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Natural Genetic Transformation in *Streptococcus gordonii*: *comX* Imparts Spontaneous Competence on Strain Wicky

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Streptococcus gordonii **Wicky becomes competent only after stimulation with conditioned medium from strain Challis as a source of competence factor (CF). A 3.2-kbp genomic fragment from Challis was found to impart spontaneous competence on Wicky by a complementation assay. Wicky clones containing the fragment secreted a heat-sensitive activity that induced competence in Wicky and in a *comA* insertion mutant of Challis. Activity was localized to a putative open reading frame, *comX*, with the potential to encode a 52-amino-acid peptide. *comX* had no similarity to known sequences, and a *comX*::*ermAM* insertion mutant of Challis transformed normally and secreted CF. These data suggest that a CF-independent pathway for competence induction exists in *S. gordonii*.**

Natural genetic transformation, the ability to recognize, process, and integrate exogenous DNA, is exhibited by several streptococcal species that are normal inhabitants of the human oral cavity and upper respiratory tract. As an early growth-phase state, competence for transformation appears to be induced in all members of a particular population via the action of competence factor (CF), a secreted cationic oligopeptide (4, 10). CF presumably interacts with receptors on noncompetent cells, inducing the expression of several new polypeptides (18, 20). These proteins most likely make up key members of the DNA recognition and processing machinery. *Streptococcus gordonii* (Challis) CF has been partially purified. The active peptide is basic, trypsin sensitive, heat resistant, and <6 kDa in size (11, 21). *Streptococcus pneumoniae* CF (also known as CSP) has been purified, and the gene *comC* has been cloned (4). The propeptide is a basic protein of 17 residues formed after cleavage of a 41-residue prepeptide with a leader sequence of the double-glycine type (5). Pneumococcal CF has no effect on *S. gordonii* (our unpublished observation). CF is most likely secreted by the ComAB transport system, a member of the ATP-binding cassette-type transporters, since mutants in this system do not produce CF but are still stimulated by it (2, 3). *comC* is not linked physically to *comAB* (4). *S. gordonii* Wicky attains normal levels of competence only after stimulation with Challis-conditioned medium as a source of CF. Previous work with Wicky demonstrated that CF action was rapid and required de novo protein synthesis (10). We report here the isolation of a fosmid genomic clone from Challis that imparts spontaneous competence on Wicky.

Library production and complementation assay. Strains and plasmids used in this study together with antibiotic designations and concentrations are described in Table 1. Total cellular DNA was isolated from Challis as previously described (15). A genomic library was constructed by ligating 40- to 50-kbp restriction fragments, prepared by partial *Sau*3AI digestion and sucrose gradient purification, to *Bam*HI-digested fosmid pFOS1 by standard procedures for double-*cos* site-containing vectors (1, 9). The library consisted of 576 members representing in excess of 2.5 genome equivalents. Individual clones were grown in Luria-Bertani–chloramphenicol broth and stored frozen in 96-well microtiter plates. Library members functioned solely as nonreplicating subchromosomal fragment vehicles that mediated transformation in Wicky via homologous recombination with the recipient genome. Fosmid pool DNA was prepared by coinoculating eight library members each into 5 ml of Luria-Bertani–chloramphenicol broth. DNA was isolated from each pool as described previously (16).

A high-efficiency marker rescue system was utilized to detect transformation in Wicky clones that previously had been transformed nonselectively with various fosmid pool DNAs (12). Wicky containing pVA1208 (WK1-1) was transformed as described previously (13) with 1 μg of fosmid pool DNA after competence induction with 20% (vol/vol) heat-inactivated (100°C, 5 min), sterile filtered (pore size, 0.2 μm, Milex-GV low protein binding; Millipore Corp., Bedford, Mass.) Challis-conditioned medium as a source of CF (19). Negative control reactions received Wicky genomic DNA alone. After transformation, cells from individual reaction mixtures were washed twice in 10 ml of brain heart infusion after centrifugation (8,000 × *g*, 30 min), suspended in 2.5 ml of Todd-Hewitt erythromycin broth, and grown for 12 h. These cultures were utilized as inocula for standard transformation reactions with pVA981 without CF stimulation. Cells from each reaction were plated in toto to single brain heart infusion-tetracycline plates and examined for transformation after 18 h. Fosmid pools exhibiting competence-stimulating activity (CSA) were reexamined by testing member DNAs individually until an active single clone was identified. Several fosmids were isolated, and one, f1A8, was chosen for further analysis.

