted ORF3 in the chromosome, as confirmed by Southern analysis, was associated with the complete loss of competence by the mutant strain. The ability of the mutant cells to bind exogenous radiolabeled DNA was reduced to the level of nonspecific binding of DNA by noncompetent cells. The chromosomal ORF3 mutation was partially complemented in *trans* by a plasmid-encoded wild-type ORF3 copy under P_{SPAC} control upon induction of the P_{SPAC} promoter. Using antiserum raised against a synthetic 14-mer oligopeptide deduced from the ORF3 sequence, an immunoreactive band of approximately the expected molecular size was obtained in Western blot (immunoblot) experiments with extracts of cells containing the plasmid-encoded inducible gene. A signal was also detected when cells harboring the chromosomal wild-type or mutant ORF3 in single copy were grown in competence medium. This signal was detected only in the light-buoyant-density (competent) cell fraction and only after the transition from the exponential to the stationary growth phase. In cell fractionation experiments with competent cell extracts, the immunoreactive protein was found in both the NaOH-insoluble and -soluble membrane fractions and was sensitive to proteinase K treatment of either protoplasts or whole cells.

Competent cells of *Bacillus subtilis* are able to bind, process, and take up exogenous transforming DNA with high efficiency. Competence develops in a programmed manner during the transition to the stationary growth phase in response to nutritional and growth stage-specific signals that are poorly understood. The developmental process is quite complex, requiring the products of many regulatory genes (8). The end products of this regulatory pathway comprise a set of proteins that are encoded by late competence genes. The DNA sequences of several of these genes have been determined (1, 23; J. Hahn and D. Dubnau, unpublished data). Most specify hydrophobic proteins that have been postulated to reside in association with the membrane and to be directly involved in the DNA binding and uptake process during transformation.

The *comG* operon is a late competence transcription unit that contains seven open reading frames (ORFs) (1, 2). These are transcribed from a single major promoter that appears to be read by the predominant (σ^A-containing) form of vegetative RNA polymerase, beginning at about the time of transition from exponential growth (t_0). *comG* ORF3, −4, and −5 specify small proteins, each consisting of about 100 amino acid residues. These three predicted proteins possess hydrophobic N termini and resemble in their amino acid sequences a class of bacterial proteins known as the *N*-methylphenylalanine (*N*-methy-Phe) pilins. The ORF3 product bears the closest relationship to these pilins. These similarities are interesting, since the pilin of *Neisseria* and *Moraxella* spp. has been implicated in genetic competence in those organisms (5, 27, 28), and pili in other gram-negative bacteria are involved in conjugational DNA transfer, although not necessarily in the transport process (19). Particularly suggestive is the ability of pilin to assemble into a tubular structure with a central pore sufficiently wide to admit single-stranded DNA.

The identification and analysis of the *comG* operon were based on the availability of a group of Tn*917lac* insertion mutants (1, 2, 16). All of these resulted in the complete loss of competence and in the inability of the culture to bind DNA. An insertion in *comG* ORF1 yielded an additional phenotypic change: unlike the wild type (15, 18), the resulting strain was unable to resolve in Renografin density gradients into light (competent) and heavy (noncompetent) cell fractions. Mutants with insertions in the other *comG* ORFs were fully able to resolve into these cell types. This difference provided evidence that ORF1 was independently required for the development of competence. Since a mutant with an insertion in the most distal ORF (ORF7) was noncompetent, this gene was also clearly required. However, since Tn*917lac* insertion is polar on downstream ORFs, it could not be concluded that ORF2 to 6 were needed for transformation.

This study establishes the importance of ORF3 for competence. An in-frame deletion in ORF3 is shown to result in a competence-deficient phenotype. The ORF3 product is shown to be expressed under competence control and to be a membrane protein, exposed on the exterior face of the cell membrane.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used in competence studies were derived from *B. subtilis* IS75 (*hisA1 leu-8 metB5*). This strain was wild type with respect to competence. Chromosomal DNA used for transformation experiments was prepared from *B. subtilis* BD1018 (*trpC2*). The strain used for preparation of ^{3}H-chromosomal DNA in DNA-binding experiments was *B. subtilis* BD204 (*his thy*).

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The bacteriophage vector for site-directed mutagenesis and integration into the *B. subtilis* chromosome was a derivative of M13mp19*cat* (14) containing a 3.5-kilobase-pair (kb) *Eco*RI fragment representing the distal part of the *B. subtilis* IS75 *comG* operon (1). Plasmid pRB20 was constructed by insertion of a 609-base-pair *Pvu*II fragment of pED32 (1), containing *comG* ORF3, into the *Sma*I site of the *Escherichia coli*-*B. subtilis* shuttle plasmid pIQ45 (31) under control of the inducible P_{SPAC} expression system. The transformable *E. coli* recipient for isolation of this recombinant plasmid was the chloramphenicol-sensitive strain AMA1004 (6).

Media. Liquid media for all *B. subtilis* experiments were VY (25 g of veal infusion [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], 1,000 ml of water) and competence medium (7). Amino acids were added at 50 μg/ml. For selection of chromosomal or plasmid-encoded drug resistance, chloramphenicol was added at 5 μg/ml. Solid medium was tryptose blood agar base (Difco). Liquid media used for *E. coli* were Luria broth (LB) or 2× yeast tryptone (22). Where appropriate, LB agar was supplemented with chloramphenicol (10 μg/ml) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml).

Isolation of DNA. Plasmid preparations from *B. subtilis* and *E. coli* strains were made by using a modification of the alkaline lysis protocol (4). Chromosomal DNA was isolated from *B. subtilis* strains as described previously (9).

Site-directed mutagenesis. For generation of the ORF3 mutation, the Muta-Gene M13 in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.) was used as instructed by the manufacturers. Template was the M13mp19*cat*-*comG* derivative described above. The mutagenic 49-mer deoxyoligonucleotide primer was purchased from Operon Technologies, Inc., San Pablo, Calif. The sequence of the primer was: 5′-CTTTTAATTACGATACC GAATTCGTCGACCATGGTTAAGGCACAAATGA-3′.

DNA sequencing. DNA was sequenced by the dideoxynucleotide chain termination method (26), using [α-^{35}S]dATP as the sequencing label. In all cases, the sequencing reactions were performed with T7 DNA polymerase Sequenase kits (U.S. Biochemical Corp., Cleveland, Ohio). The sequencing reaction products were resolved on 6% polyacrylamide–8 M urea sequencing gels. Gels were run at 1,500 V and between 50 and 55°C, fixed in 10% methanol–10% acetic acid in water, vacuum dried on Whatman 3MM paper, exposed to either Fuji RX or Kodak X-OMAT AR film, and visualized by autoradiography.

Southern hybridization. Chromosomal *B. subtilis* DNA was hybridized to the radiolabeled probe as described previously (21). The probe was prepared from *Eco*RI-digested M13mp19*cat*-*comG* DNA labeled with [^{32}P]dATP to a specific activity of 10^8 cpm/μg, using the nick translation reagent kit supplied by Bethesda Research Laboratories, Inc., Gaithersburg, Md. The [^{32}P]dATP (3,000 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill.

