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Natural Transformation of *Gallibacterium anatis*

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***Gallibacterium anatis* is a pathogen of poultry. Very little is known about its genetics and pathogenesis. To enable the study of gene function in *G. anatis*, we have established methods for transformation and targeted mutagenesis. The genus *Gallibacterium* belongs to the *Pasteurellaceae*, a group with several naturally transformable members, including *Haemophilus influenzae*. Bioinformatics analysis identified *G. anatis* homologs of the *H. influenzae* competence genes, and natural competence was induced in *G. anatis* by the procedure established for *H. influenzae*: transfer from rich medium to the starvation medium M-IV. This procedure gave reproducibly high transformation frequencies with *G. anatis* chromosomal DNA and with linearized plasmid DNA carrying *G. anatis* sequences. Both DNA types integrated into the *G. anatis* chromosome by homologous recombination. Targeted mutagenesis gave transformation frequencies of $>2 \times 10^{-4}$ transformants CFU^{-1}. Transformation was also efficient with circular plasmid containing no *G. anatis* DNA; this resulted in the establishment of a self-replicating plasmid. Nine diverse *G. anatis* strains were found to be naturally transformable by this procedure, suggesting that natural competence is common and the M-IV transformation procedure widely applicable for this species. The *G. anatis* genome is only slightly enriched for the uptake signal sequences identified in other pasteurellaceaen genomes, but *G. anatis* did preferentially take up its own DNA over that of *Escherichia coli*. Transformation by electroporation was not effective for chromosomal integration but could be used to introduce self-replicating plasmids. The findings described here provide important tools for the genetic manipulation of *G. anatis*.**

The species *Gallibacterium anatis* is of particular interest in veterinary medicine because it infects the reproductive organs of laying hens, reducing egg production (19, 28, 29). The aim of this study was to establish molecular tools for genetic manipulation of *G. anatis* to facilitate the study of gene function.

A prerequisite for genetic manipulation in bacteria is the introduction of DNA into the cytoplasm. *Gallibacterium* has been recently defined as a genus in the Gram-negative family *Pasteurellaceae* (10), which includes several genera (*Haemophilus*, *Aggregatibacter*, and *Actinobacillus*) that are naturally competent to actively take up DNA from their surroundings. This ability has been exploited in the construction of targeted mutants (1, 4, 38, 40).

Competence and its regulation have been extensively studied in *Haemophilus influenzae*. *H. influenzae* is not constitutively competent, but cells become competent when nutrients are limited. Competence is induced in laboratory cultures when they are transferred from rich medium to starvation medium, from aerobic conditions to anaerobic conditions, and during growth in rich media when cells enter late exponential phase and early stationary phase (14, 15, 31). Two regulatory proteins, Sxy (also called TfoX) and cyclic AMP (cAMP) receptor protein (CRP), are essential for the induction of competence in *H. influenzae* (8, 41). Together they induce the expression of 25 competence genes collectively called the competence regulon (32). The DNA-binding capacity of CRP is activated when the intracellular level of cAMP rises as a response to the absence of preferred sugars, allowing the active cAMP-CRP complex to form. The genes in the competence regulon share a specific promoter sequence (CRP-S); binding of activated CRP at this sequence requires Sxy and induces transcription by recruiting RNA polymerase (6, 32).

The competence regulon includes the genes required for the two steps that transport DNA across the outer and inner membranes. Double-stranded DNA (linear or circular) is first transported across the outer membrane into the periplasm through retraction of type 4 pseudopili. Circular DNA remains in the periplasm, but linear DNA is further processed, with one strand degraded to nucleotides and the other transported across the inner membrane. In the cytoplasm, most of this single-stranded DNA is degraded, but if sequence similarity permits, it can be integrated into the chromosome by homologous recombination, resulting in natural transformation (reviewed in reference 26). Competent members of the *Pasteurellaceae* typically do not efficiently take up DNA from species outside this family, because the initial uptake step shows a strong preference for DNA fragments carrying uptake signal sequences (USSs) specific to the *Pasteurellaceae* (33).