Subcloning and phenotype analysis. A sublibrary of *Bam*HI fragments from the genomic insert of f1A8 was prepared in pDL277, placed into Wicky, and screened for spontaneous competence as described above by transformation with pCC22 and selection for erythromycin resistance (Em^r). Colonies were examined for insert-bearing plasmids corresponding to restriction fragments present in the original fosmid. All positive clones contained the *Bam*HI-E fragment of f1A8. This fragment was subcloned into both pBluescriptIIKS(+) (Stratagene, La Jolla, Calif.) and pDL277 to yield pCC28 and pCC29, respectively. pCC29 was found to impart normal levels of competence on host Wicky cells, and filtered supernatants

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source[b]
S. gordonii		
Challis	CF^+	
Ch2	Challis Rf^r	
Ch5	Challis Sm^r	
ComA1	Ch2 *comA*::*ermAM* (*Eco*RV), Xfo^-	
ComB1	Ch2 *comB*::*ermAM* (*Nsi*I), Xfo^-	
ComX1	Ch2 *ermAM* at nt 3040–3050, Xfo^+	
Orf-M,O1	Ch2 *ermAM* at nt 3152–3159, Xfo^+	
Wicky	CF^-	
WK1	Wicky Rf^r	
WK1-1	WK1(pVA1208), Em^r	
WK1-2	WK1(pCC29), Sp^r Xfo^+	
WK1-3	WK1(pCC44), Sp^r Xfo^+	
WK1-4	WK1(pCC48), Sp^r Xfo^+	
WK1-5	WK1(pCC107), Sp^r Xfo^+	
WK1-6	WK1(pCC108), Sp^r Xfo^+	
WK1-7	WK1(pCC109), Sp^r Xfo^-	
PK488	Xfo^-	
E. coli DH10B	F^- *mcrA* Δ(*mrr hsdRMS mcrBC*) *endA recA*	Gibco-BRL
Plasmids		
pBluescriptIIKS(+) (pBS)	Ap^r	Stratagene
pDL277	Sp^r	D. LeBlanc
pKSerm2	Ap^r Em^r, 922-bp *ermAM* cassette	
pVA1208	Em^r, streptococcal rep^+	12
pVA981	Tc^r, streptococcal rep^-	12
pFOS1	fosmid, Cm^r	9
f1A8	pFOS1 with 40-kbp Ch2 insert, CSA^+	
pCC22	pDL277 *Sma*I (*ermAM*, blunt)	
pCC28	pBS (*Bam*HI-E) of f1A8	
pCC29	pDL277 (*Bam*HI-E) of f1A8,CSA^+	
pCC32	pBS(*ermAMp*) *Eco*RI-*Hin*dIII	
pCC44	pDL277(*ermAMp*::*comB*)	
pCC48	pDL277 (1,976-bp *Ava*I-*Bam*HI fragment of *Bam*HI-E)	
pCC51	pCC28 *Nsi*I (*ermAM*, *Pst*I)	
pCC53	pCC28 *Eco*RV (*ermAM*, blunt)	
pCC84	pKSerm2 with 352-bp *Eco*RI-*Pst*I and 123-bp *Bam*HI-*Xba*I PCR fragments	
pCC91	pKSerm2 with 239-bp *Eco*RI-*Pst*I and 232-bp *Bam*HI-*Xba*I PCR fragments	
pCC107	pDL277 with bp 1–3290 (PCR) (Fig. 2)	
pCC108	pDL277 with bp 1–3176 (PCR) (Fig. 2)	
pCC109	pDL277 with bp 1–2983 (PCR) (Fig. 2)	

[a] Abbreviations: Rf^r, Ap^r, Sp^r, and Cm^r, resistant to rifampin, ampicillin, spectinomycin, and chloramphenicol, respectively; *ermAMp*, *ermAM* promoter fragment; Xfo^+, transformation positive; Xfo^-, transformation negative; rep^+, streptococcal replication proficient; rep^-, replication deficient. Antibiotic concentrations used were as follows: for *S. gordonii*, erythromycin, 10 μg/ml; tetracycline, 10 μg/ml; streptomycin, 500 μg/ml; rifampin, 100 μg/ml; and spectinomycin, 800 μg/ml; and for *E. coli*, erythromycin, 300 μg/ml; spectinomycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml).