Transduction and transformation. Preparation of PBS1 transducing lysates and transduction of *B. subtilis* strains was carried out as described previously (11). For studies of transformation frequency, *B. subtilis* strains were grown in the one-step competence regimen (3) or were made competent according to the two-step procedure (9). The latter method was always used before Renografin separation. The qualitative plate test for transformability was carried out as described previously (16). *E. coli* competent cells were prepared as described previously (21).

DNA binding and uptake. The capability of *B. subtilis* cells to bind and take up exogenous macromolecular DNA was measured as described previously (10). The cells were incubated with chromosomal DNA (1 μg/ml) prepared from *B. subtilis* BD204 grown in the presence of 3 mCi of [^{3}H]thymidine (DuPont Co., Wilmington, Del.) per 75 ml of minimal medium containing 1.7 μg of cold thymidine per ml. The specific activity of the transforming DNA was 5×10^5 cpm/μg.

Separation of competent cells on renografin gradients. Competent cultures were resolved in Renografin gradients as described by Haseltine Cahn and Fox (18) and modified by Joenje et al. (20), using 5-ml gradients. The latter were centrifuged in an SW50.1 rotor at 15,000 rpm for 30 min at 20°C.

Subcellular fractionation. *B. subtilis* cells were harvested from 10-ml cultures, washed with STM buffer (25% sucrose, 50 mM Tris hydrochloride [pH 8.0], 50 mM NaCl, 5 mM $MgCl_2$) and resuspended in 0.5 ml of STM buffer. For preparation of protoplasts, lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added to 300 μg/ml, and the cells were incubated for approximately 30 min at 37°C. The protoplasting was followed by microscopy. The protoplasts were sedimented by centrifugation (5 min, 15,000 rpm), and the supernatant (cell wall fraction) was added to 0.5 volume of 3× sample buffer (0.5 M Tris [pH 6.8], 10% sodium dodecyl sulfate [SDS], 0.5 M EDTA, 30% glycerol, 0.02% bromphenol blue, 1.5 mM β-mercaptoethanol). The pellet was suspended in 0.5 ml of TM buffer (50 mM Tris hydrochloride [pH 8.0], 5 mM $MgCl_2$). Phenylmethylsulfonyl fluoride (PMSF; Sigma) was added to 1 mM, and DNase I (Sigma) was added to 20 μg/ml. The sample was allowed to clear at room temperature for 10 min and subsequently centrifuged (5 min, 15,000 rpm). The supernatant (cytoplasmic fraction) was added to 0.5 volume of 3× sample buffer. The pellet (membrane fraction) was either solubilized in 50 to 200 μl of 1× sample buffer or suspended in 0.2 ml of 0.1 N NaOH (25). In the latter case, the sample was briefly vortexed and centrifuged for 5 min at 15,000 rpm. The supernatant (NaOH-soluble membrane fraction) was added to 0.5 volume of 3× sample buffer. The pellet (NaOH-insoluble membrane fraction) was dissolved in 50 μl of 1× sample buffer. Samples were withdrawn from the cytoplasmic and NaOH-soluble cell fractions before addition of the sample buffer for determination of the protein concentration, using the Bio-Rad protein assay kit according to the microassay procedure. Before electrophoresis, all samples were incubated at 100°C for 3 min.

Proteinase K treatment of protoplasts or whole cells. Whole cells or protoplasts were treated with 2.5 mg of proteinase K per ml in STM for 30 min at room temperature. PMSF was added to 4 mM, and the protoplasts or cells were washed three times by centrifugation in STM containing 4 mM PMSF. These samples were then subject to further fractionation as described above.

SDS-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). The cell extracts were separated on 15% SDS-polyacrylamide gels as described previously (17). The gels were either Coomassie blue stained or used for immunological detection of the ORF3 protein. Protein molecular weight standards (prestained and not prestained), run on these gels, were from Bethesda Research Laboratories. The protein standards were ovalbumin, carbonic anhydrase, β-lactoglobulin, lysozyme, bovine trypsin inhibitor, and insulin. The ORF3 antiserum was raised in rabbits against a synthetic 14-mer oligopeptide deduced from the predicted amino acid sequence of the ORF3 protein (Fig. 1) and was

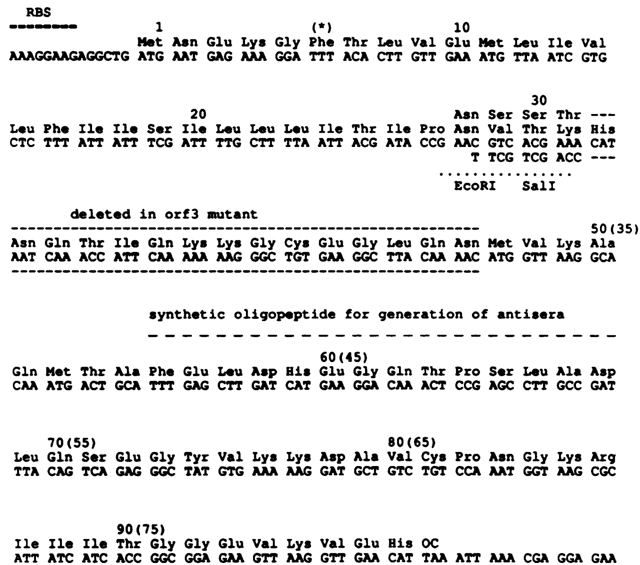

FIG. 1. Nucleotide sequences and predicted amino acid sequences of *comG* wild-type and mutant ORF3. By using the indicated mutagenic oligonucleotide, an in-frame deletion of 45 nucleotides was generated in the wild-type ORF3 sequence, removing the coding sequence for amino acids 32 to 46. at the same time, *Eco*RI and *Sal*I sites were introduced, altering amino acid residues 29 to 31. Also indicated is the sequence to which a rabbit antiserum was raised by using a synthetic 14-mer oligopeptide corresponding to amino acids 55 to 68 of the wild-type ORF3. The probable ribosome-binding site (RBS) for ORF3 is shown. *, Position of the conserved Phe residue that is N methylated in certain gram-negative pilins.

prepared by Berkeley Antibody Co. Inc., Richmond, Calif. The oligopeptide (CFELDHEGQTPSLAD-amide) was purchased from Multiple Peptide Systems, Inc., San Diego, Calif., and was coupled to keyhole limpet hemocyanin. The Western blotting was performed essentially as described previously (17). The proteins separated on SDS-polyacrylamide gels were transferred onto a nitrocellulose membrane of 0.45-μm pore size (Schleicher & Schuell, Inc., Keene, N.H.) by the wet transfer technique. The membrane was blocked with 2.5% nonfat skim milk as described previously (17) for 30 min at room temperature. The ORF3 antiserum (diluted 1:1,000) was preincubated in 10 ml of 2.5% nonfat skim milk with cells of *B. subtilis* IS75 (10 mg [wet weight] per ml) grown in complex medium (VY). The immunological reaction was visualized by the alkaline phosphatase staining technique (17). The secondary antiserum (goat anti-rabbit) was a product of Calbiochem Co., San Diego, Calif. The chromogenic substances 5-bromo-4-chloro-3-indolylphosphate and *p*-nitroblue tetrazolium chloride were obtained from U.S. Biochemical Corp.