In *Pasteurellaceae* lacking natural transformation, targeted mutants have been constructed by introducing DNA into cells by either conjugation or electroporation. However, this is not always particularly efficient, and genetic manipulation in, e.g., *Mannheimia haemolytica* and *Pasteurella multocida* is challenging; in these species very few double-crossover mutants have been constructed. The goal of this work was to develop methods for transformation of *G. anatis*.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference
G. anatis		
12656-12	Wild-type strain	2
12656-12 Nal^r	12656-12 spontaneous Nal^r mutant	This study
Δ*gtxA*	12656-12 Δ*gtxA*::Km^r	21
Δ*gtxBD*	12656-12 Δ*gtxBD*::Km^r	20
F149^T	Wild-type strain	10
10672/6	Wild-type strain	10
10T4	Wild-type strain	2
21K2	Wild-type strain	2
24T10	Wild-type strain	2
Avicor	Wild-type strain	20
4895	Wild-type strain	20
07990	Wild-type strain	20
Plasmids		
pDO6	pWSK29 *gtxA*::Km^r; 9.8 kb	21
pT2	pBluescript *gtxBD*::Km^r; 6.5 kb	20
pBA1100	*E. coli-P.multocida* shuttle vector; Km^r; 4.5 kb	16

MATERIALS AND METHODS

Bacterial strains and DNA. The *G. anatis* strains used in this study are listed in Table 1. The strains were cultured at 37°C in brain heart infusion (BHI) broth or on BHI agar (Oxoid) plates supplemented with 5% (vol/vol) horse or cow blood. Broth cultures were incubated with agitation. Growth on plates was improved by incubation in closed plastic bags, an effect that may be due to increased CO_2 concentration and/or increased humidity. A spontaneous nalidixic acid-resistant (Nal^r) mutant of *G. anatis* 12656-12 was isolated by diluting an exponentially growing culture 1:1 in medium with 10 μg nalidixic acid ml^{-1}, incubating it overnight, and plating it on agar plates with 30 μg nalidixic acid ml^{-1}. Chromosomal DNA was purified with the DNeasy blood and tissue kit (Qiagen), including the RNase treatment. Plasmids (listed in Table 1) were purified from *Escherichia coli* DH5α and Top10 with Qiagen plasmid minikit (Qiagen). DNA concentrations were measured using the NanoDrop 2000 spectrophotometer (λ = 260) and gel electrophoresis.

M-IV medium induction of competence. Induction of competence by incubation in M-IV medium (15) was performed as described by Poje and Redfield (30). Briefly, an overnight culture of *G. anatis* was diluted 1:100 in BHI, grown to an optical density at 600 nm (OD_{600}) of 0.2 (±0.02), washed once in M-IV medium, and incubated in M-IV medium at 37°C for 100 min. The cells were then used for transformation or stored in 15% (vol/vol) glycerol at −80°C for later use as described previously (30).

Transformation procedure. Cells (typically 0.25 ml) were incubated with DNA for 20 min at 37°C, using a DNA concentration of either 1 μg ml^{-1} (chromosomal DNA and circular plasmid) or 0.5 μg ml^{-1} (linearized plasmids). Two volumes of BHI were then added, and the cells were incubated for 1 h (37°C) before being serially diluted and plated on nonselective and selective plates (usually nalidixic acid or kanamycin at 4 μg ml^{-1}). Transformation frequencies were calculated as CFU ml^{-1} on selective plates divided by CFU ml^{-1} on nonselective plates. Avoiding false positives with some strains required higher antibiotic concentrations: 6 μg nalidixic acid ml^{-1} for strain Avicor and 6 μg kanamycin ml^{-1} for strains F149^T, 10672/6, 4895, 07990, and Avicor. Transformation with circular plasmid DNA was performed by osmotic shock by incubating the M-IV-competent cells in 30% (vol/vol) glycerol for 10 min after the incubation with DNA, as described by Poje and Redfield (30).