[b] Entries lacking reference or source designations were either laboratory stocks or strains or plasmids constructed in this study.

from these cells exhibited a low-level heat-labile (100°C, 5 min) activity that induced competence levels in Wicky and ComA1 25% of wild type (see below). Introduction of pCC29 into *S. gordonii* PK488, a strain that does not naturally transform under any condition, did not induce competence. When Ch5 DNA (streptomycin resistant [Sm^r]) was used to test the pCC29-derived supernatant, it appeared that all activity was lost after heat treatment. Only when the high-efficiency tetracycline resistance (Tc^r) marker system was utilized could we determine that heating destroyed about 75% of the activity. No change in levels of activity was seen with heat-inactivated Challis supernatant. Crude sonic extracts of plasmid host DH10B containing either f1A8 or pCC28 failed to induce competence in Wicky, suggesting that the activity was not expressed directly in *Escherichia coli*. On the basis of its heat sensitivity, we designated the new activity CSA in order to differentiate it from genuine CF, which is heat resistant.

DNA sequence analysis. The DNA sequence was obtained with Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio) with custom walking primers on plasmid templates prepared with anion-exchange resin (Qiagen, Inc., Chatsworth, Calif.). Sequence assembly and analysis were performed with Assembly-LIGN and MacVector (International Biochemicals, Inc., New Haven, Conn.). The sequence of the region immediately 3′ of *comB* in *Bam*HI-E was independently confirmed by a commercial sequencing service (Sequetech, Mountain View, Calif.). Database searches were conducted with the BLAST network system (NCBI-NLM, Bethesda, Md.). Protein alignments were performed with GAP (Wisconsin Package, version 8.0, for VMS-VAX; Genetics Computer Group, Madison, Wis.).

Two large open reading frames (ORFs) representing the *S. gordonii comAB* homologs were identified in the *Bam*HI-E fragment (Fig. 2). *comA* was severely truncated and found to be missing 186 amino acids of its N-terminal sequence on the basis of an alignment with the *S. pneumoniae* sequence. *comB* was complete in the clone. A comparison of the *S. pneumoniae* and *S. gordonii* ComA sequences revealed that the peptides were 90% similar and 82% identical (6). A comparison with ComB displayed 71% similarity and 55% identity (7). Pneumococcal ComA and ComB were previously identified as members of the ATP-binding cassette family of bacterial transporters and are believed to be responsible for secretion of mature CF (6, 7). In addition to having general signature domains indicative of the ATP-binding cassette class, such as the Walker motif (24), the Challis ComAB system is very similar to the transporter for the nonlanthionine-containing bacteriocin lactococcin-A (23). ΔComA exhibited 78% similarity and 63% identity to LcnC. ComB showed 53% similarity and 33% identity to LcnD.

The 319-nucleotide (nt) region 3′ of *comB* exhibited no significant similarity to known sequences. Analysis of the region revealed three small putative ORFs (Fig. 1). *orfM* and *comX* partially overlapped each other and were in the same transcriptional sense as *comAB*. *orfM* potentially encodes a 43-residue peptide with a theoretical pI of 3.8. *comX* has the potential to encode a 52-residue peptide with a pI of 10.2. *orfO* was antisense to *comAB*, partially overlapped *comX* and *orfM*, and could direct the synthesis of a 53-residue peptide with a pI of 12.5. Only *orfM* was found to have a typical streptococcal ribosome binding site. None of the potential peptides contained leader processing sites indicative of known lanthionine- or nonlanthionine-containing bacteriocins, although all three contained internal proline and glycine residues that could provide processing sites analogous to those of the lanthionine-containing class (8). High-stringency DNA hybridization anal-