RESULTS

Site-directed mutagenesis of *comG* ORF3. To test the significance of *comG* ORF3 for competence, the ORF3 protein was inactivated by an in-frame deletion that was not likely to exert a polar effect on the remaining ORFs of this operon.

The template for mutagenesis was an M13mp19*cat* derivative (14) harboring the distal 3.5-kb *Eco*RI fragment of the *comG* operon including ORF2 to -7 (1). The mutagenic primer was a synthetic 49-mer deoxyoligonucleotide with 20 nucleotides complementary to the template on each end. The regions of complementarity on the template were separated by 54 nucleotides within ORF3. The correct annealing and subsequent replication of the mutant DNA strand were expected to remove the coding region for 15 amino acids (residues 32 to 46) out of the central portion of the ORF3 protein. Furthermore, the sequence of amino acids 29 to 31 was changed by introduction of *Eco*RI and *Sal*I restriction sites adjacent to the deletion (Fig. 1).

After mutagenesis as described in Materials and Methods, replicative form DNA from the putative M13mp19*cat-comG* clones was screened for the presence of additional *Eco*RI and *Sal*I sites. Of six clones tested, three were identified as mutants. Subsequently, the entire *comG* ORF3 region of the mutant clones was sequenced to ensure the desired changes and to exclude the presence of any other mutation in ORF3 that might have been introduced during the mutagenesis. One of the clones that matched these requirements was used for further experiments. The nucleotide sequence of the mutagenized region of this clone is shown in Fig. 1. Interestingly, one of the clones sequenced contained an additional single-nucleotide deletion in the primer region (not shown). This finding underscored the importance of the postmutagenesis sequencing.

Substitution of the chromosomal wild-type copy by the mutant copy of *comG* ORF3. The M13mp19*cat-comG* phage obtained after mutagenesis carried the ORF3 deletion flanked by 1.4- and 2.1-kb segments of the *comG* operon. This phage cannot replicate autonomously in *B. subtilis* cells and therefore could be used directly for integration into the *B. subtilis* chromosome by Campbell-like homologous recombination with selection for Cm^r (14). The substitution was carried out in two steps (Fig. 2). First, the mutant M13mp19*cat-comG* vector was integrated into the *B. subtilis* wild-type chromosome by a single reciprocal (Campbell-like) recombination. The second step resulted in excision of the heterologous M13mp19*cat* region and of the redundant *comG* sequence introduced during the first step.

Competent cells of *B. subtilis* IS75 (wild type with respect to competence) were transformed with replicative-form DNA of M13mp19*cat-comG* harboring the ORF3 deletion. The clones in which recombination between one of the flanking regions of the mutant *comG* segment and the chromosomal wild-type *comG* operon had occurred were selected on chloramphenicol plates. The integrant clones could be divided into two classes on the basis of whether the recombination had occurred 5′ or 3′ to the *comG* ORF3 deletion (a or b in Fig. 2A). In this way, constructs were generated in which the ORF3 mutation was embedded in the entire *comG* operon (a) or in which the wild-type *comG* operon was reconstituted and the mutation remained in the nonfunctional 3.5-kb *comG* segment (b). In the former case, the recombinant clones were expected to be competence deficient (if ORF3 was important for competence), whereas in the latter, they were expected to retain normal competence. The latter prediction assumed that ORF7 was the most distal ORF of the *comG* operon (2). The qualitative plate test for competence, performed with several of the Cm^r clones obtained, yielded both expected phenotypes (not shown). In a control experiment, an analogous M13mp19*cat-comG* vector carrying the 3.5-kb *comG* wild-type region was used for integration into the *B. subtilis* wild-type chromosome. As expected, only competence-proficient Cm^r clones were obtained. This experiment confirmed that the essential region of the *comG* operon did not extend beyond the *Eco*RI site downstream of ORF7.

In the second step of the substitution experiment, the Cm^r

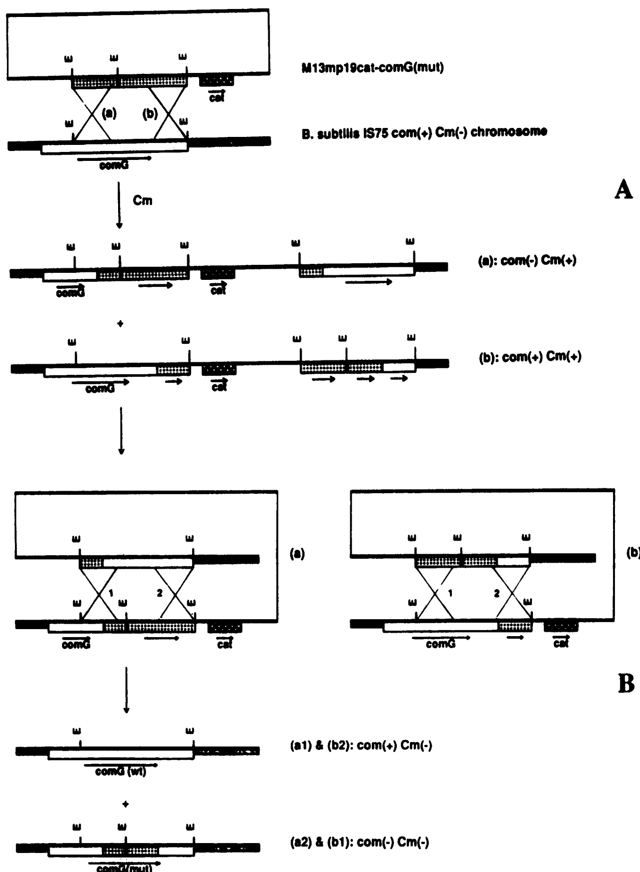

FIG. 2. Strategy for replacement of wild-type ORF3 by the mutant (deleted) allele. (A) The in vitro-mutagenized M13mp19*cat*-mutant ORF3 phage DNA (replicative form) was used to transform IS75, with selection for Cm^r. Single-reciprocal recombination events are predicted to yield Com$^-$ (a) or Com$^+$ (b) transformants carrying duplicated *comG* DNA. (B) During growth in the absence of chloramphenicol, the duplications permit excision to occur, leaving in the chromosome either a wild-type ORF3 (a1 and b2) or a mutant allele (a2 and b1). *cat* sequences are indicated by marbled boxes, the wild-type chromosomal *comG* operon is indicated by empty boxes, and the cloned *comG* sequence is indicated by stippled boxes. The ORF3 deletion mutation is indicated by a vertical bar. M13mp19 DNA is depicted by a black line, and non-*comG B. subtilis* chromosomal DNA is indicated by shaded boxes.

clones obtained in the first step were screened for the spontaneous loss of antibiotic resistance in the absence of selective pressure. Twenty independent Cm^r clones, including representatives of both competence phenotypes obtained in step 1, were passaged four times in VY without antibiotic. Serially diluted cultures were plated on tryptose blood agar base without chloramphenicol and subsequently replica plated on tryptose blood agar base supplemented with chloramphenicol. Cm^s clones occurred with a frequency of about 10^{-2}.