Transformation competition experiments. Competition experiments were performed essentially as previously described (33). A total of 0.25 ml of competent *G. anatis* 12656-12 cells was incubated with a mixture of isogenic Nal^r chromosomal DNA (0.5 μg ml^{-1}) and various concentrations (0.5, 2, and 4.5 μg ml^{-1}) of competing chromosomal DNA from either *G. anatis* 12656-12 (wild type) or *E. coli* DH5α. In parallel, cells were incubated with *G. anatis* 12656-12 Nal^r DNA (1, 2.5, or 5 μg ml^{-1}) without any competing DNA. The Nal^r transformation frequencies from these experiments were used to calculate relative transformation frequencies (transformation frequency in the absence of competing DNA divided by transformation frequency in the presence of competing DNA).

Electroporation of *G. anatis*. An overnight (16 h) culture was diluted 1:100 in BHI and incubated at 37°C with shaking until the OD_{600} reached 0.7 (±0.1). The culture was incubated on ice for 15 min, and cells were pelleted by centrifugation at 4°C and washed three times in cold 9.3% (wt/vol) sucrose, with centrifugation at 2,000 × *g*, 2,500 × *g*, and 3,000 × *g*. Following the last centrifugation step, the pellet was resuspended in the liquid remaining after the supernatant had been decanted (approximately 200 μl). Aliquots of 65 μl were mixed with DNA (0.5 μg or 1 μg) and transferred to precooled electroporation cuvettes (gap size, 1 mm). Electroporation was performed with a Gene Pulser II/Pulse Controller Plus (Bio-Rad) with the settings 2.2 kV, 400 Ω, and 25 μF, resulting in time constants of 8 to 10 ms. A total of 1 ml of prewarmed (37°C) BHI was immediately added, and the cells were incubated for a minimum of 2 h at 37°C before selection of transformants on plates containing 15 μg kanamycin ml^{-1}. Transformation efficiency was calculated as the total number of transformants divided by μg DNA added.

Identification of *G. anatis* homologs of proteins of the competence regulon. The predicted amino acid sequences of *H. influenzae* competence proteins (26, 32) were used in BLASTP searches against predicted proteins from the complete genome of *G. anatis* UMN176 (18) and the unassembled genome sequences of *G. anatis* 12656-12 (A. M. Bojesen, unpublished data) and F149^T (T. J. Johnson, unpublished data). The genome sequences of strains 12656-12 and F149^T had been obtained by 454 sequencing; they consisted of 141 and 71 contigs and covered 2.6 and 2.5 Mb, respectively. When the search resulted in only weak BLAST matches (*E* [Expect value] < 0.0001), orthologs from other *Pasteurellaceae* (26) were used as queries. The candidates were subsequently used in a reciprocal search in *H. influenzae* KW20. CRP-S sequences (6) were identified by visual scanning of the 200 nucleotides (nt) upstream of start codons of predicted competence transcription units.

Analysis of uptake signal sequences. For sequence analysis, genome sequences of *G. anatis* UMN179, *H. influenzae* KW20, *Actinobacillus pleuropneumoniae* serovar 7 strain AP76, and *E. coli* K-12 substrain W3110 were downloaded from GenBank. To examine the abundance and distribution of *k*-mers (nucleotide subsequences of length = *k*) in reference DNA sequences and to extract subsequences, the R statistical programming language and its add-on package SeqinR (9) were used. Expected occurrences (Bernoulli) of USS cores (*Hin* type [5′-AAGTGCGGT-3′] and *Apl* type [5′-ACAAGCGGT-3′]) in each genome sequence were calculated as $(x/2)^y \times [(1-x)/2]^{(9-y)} \times z$, where x is the fraction of GC bases in the genome, y is the number of GC bases in the 9-bp core USS, and z is the genome size in base pairs. To account for occurrences on the reverse strand, this value was then multiplied by 2. Sequences flanking perfect matches to the core *Hin*-type and *Apl*-type USSs on both forward and reverse strands were extracted and examined using WebLogo (11). The count function in SeqinR was used to determine the occurrences of each *k*-mer in each genome ($k = 9$ in the analyses shown), and these values were normalized to densities per kilobase. The Gibbs motif sampler (37) was used to search the complete genome sequence of *G. anatis* UMN179 (18) for abundant motifs as described by Maughan et al. (27), using the prior settings described for *H. influenzae*.