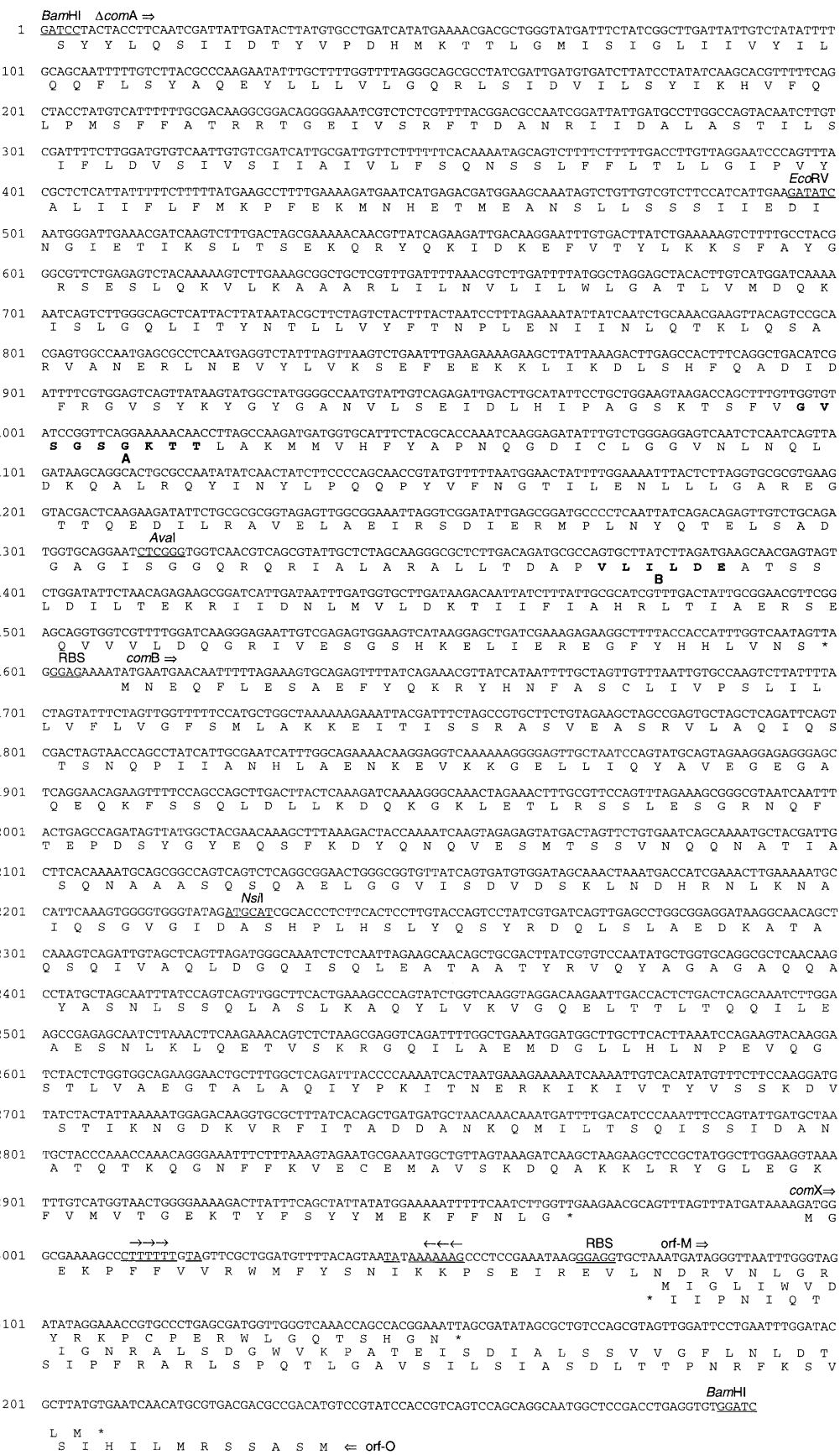

FIG. 1. Sequence analysis of *Bam*HI-E. Complete nucleotide sequence and deduced peptides are displayed together with relevant subsequence information. Restriction sites, putative ribosome binding sites (RBS), and inverted repeats are underlined. The A and B sites of the Walker motif are shown in boldface type.