Assuming the excision of the heterologous M13mp19*cat* sequence together with the duplicated, nonfunctional, distal 3.5-kb *comG* region of the Cm^r clones by homologous intrachromosomal recombination at site 1 or 2 in Fig. 2B, two classes of Cm^s clones could be expected, with one class carrying the ORF3 deletion in an otherwise intact *comG* operon and the other class containing the reconstituted wild-type *comG* operon. Because of the *Eco*RI and *Sal*I sites introduced simultaneously with the deletion, the expected

TABLE 1. Complementation of the chromosomal ORF3 mutation in *trans* by a plasmid-encoder wild-type ORF3

Strain	CFU/ml (10^8)	Transformants/ml[a]	Efficiency[b]
Wild type	9	6×10^5	7×10^{-4}
ORF3 mutant	5	0	0
ORF3 mutant with pRB20	4	8×10^3	2×10^{-5}

[a] The *B. subtilis hisA1 leu-8 metB5* strains were grown through the one-step competence regimen (3). Chromosomal (Leu$^+$) DNA was added at a concentration of 1 μg/ml. The selection was for leucine prototrophy. The pRB20-containing strain was induced with 1 mM IPTG.

[b] Transformants per milliliter divided by CFU per milliliter.

chromosomal structures could be verified by Southern hybridization. Chromosomal DNA of 10 independent Cm^s clones was prepared and digested with *Eco*RI. The DNA was probed with *Eco*RI-treated replicative-form DNA of M13mp19*cat-comG* containing the mutant 3.5-kb *comG* fragment, radiolabeled by nick translation with [^{32}P]dATP as described in Materials and Methods. The Cm^s clones harbored either a hybridizing 3.5-kb *Eco*RI fragment or two *Eco*RI fragments of 1.4 and 2.1 kb (not shown). None of these clones showed a signal corresponding to fragments derived from the M13mp19*cat* vector. This pattern as well as that of *Sal*I-restricted chromosomal DNA of these clones was in full agreement with the predicted chromosomal structure outlined in Fig. 2B.

Biological effects of the ORF3 in-frame deletion. The effect of the in vitro-generated deletion in *comG* ORF3 on the development of competence was tested both qualitatively and quantitatively. The qualitative plate test revealed that of the 10 clones tested, 5 were competence proficient. As expected, these five competent clones all lacked the in vitro-generated deletion. Four of the remaining five noncompetent strains were shown by Southern hybridization to be deletants. A single clone showed the signal of the wild-type 3.5-kb *Eco*RI fragment but was competence deficient. A possible explanation is the presence of a spontaneous mutation outside of ORF3, introduced during mutagenesis, leading to a loss of competence. This clone was not tested further. The results of the quantitative test for competence, performed on one of the mutant clones, indicated that the in-frame deletion led to a complete loss of competence (Table 1). The DNA-binding capability of this clone was then tested.

The Tn*917lac* insertional mutants in the *comG* locus, characterized previously, were found to be deficient in binding of exogeneous macromolecular DNA (16). However, in these experiments, polar effects of the transposon insertions on the downstream ORFs of *comG* could not be excluded. To test the role of the ORF3 product alone in DNA binding and uptake, a culture of the in-frame deletion mutant, constructed and characterized as described above, was grown through the one-step competence procedure and incubated with [^{3}H]DNA prepared from *B. subtilis* BD204 as described in Materials and Methods. After incubation, equal amounts of the samples either were treated with DNase I or remained untreated. The amounts of radioactivity associated with the respective treated cells are shown in Table 2. The results indicated that the strain with the ORF3 deletion was impaired in DNA binding by at least 1 order of magnitude as compared with the wild-type strain. In a control variant of this experiment, a complex medium (VY) was used in parallel with the glucose mineral salts competence medium for growth of the wild-type cells. It was shown previously

TABLE 2. Binding and uptake of DNA by *B. subtilis*[a]

Strain/medium	DNase I	cpm/ml	Cells/ml (10^8)	cpm/10^8 cells
Wild type/CM[b]	−	1,199	2.8	428
	+	768		274
ORF3 mutant/CM	−	97	2.2	44
	+	62		28
Wild type/VY	−	285	4.8	59
	+	248		52

[a] Competent cells were prepared by the one-step procedure. [^{3}H]DNA was isolated from *B. subtilis his thy* with a specific activity of 5×10^5 cpm/μg. Bound radioactivity was measured as described in Materials and Methods. The control (wild type) grown in complex medium (VY) exhibited DNA binding unrelated to competence.

[b] CM, Competence medium.

that the *comG* operon as well as the other characterized late-competence operons are not transcribed and that competence is expressed at extremely low levels in complex media (3). The amount of radioactivity associated with the wild-type cells grown in VY was similar to the level of radioactivity associated with the ORF3 mutant cells grown in the competence regimen. Therefore, this level of cell-bound DNA is probably not competence related.

Complementation of the ORF3 mutation in *trans*. To further ensure that in the mutant clones the *comG* ORF3 mutation was responsible for loss of competence, a complementation experiment was carried out. For this purpose, a 609-base-pair *Pvu*II-generated DNA fragment of the wild-type *comG* operon, encompassing the intact ORF3 together with parts of the upstream and downstream ORF2 and -4, was isolated from a pUC18 derivative carrying a 3.5-kb *Hin*dIII fragment of the *comG* operon including ORF2 to -7 (1). The *Pvu*II fragment was cloned into the *E. coli-B. subtilis* shuttle plasmid pIQ45 (31), under control of the inducible P_{SPAC} expression system. The resulting plasmid, containing the ORF3 insert in the appropriate orientation for transcription from the P_{SPAC} promoter, was identified by restriction analysis and designated pRB20. In this construct, the ORF3 was preceded by its native translation initiation signals. After primary cloning steps in *E. coli* and wild-type *B. subtilis* strains, the plasmid was introduced into the competence-deficient ORF3 deletion mutant strain by transduction with phage PBS1. The mutant strain containing pRB20 was tested for competence as described above. The qualitative plate test revealed a partial complementation of competence of the pRB20-containing strain upon induction of P_{SPAC} with 1 mM isopropyl-β-D-thiogalactoside (IPTG). Without the inducer, the number of leucine prototrophic transformants was at the level of spontaneous background revertants (not shown). The quantitative test showed that after addition of IPTG, the competence of this strain was restored from 0 to approximately 3% of the wild-type level (Table 1). Given the complex regulation of competence and the likelihood that it involves the assembly of a DNA uptake machine (8), it was not surprising to obtain partial complementation under the conditions of this experiment. These results indicated that the expression of a plasmid-encoded wild-type copy of *comG* ORF3 did complement the competence deficiency of a *B. subtilis* strain harboring an in-frame deletion in ORF3. This finding implies that expression of the remaining ORFs of the *comG* operon was probably not affected markedly by the in-frame deletion in ORF3. We cannot completely exclude the presence of additional unintended mutations in *comG*. If present, however, these have a relatively minor