Nucleotide sequence accession numbers. The nucleotide sequences of the predicted competence regulon of *G. anatis* 12656-12 have been deposited in GenBank under accession numbers JQ429391 to JQ429404.

TABLE 2 *G. anatis* homologs of *H. influenzae* competence proteins and regulators[a]

H. influenzae		*G. anatis* 12656-12			*G. anatis* UMN179	
Category and homolog (locus ID)	Length (aa)	Homolog	Length (aa)	Sequence ID (%)[b]	Locus ID	Length (aa)
Regulation						
CRP (HI_0957)	224	CRP	219	74	UMN179_00941	218
Sxy (HI_0601)	217	Sxy	214	34	UMN179_00887	214
DNA uptake						
ComA (HI_0439)	265	ComA	272	23	UMN179_01492	272
ComB (HI_0438)	168	ComB	184	28	UMN179_01491	184
ComC (HI_0437)	173	ComC	176	22	UMN179_01490	176
ComD (HI_0436)	137	Absent			Absent	
ComE (HI_0435)	445	ComE	380	38	UMN179_01489	380
ComF/Com101 (HI_0434)	228	ComF	232	52	UMN179_01573	232
ComE1 (HI_1008)	112	ComE1	109	44	UMN179_01573	109
PilA (HI_0299)	148	PilA	153	51	UMN179_01819	153
PilB(HI_0298)	464	PilB	469	58	UMN179_01820	469
PilC (HI_0297)	406	PilC	402	37	UMN179_01821	402
PilD (HI_0296)	230	PilD	209	23	UMN179_01822	213
PulG/ComN (HI_0938)	170	ComN	177	32	UMN179_01010	177
PulJ/ComO (HI_0939)	238	ComO	240	35	UMN179_01009	240
ComP (HI_0939)	227	ComP	217	25	UMN179_01008	217
ComQ (HI_0941)	101	ComQ	98	29	UMN179_01007	98
Rec2 (HI_0061)	788	Rec2	794	37	UMN179_00665	794
PilF2 (HI_0366)	179	PilF2	186	41	UMN179_00438	186
DNA processing						
ComM (HI_1117)	509	ComM	512	75	UMN179_01347	512
DprA (HI_0985)	373	DprA	398	49	UMN179_01231	398
RadC (HI_0952)	234	RadC	220	58	UMN179_00946	221
Ssb (HI_0250)	168	Ssb	171	61	UMN179_01039	171
LigA (HI_1182)	185	LigA	275	38	UMN179_00865	275
Unknown function						
HI0660 (HI_0660)	119	Absent			Absent	
HI0659 (HI_0659)	98	Absent			Absent	
HI1631 (HI_1631)	190	Absent			Absent	

[a] ID, identity; aa, amino acid.
[b] Sequence identity between *G. anatis* 12656-12 and *H. influenzae* Rd KW20 predicted proteins.