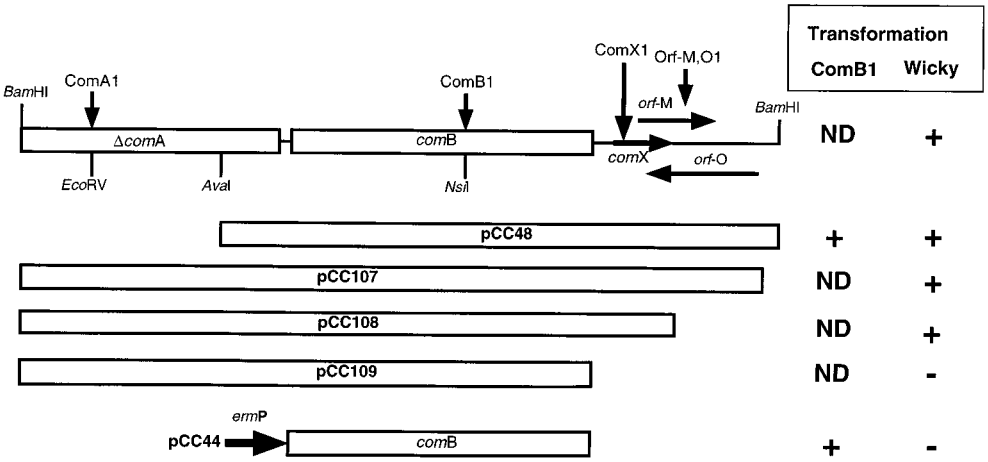

FIG. 2. ORF structure and subclones of *Bam*HI-E. Downward-pointing arrows show the sites of *ermAM* insertions for the generation of ComA1, ComB1, ComX1, and Orf-M,O1. Subclones are indicated as open rectangles with plasmid numbers, and phenotypes in either ComB1 or Wicky backgrounds appear at the right. +, spontaneous competence; −, no competence; ND, not determined.

ysis of Wicky genomic DNA with *comA*, *comB*, and *comB* 3′ region-specific probes indicated that these loci are present in this strain (data not shown). There was no similarity between *S. gordonii* and *S. pneumoniae* sequences in this region.

Insertion mutagenesis and deletion analysis. *comA* was interrupted by inserting a blunt-ended *ermAM* cassette into the *Eco*RV site at nt 495 (Fig. 1) (with pCC53). *comB* was interrupted by placing *ermAM* as a *Pst*I fragment into the *Nsi*I site at nt 2224 (Fig. 1) (with pCC51). *orfM* and *-O* were simultaneously disrupted by placing *ermAM* between bp 3152 and 3159 (Fig. 1). The targeting vector (pCC84) for this mutagenesis was constructed with two PCR fragments with Ch5 DNA as the template and a high-fidelity thermal DNA polymerase (*Pfu*; Stratagene). All PCR primers used in this study are listed in Table 2. The left fragment was 352 bp in length and was synthesized as an *Eco*RI-*Pst*I product with primers 5 and 6. It was cloned into *Eco*RI-*Pst*I-cleaved pKSerm2. The right fragment was made as a 123-bp *Bam*HI-*Xba*I product with primers 7 and 8. *comX* was inactivated in a similar fashion with primers 5 and 9; 8 and 10 yield fragments of 239 bp (left) and 232 bp (right) (with pCC91). Final constructs were linearized and used to transform Challis to Em^r as described previously (15). The resulting mutants were designated ComA1, ComB1, ComX1, and Orf-M,O1. ComA1 and ComB1 had phenotypes identical to that of Wicky and could be complemented to normal levels of competence with Challis culture supernatant. Supernatants from ComA1 had no effect on Wicky. Likewise, Wicky supernatants did not stimulate ComA1. Because *comA* is severely truncated in *Bam*HI-E, it is highly likely, on the basis of what is understood about the lactococcin-A system, that this ORF is inactive in pCC29 (5). Deletion analysis of the fragment, specifically the pCC48 construct (Fig. 2), confirmed this assumption. ComX1 and Orf-M,O1 exhibited normal levels of transformation.

Our initial opinion based on the sequence data was that Wicky might be a natural *comB* mutant. To test this hypothesis, we constructed an expression plasmid in which *comB* was placed under both transcriptional and translational control of the constitutive promoter of *ermAM. ermAM* is expressed well in streptococcal backgrounds (reference 14 and our unpublished observations). A promoter element from *ermAM* was synthesized via *Pfu* PCR with primers 1 and 2 (Table 2) (17). pKSerm2 served as the template. The 135-bp product (*ermAMp*) was digested with *Eco*RI and *Hin*dIII and placed into identically cleaved pBluescriptIIKS(+) to yield pCC32. A *comB* gene cassette was synthesized with primers 3 and 4. Ch5 DNA served as the template. The resulting fragment was digested with *Nco*I-*Xho*I and cloned into identically cut pCC32. The *ermAMp*::*comB* fusion was released by digestion with *Xho*I, treated with the Klenow fragment, and subsequently digested with *Eco*RI. The final fragment was placed into *Eco*RI-*Sma*I-digested pDL277 to yield pCC44. The plasmid was found to complement ComB1 to wild-type levels, proving that the fusion was active. When pCC44 was placed in Wicky, however, no transformation was detected. These observations suggested that sequence information 3′ of *comB* either alone or in concert with *comB* was responsible for complementation and that a cryptic promoter element was present elsewhere within *comA* or *comB* to drive transcription of this region.