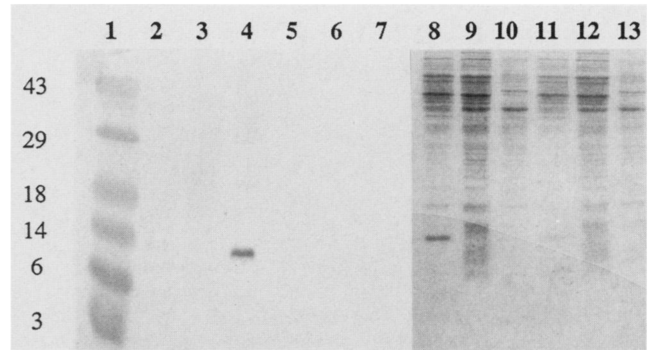

FIG. 3. Inducible synthesis of the ORF3 protein in *B. subtilis*(pRB20). Lanes 2 to 7 are from a Western blot, prepared by using anti-peptide ORF3 antiserum. Lanes 8 to 13 are from a similar gel with the same samples, stained with Coomassie blue. Lanes 2, 5, 8, and 11, Cell wall fraction released by lysozyme treatment; lanes 3, 6, 9, and 12, cytoplasmic fraction released by osmotic shock of protoplasts; lanes 4, 7, 10, and 13, membrane fraction. Lanes 2 to 4 and 8 to 10 are from cells induced with IPTG; lanes 5 to 7 and 11 to 13 are from uninduced cells. Material from equivalent amounts of initial cell suspension (about 2×10^8 CFU) was loaded on each lane. Lane 1 contains prestained molecular size standards; approximate molecular sizes are given in kilodaltons on the left.

effect, and it is clear that *comG* ORF3 must be an essential competence gene.

Immunological detection of the ORF3 protein. To further characterize the ORF3 gene product at the protein level, antiserum was raised in rabbits against a synthetic 14-mer oligopeptide, deduced from the predicted amino acid sequence of the ORF3 polypeptide, as described in Materials and Methods. The region from which the sequence of this relatively hydrophilic oligopeptide was derived (Fig. 1) was distal to the in vitro-generated deletion in *comG* ORF3.

At first, immunoblotting was carried out with cell extracts of the wild-type *B. subtilis* IS75 strain, containing the plasmid-encoded copy of the wild-type ORF3 gene under P_{SPAC} control (pRB20). The cells were grown in VY and either were induced with IPTG at the mid-log growth phase or remained uninduced. Subsequently, the cells were harvested and fractionated into cell wall, cytoplasmic, and membrane fractions as outlined in Materials and Methods. Samples of the fractions, representing equal amounts of cells, were separated by SDS-polyacrylamide gel electrophoresis and blotted with the antiserum. The immunological reaction was visualized by alkaline phosphatase staining (Fig. 3). An immunoreactive signal of approximately the expected molecular weight was detected only in extracts prepared from induced cells. (The molecular weight of the ORF3 protein, calculated from its DNA sequence, was 10,851. The signal on Western blots migrated as expected for a protein with a molecular weight of about 13,000.) These results suggested that the predicted ORF3 protein was actually translated in vivo. Furthermore, subcellular fractionation revealed that the immunological signal was present predominantly in the membrane fractions of the induced cells. A faint signal of the same size found in the other fractions in some experiments was probably due to cross-contamination during the fractionation procedure, as the result of cell lysis.

Detection of the ORF3 signal in competent cells. Extracts from *B. subtilis* strains containing the chromosomal copy of either the wild-type or the mutant ORF3 gene were tested in Western blot experiments. The strains were grown through

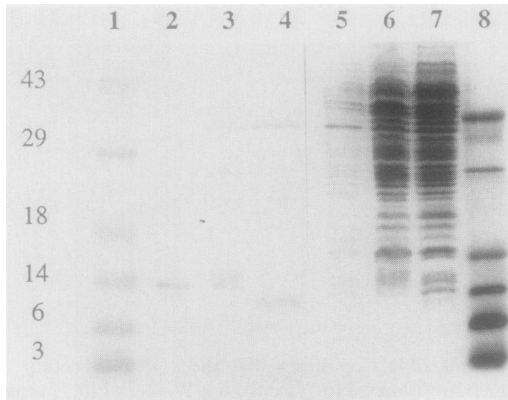

FIG. 4. Expression of mutant and wild-type ORF3 protein under competence control. Lanes 1 to 4 are from a Western blot; lanes 5 to 8 are from a similar gel run on the same samples and stained with Coomassie blue. All samples consisted of membrane fractions. Lanes 3 and 6, Wild-type strain; lanes 4 and 7, mutant strain. These samples were from cultures grown in competence medium, using strains carrying only a single chromosomal copy of the *comG* operon expressed under competence control. Lanes 2 and 5, Strain with the plasmid (pRB20)-encoded wild-type ORF3, grown in LB medium and induced with IPTG. Lanes 1 and 8 contain prestained and nonprestained, respectively, molecular size standards; the approximate molecular sizes are given in kilodaltons on the left.

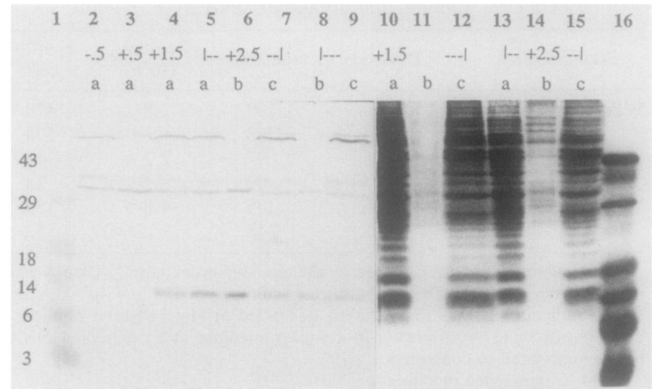

FIG. 5. Kinetics of ORF3 expression during the development of competence. *B. subtilis* IS75 was grown through the one-step competence regimen. At various times, samples were withdrawn and fractionated. The sampling times are indicated in hours before (−) or after (+) the time of transition from exponential to stationary phase (t_0). The total membrane fractions (lanes a) were treated with 0.1 N NaOH, yielding NaOH-insoluble (lanes b) and -soluble (lanes c) subfractions. Samples from equal amounts of total membrane protein were loaded on all lanes. Lanes 1 to 9 are from an immunoblot; lanes 10 to 16 are from a Coomassie blue-stained gel. Lanes 1 and 16 contain prestained and nonprestained, respectively, molecular size standards; approximate molecular sizes are given in kilodaltons on the left.

the competence regimen, and extracts were prepared from stationary-phase cultures. The membrane fractions of both strains exhibited the expected immunological signals (Fig. 4). The wild-type ORF3 protein, expressed from the intact *comG* operon, migrated at the same position as did the plasmid-encoded wild-type ORF3 protein expressed from the P_{SPAC} system. This finding indicated that the same ORF3 protein was expressed from pRB20 as from the host chromosome. Because of the 15-amino-acid deletion, the mutant ORF3 protein (calculated molecular weight, 9,118) was expected to migrate faster than the wild-type protein. This was observed (Fig. 4). The detection of an immunological signal upon expression of the chromosomal mutant ORF3 gene under competence control proved that the in vitro-generated deletion, located upstream of the epitope to which the antiserum was raised, was in frame. The absence of the wild-type protein in the mutant extract demonstrated that the signal detected did represent the ORF3 product. The mutant protein was found in the same subcellular fraction as the wild-type protein, suggesting that the deleted 15 amino acids were not needed for association with the membrane.

Time course of ORF3 expression. It was known from previous experiments that the *comG* operon was transcribed only after the culture had departed from the exponential growth phase (3). To study the translational pattern of the ORF3 protein, a time course experiment was carried out. *B. subtilis* IS75 containing the wild-type *comG* operon was grown through the competence regimen, and samples of the culture were withdrawn at the times indicated in Fig. 5. The immunoreactive signal was detected in the membrane fractions only after the departure of the culture from the exponential growth phase. This result indicated that the translation of the ORF3 protein approximately followed the kinetics of transcription of the *comG* operon.

Subcellular localization of the ORF3 signal. The subcellular localization of the ORF3 protein was further investigated in the strain containing the wild-type *comG* operon on the chromosome (Fig. 5) and in the strain containing in addition the plasmid-encoded (pRB20) wild-type copy of the ORF3 gene under P_{SPAC} control (Fig. 6). The chromosomal ORF3 gene was expressed under competence control in glucose minimal salts medium, whereas expression of the plasmid-encoded ORF3 gene was induced with IPTG in complex medium. In the latter case, the chromosomal *comG* operon (as well as the other characterized late *com* genes) was not transcribed (3). The membrane fractions of these strains were further fractionated into NaOH-soluble and insoluble subfractions as described in Materials and Methods. Pro-

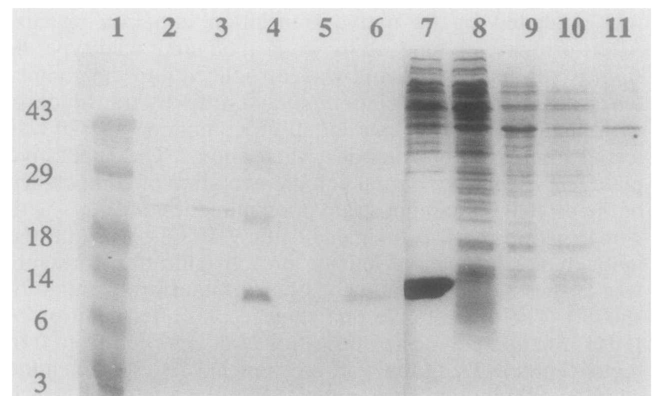

FIG. 6. Membrane localization of the ORF3 protein in *B. subtilis*(pRB20). After induction with IPTG, membrane fractions were prepared and treated with 0.1 N NaOH, yielding NaOH-soluble (lanes 5 and 10) and -insoluble (lanes 6 and 11) subfractions. Other lanes: 2 and 7, cell wall fraction released upon lysozyme treatment; 3 and 8, cytoplasmic fraction, released after osmotic lysis of protoplasts; 4 and 9, total membrane fraction. Samples corresponding to equivalent amounts of initial cell suspension were loaded on all lanes. Lanes 1 to 6 are from an immunoblot; lanes 7 to 11 are from a Coomassie blue-stained gel. Lane 1 contains prestained molecular size standards; approximate molecular sizes are given in kilodaltons on the left.

teins loosely associated with cell membranes are usually soluble in 0.1 N NaOH, whereas integral membrane proteins remain in the NaOH-insoluble fraction (25). In the strain containing pRB20, the signal was associated with the NaOH-insoluble subfraction of the cell membranes, whereas the NaOH soluble subfraction, containing most of the proteins isolated with the membrane fractions, did not contain a significant amount of immunoreactive ORF3 protein (Fig. 6). This finding indicated that when specified under P_{SPAC} control, ORF3 protein was an integral membrane protein. We have suggested previously that a good candidate for a membrane-spanning region was in the N-terminal portion of the ORF3 protein (residues 5 to 27), which displayed a pronounced hydrophobic profile (1). The central and C-terminal moieties of the protein (residues 28 to 98) are largely hydrophilic and therefore probably in contact with the aqueous phase. Interestingly, in the analogous experiment in which the ORF3 gene was expressed under competence control, both the NaOH-soluble and -insoluble membrane subfractions gave rise to immunoreactive signals of approximately equal intensities (Fig. 5). It should be noted that in the case of P_{SPAC}-driven expression in noncompetent cells, the ORF3 protein was substantially overproduced. Thus, the partition of protein into NaOH-soluble and -insoluble components in competent cells was not due to limited capacity of the membrane to accommodate the ORF3 product. The difference in NaOH solubility might indicate that in the component cell, the ORF3 protein was assembled in the membranes in such a way that some of the molecules were membrane spanning whereas the remainder were more loosely associated with the membranes. This suggests the hypothesis that under competence control, when the *comG* operon (and other late *com* operons) were transcriptionally activated, the ORF3 protein was organized on the cell surface in a specific way, different from the spontaneous insertion into the membrane characteristic of the ORF3 product in the absence of other competence proteins. To further clarify the localization of the ORF3 product, additional experiments were carried out.

The ORF3 protein is exposed on the outer surface of the membrane. Protoplasts were prepared from *B. subtilis* strains carrying a chromosomal *comG* operon with either the wild-type or the mutant ORF3 gene after growth of the cultures through the competence regimen. Before lysis by osmotic shock, samples of the protoplasts were treated with proteinase K as described in Materials and Methods, whereas the control sample remained untreated. After the protease treatment, the protoplasts were fractionated under standard conditions as described above. No ORF3-related signal was detected in the membrane fractions of either strain after protease treatment of the protoplasts, whereas the signal was present in the untreated controls (Fig. 7). Lanes 13 and 14 in Fig. 7 show Coomassie blue-stained cytoplasmic fractions from proteinase K-untreated and -treated protoplasts. The similarity of these two lanes suggests that the membranes remained intact during the protease treatment. These findings, together with the previous results, suggested that a portion of the total pool of ORF3 protein molecules (the NaOH-insoluble fraction) was membrane localized, with the hydrophobic N terminus of the protein integrated in the membrane and with the hydrophilic part of the protein (containing the epitope for the antiserum) exposed outside the cell membrane. The remaining ORF3 protein molecules (the NaOH-soluble fraction) were apparently associated with the exterior face of the membrane. The 15-amino-acid deletion in the mutant ORF3 protein did not appear to alter the main features of this structural organization.

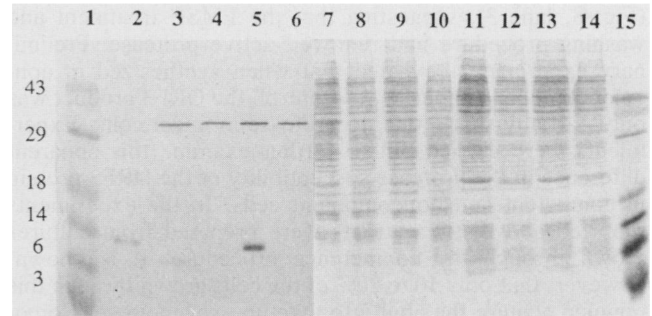

FIG. 7. Susceptibility of ORF3 protein to proteinase K treatment of protoplasts. Strains with single chromosomal copies of either wild-type ORF3 or the mutant allele were grown through the one-step competence regimen to t_2. Protoplasts were prepared, and equal samples were incubated with (lanes 4, 6, 8, 10, 12, and 14) and without (lanes 3, 5, 7, 9, 11, and 13) proteinase K treatment as described in Materials and Methods. These samples were then fractionated into membrane (lanes 3 to 10) and cytoplasmic (lanes 11 to 14) fractions. Lane 2 contains membrane from a strain carrying the plasmid-encoded wild-type protein after IPTG induction and without protease treatment. Lanes 3, 4, 7, 8, 11, and 12 contain samples from the ORF3 chromosomal mutant strain; lanes 5, 6, 9, 10, 13, and 14 are from the wild type. Lanes 1 to 6 are from an immunoblot; 7 to 15 are from a Coomassie blue-stained gel. Lanes 1 and 15 contained prestained and nonprestained, respectively, molecular size standards; approximate molecular sizes are given in kilodaltons on the left.