Nested deletions of *Bam*HI-E differing only in the *comB* 3′ region, and thus conserving any cryptic 5′ promoter elements, were synthesized as *Bam*HI fragments via PCR and subcloned into pDL277 (Fig. 2 and Table 1). pCC107 was synthesized with primers 11 and 12 and contained a sequence from the left *Bam*HI site to the beginning of *orfO*. pCC108, made with primers 11 and 13, contained the sequence extending to the 3′ end of *comX*. pCC109, made with primers 11 and 14, contained only the *comAB* sequence. At least five independent clones for each construct were pooled for testing in Wicky. Only pCC107 and pCC108 induced competence. Attempts to subclone PCR cassettes of *comX*, *orfM*, and *orfO* in *ermAM* expression vectors have been unsuccessful.

Conclusions. We suggest that *comX* is responsible for CSA in Wicky either by directly encoding the activity or by supplying a function required for expression. Putative ComX exhibits several properties indicative of a streptococcal CF, such as small size and basic pI. Unlike CF, CSA is heat sensitive. Presently, it is not clear how *comX* is expressed. The ORF is 27 bp distal to and in frame with *comB*. Conceivably, an atypical ribosome binding site or translational coupling event with *comB* could produce sufficient activity. The possibility also exists that the region containing *comX* functions as an operator sequence by titrating a regulatory molecule. This scenario is unlikely on the basis of the single-copy nature of the fosmid transformation assay. Phenotypic silence of *comX*::*ermAM* insertion mutants of Challis and the thermolability of CSA pro-

TABLE 2. PCR primers

Primer no.	Sequence[a]
1	.....5′gcgaattCTTAGAAGCAAACTTAAGAG3′ (20[b])
2	.....5′gcgaagcttccatggAATCACTCCTTCTTAATTAC3′ (91[b])
3	.....5′gcgccatgGAACAATTTTTAGAAAGTGCAGAG3′ (1640)
4	.....5′gcgctcgagTAAACTGCGTTCTTCAACC3′ (2964)
5	.....5′gccgaattcTGCTACCCAAACCAAACAGG3′ (2820)
6	.....5′gcgctgcagATTTCCGTGGCTGGTTTGAC3′ (3133)
7	.....5′cgcggatccTATAGCGCTGTCCAGCGTAG3′ (3178)
8	.....5′gcgtctagACCTCAGGTCGGAGCCATTG3′ (3264)
9	.....5′gcgctgcagCTGTAAAACATCCAGCGAAC3′ (3021)
10	.....5′cgcggatccAAAGCCCTCCGAAATAAGGG3′ (3069)
11	.....5′cgcgGATCCTACTACCTTCAATCG3′ (20)
12	.....5′cgcGGATCCACACCTCAGGTCGGAG3′ (3270)
13	.....5′cgcggatccACGCTGGACAGCGCTATATC3′ (3157)
14	.....5′cgcggatcCTAAACTGCGTTCTTCAACC3′ (2964)

[a] Lowercase letters are nonhomologous bases that served as clamps and restriction sites for cloning (see the text). Numbers in parentheses indicate the 3′ base position in Fig. 1 for each primer.

[b] The 3′ base position of the Martin et al. sequence (17).

duced in Wicky clones suggests that an alternate pathway to competence induction exists in *S. gordonii*. Multiple signal transduction pathways have been described for *Bacillus subtilis* transformation (22). Future work will concentrate on transcriptional analyses of the *comABX* region as well as detection and subcellular localization of ComX in Challis.

Nucleotide sequence accession number. The sequence of the *Bam*HI-E fragment was deposited in GenBank under accession number U40139.

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