In a subsequent series of experiments, incubation with proteinase K was performed with intact competent *B. subtilis* cells, before protoplasting. Under these conditions, the ORF3 protein (both the wild-type and mutant forms) was largely susceptible to protease treatment (Fig. 8). A control, performed by mixing proteinase K-treated and -untreated cells after washing with PMSF and before fractionation, showed that the signal from the untreated cells survived

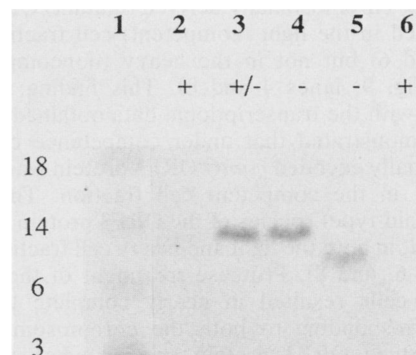

FIG. 8. Proteinase K treatment of intact competent cells. *B. subtilis* strains with a chromosomal wild-type (lanes 2 to 4) or mutant (lanes 5 and 6) copy of *comG* ORF3 were grown to t_2 in competence medium. Samples were treated with proteinase K before protoplasting and cellular fractionation (lanes 2 and 6). Control samples were treated identically in the absence of proteinase K (lanes 4 and 5). A further control consisted of equal samples of the treated and untreated wild-type cells mixed after washing in PMSF-containing buffer and before fractionation (lane 3). In all cases, the membrane fraction was loaded on the gel. Lane 1 contained prestained molecular size standards; molecular sizes are given in kilodaltons on the left.

(Fig. 8, lane 3), suggesting that the PMSF treatment and washing procedure had removed active protease. Preliminary experiments revealed that when synthesized in noncompetent cells under P_{SPAC} control, the ORF3 product was only partially susceptible to protease in intact cells. Experiments were carried out to further examine this apparent difference in the protease susceptibility of the ORF3 protein in competent and noncompetent cells. In the experiments reported above, the extracts were prepared from cultures grown through the competence procedure. It is known, however, that only 10 to 20% of the cells grown through this regimen acquire the ability to take up exogenous macromolecular DNA. This fraction of cells is morphologically and physiologically distinct from the major cell fraction and can be separated on Renografin density gradients as the lighter fraction (15, 18). It was shown previously that the *comG* operon was transcribed only in the light cell fraction (1). To further test the hypothesis that the ORF3 protein is organized differently in competent and noncompetent cells and to demonstrate the cell-type-specific expression of *comG* ORF3, Renografin separation experiments were carried out. The *B. subtilis* strain carrying a chromosomal *comG* operon with ORF3 deletion, as well as the plasmid with the wild-type copy of the ORF3 gene under P_{SPAC} control (pRB20), was grown through the two-step competence regimen (9), and expression of the plasmid-encoded copy of the ORF3 gene was induced with IPTG at the time point of the shift to the second step. To generate approximately equal amounts of the mutant chromosomal and plasmid-encoded wild-type ORF3 proteins per competent cell, a suboptimal concentration (0.1 mM) of the inducer was used. When the culture reached competence, the cells were harvested and separated on Renografin gradients as described in Materials and Methods. Both the light and heavy cell fractions were treated with proteinase K before the preparation of protoplasts. As a control, samples of both cell fractions remained untreated with protease. After fractionation, the samples were tested by Western blotting as described earlier. Because of their differing molecular weights, the wild type (plasmid encoded) and mutant (competence controlled) ORF3 proteins could be discriminated from one another. In the untreated control samples, the chromosomally derived (mutant) ORF3 protein was detected in the light (competent) cell fraction (Fig. 9, lanes 2 and 6) but not in the heavy (noncompetent) cell fraction (Fig. 9, lanes 4 and 8). This finding was in full agreement with the transcriptional data obtained previously (3) and demonstrated that under competence control, the chromosomally encoded *comG* ORF3 protein was expressed exclusively in the competent cell fraction. The plasmid-encoded (wild-type) species of the ORF3 protein was found, as expected, in both the light and heavy cell fractions (Fig. 9, lanes 2, 4, 6, and 8). Protease treatment of the separated competent cells resulted in nearly complete loss of the signals corresponding to both the chromosomal and the plasmid-derived ORF3 protein species when these were present in approximately equal amounts (Fig. 9, lanes 2 and 3). When the plasmid-encoded species was overproduced, however, a residual signal was clearly visible (Fig. 9, lane 7). These findings imply that in competent cells, the ORF3 protein, whether it is expressed from a gene on the chromosome or on a plasmid, is organized in the membrane in such a way that it is nearly completely susceptible when intact cells are treated with protease. In contrast, the ORF3 species in the separated noncompetent cell fraction appeared to be more resistant to treatment of the intact cells with proteinase K (Fig. 9, lanes 4, 5, 8 and 9). This difference in protease susceptibility lends support to the hypothesis that during the development of competence, the ORF3 protein is organized in the membrane differently than when present in noncompetent cells.

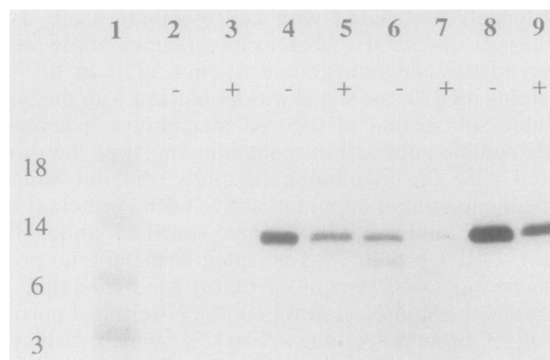

FIG. 9. Proteinase K treatment of competent and noncompetent Renografin-separated cells. The strain carrying both the plasmid-encoded wild-type ORF3 and the mutant ORF3 in the chromosome in single copy under competence control was grown to competence and separated on Renografin gradients into light (competent) and heavy (noncompetent) cell fractions. Samples of the separated cells were treated with proteinase K before protoplasting and subcellular fractionation (lanes 3, 5, 7, and 9). Other samples were treated identically in the absence of proteinase K (lanes 2, 4, 6, and 8). Expression of the plasmid-encoded wild-type ORF3 protein was induced with 0.1 mM (lanes 2 to 5) or 0.3 mM (lanes 6 to 9) IPTG. Lanes 2, 3, 6, and 7 contain membrane fractions from competent (light-buoyant-density) cells; lanes 4, 5, 8, and 9 contain membrane fractions from noncompetent (heavy) cells. Lane 1 contains prestained molecular size markers; molecular sizes are given in kilodaltons on the left.

DISCUSSION

The development of natural competence in *B. subtilis* is a complex process. To date, the products of about 20 transcription units are known to be involved (8). On the basis of their times of expression, these have been designated as early or late competence genes. Transcription of early transcription units occurs during the exponential growth phase, whereas transcription of the late operons is measurable only after the cultures have departed from exponential growth. The early genes, including *comA*, *comB*, *comP*, *degU*, *degS*, *sin*, *abrB*, *spo0H*, and *spo0A*, are involved in the regulation of competence (8, 29; Y. Weinrauch, J. Hahn, and D. Dubnau, unpublished data), whereas the late genes are most likely required for assembly of the DNA binding and uptake machinery.

comG is a late-expressed competence operon, transcriptionally activated after t_0. Determination of its nucleotide sequence revealed seven ORFs, and mapping of transcriptional start and termination sites revealed the polycistronic character of this operon, which was transcribed from a single major promoter (1). The use of Tn*917lac* insertions to define and characterize the *comG* operon (1–3, 16) did not allow conclusions about the essentiality of ORF3 for competence because of the polar nature of the transposon insertions. We have now shown that the ORF3 product is required for competence and is specifically needed for DNA binding, although the latter may be an indirect requirement. We have also shown that as expected, the ORF3 protein is synthesized under competence control in the competent cell fraction and after t_0.

Comparison of the predicted amino acid sequences of the *comG* proteins with the translated DNA sequence data base demonstrated that the *comG* ORF3, ORF4, and ORF5 proteins shared a significant similarity to a class of pilins from various species of gram-negative *Bacteroides*, *Moraxella*, *Neisseria*, and *Pseudomonas* spp. (1). This similarity was confined to the N-terminal regions (residues 4 to 28 in the ORF3 protein) and was most pronounced in the case of the predicted ORF3 protein. In general, the N-terminal portions of these pilins are most highly conserved (12). In the three *comG* proteins a phenylalanine residue at positions 6 to 8 was present, which also completely conserved in all members of the class of gram-negative so-called *N*-methyl-Phe pilins. This Phe residue is posttranslationally N methylated in the mature pilins of gram-negative origin, and a short N-terminal 6- or 7-amino-acid extension is removed (24). As a result, these proteins are referred to as *N*-methyl-Phe pilins. It is not known whether the ORF3, ORF4, or ORF5 protein is processed in *B. subtilis* or whether the conserved Phe residues are N methylated. *N*-Methyl-Phe pilin may be necessary for the development of competence. Naturally occurring piliated strains of *Moraxella* and *Neisseria* spp. were found to be about 3 orders of magnitude more competent than nonpiliated strains (5, 28). Furthermore, inactivation of a chromosomal pilin gene of *Neisseria gonorrhoeae* resulted in competence deficiency (27). From the results of the study described here, it can be concluded that the pilin-related ORF3 protein is essential for the development of competence in the gram-positive *B. subtilis*.

In gram-negative organisms, the pilin proteins constitute the major component of pili. Pili are flexible multisubunit filamentous cell appendages that are involved in such processes as adhesion of the bacteria to surfaces or adsorption of bacteriophages. It has been concluded that the pili of *Pseudomonas aeruginosa* are assembled from a helical array of pilin subunits with fivefold symmetry and with an outer diameter of 5.2 nm and a central pore of 1.2 nm in diameter (13). In this model, the major forces holding the subunits together are hydrophobic interactions of pilin dimers involving residues in the N-terminal regions of the pilin subunits (30). The hydrophobic region around tyrosine residues 24 and 27 of mature pilin is believed to be at the dimer-dimer interface, although these residues are themselves certainly not sufficient to account for this interaction, and there is no evidence demonstrating their essentiality for pilin assembly. Although the region of amino acid similarity of the ORF3 protein to the *N*-methyl-Phe pilins ends immediately before tyrosine 24 (3), the N termini of the ORF3 as well as the ORF4 and ORF5 proteins of *B. subtilis* display a pronounced hydrophobic character. To our knowledge, no structure resembling a pilus has been described in *B. subtilis*. The assembly of such a structure from ORF3 protein subunits is unlikely, since this protein has a predicted molecular weight of about 10,800, whereas that of the related pilins of gram negative origin is about 15,000. Also, it is likely that amino acid residues other than those in the conserved N terminus are involved in assembling the pilus (30).

From the results of our study, it can be concluded that the ORF3 protein is membrane localized, whether synthesized in single copy under competence control or overproduced in noncompetent cells. Thus, the protein (presumably the hydrophobic N terminus) possesses sufficient information to specify membrane association. When encoded on a plasmid in noncompetent cells, the ORF3 protein is apparently an integral membrane protein, as judged by its NaOH insolubility, and is accessible to proteolytic degradation in protoplasts. We suggest, therefore, that the N-terminal hydrophobic segment of the ORF3 protein, encompassing residues 5 to 27, is buried in the membrane and that the remainder of the molecule, including the epitope for recognition by the antiserum, is exterior to the membrane.

The existence of a specifically competence related organization of the ORF3 protein may be inferred from several findings. When expressed under competence control, the immunoreactive signal was found in the NaOH-insoluble as well as in the NaOH-soluble membrane fractions, indicating that both integral membrane and loosely membrane-associated protein was present. In contrast, expression of the plasmid-located ORF3 gene under P_{SPAC} control resulted in exclusively integral membrane localization of the ORF3 protein (Fig. 6). After separation of competent and noncompetent cells on Renografin gradients, the ORF3 signal was sensitive to proteinase K treatment of intact competent cells whether the protein was encoded by the chromosomal or the plasmid-borne gene (Fig. 9). At the same time, in the noncompetent cell fraction, the chromosomal *comG* ORF3 gene was not expressed, and the plasmid-borne ORF3 protein appeared to be more resistant to the proteinase K treatment. Thus, we tentatively conclude that the ORF3 protein is more accessible to exogenous protease in competent cells than when assembled into the membrane in noncompetent cells. In preliminary experiments, the accessibility of ORF3 protein to proteinase K in intact competent cells was not altered detectably in mutant strains with transposon insertions in *comC*, *comD*, or *comE* or in *comG* ORF4 (not shown). Thus, these genes do not appear to influence the increased protease susceptibility.

On the basis of these data, we suggest that in competent cells, some of the ORF3 molecules (NaOH insoluble) are organized as transmembrane protein in the membrane, perhaps serving to anchor additional molecules (NaOH soluble) that are organized into a competence-related structure, possibly traversing the cell wall and providing all or part of an aqueous channel for DNA binding and uptake. The ability of *Pseudomonas* pilin to form a structure with a central channel wide enough to accommodate single-stranded DNA is certainly suggestive in this connection. This channel would provide a cell wall discontinuity, allowing access of proteinase K to the ORF3 protein. It must be stressed that although consistent with the available evidence, this model is highly speculative, and other arrangements of the ORF3 protein are certainly possible. Also, the structure formed must be considerably more complex than suggested by this description, since we know of about nine additional late competence proteins that are likely to play a role, most of which are predicted to be substantially hydrophobic on the basis of DNA sequence information.

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