RESULTS

Identification of a putative competence regulon in *G. anatis*. Most of the 25 proteins encoded by the *H. influenzae* competence regulon are conserved among the *Pasteurellaceae* and also in *Enterobacteriaceae* and *Vibrionaceae* (6). However, some noncompetent strains and species have mutations in one or more of the competence genes, which likely explains their inability to transform (6, 25). To investigate whether *G. anatis* has the genes required for DNA uptake, we searched the provisional genome sequence of *G. anatis* 12656-12 for homologs of the proteins encoded by the *H. influenzae* competence regulon as well as for the competence regulators Sxy and CRP. This search identified apparently intact homologs of both regulators and most of the competence regulon proteins known to contribute to DNA uptake and transformation (Table 2). Examination of the DNA sequences upstream of the competence regulon transcriptional units revealed elements resembling the CRP-S motifs identified in other bacteria, supporting the inclusion of the identified genes in a *G. anatis* competence regulon (6) (motif shown in Fig. 1a).

We found no homologs of the gene for HI0660, HI0659, or HI1631 or of *comD*. Of these genes, only the gene for HI0659 and *comD* are required for *H. influenzae* transformation (S. Sinha, unpublished data). However, naturally competent *Aggregatibacter actinomycetemcomitans* lacks a homolog of the gene for HI0659, so this gene is not generally required for transformation in *Pasteurellaceae* (6).

The absence of *comD* is surprising. The *comABCDE* operon is found not only in other *Pasteurellaceae* but also in many other proteobacteria (6, 13). However, in *G. anatis*, *comC* and *comE* overlap by one nucleotide and there is no evidence for the presence of *comD* elsewhere on the genome (Fig. 1b). As in all *Pasteurellaceae* except *H. influenzae*, *comF* is carried separately from the *comABCDE* operon, under its own CRP-S promoter (6) (Fig. 1b).

The same competence genes were identified in the published genome sequence of *G. anatis* UMN179 (18) and in the provisional genome sequence of another *G. anatis* strain, F149^T. UMN179 and F149^T had homologs of all the proteins found in 12656-12, most with sequence identity above 95% (Table 2 and data not shown). Like 12656-12, UMN179 and F149^T contained a *comABCE* operon without *comD*.

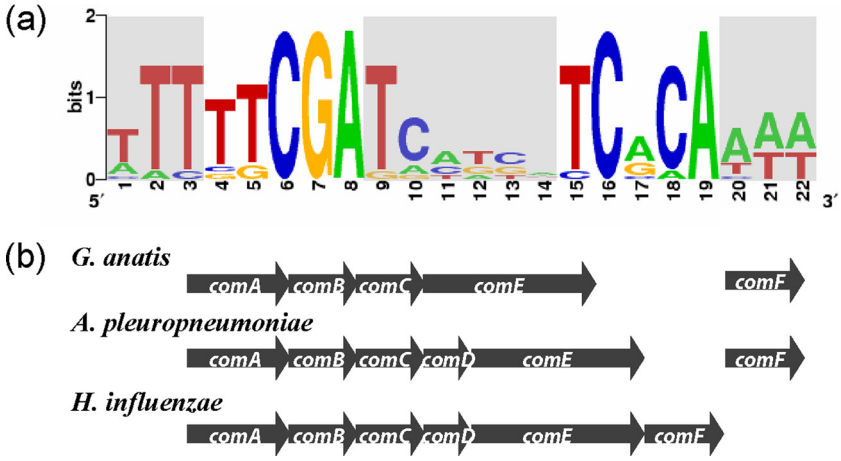

FIG 1 *G. anatis* competence regulon. (a) Sequence logo of all predicted *G. anatis* CRP-S sites (based on sites identified upstream of *comA*, *comF*, *comE1*, *pilA*, *comN*, *rec2*, *pilF2*, *comM*, *dprA*, *radC*, *ssb*, and *ligA*). (b) Organization of *comABCDEF* genes in *G. anatis*, *A. pleuropneumoniae*, and *H. influenzae*.

Induction of natural competence in *G. anatis*. The presence of a homologous competence regulon and the competence regulators Sxy and CRP suggested that *G. anatis* may be naturally competent like *H. influenzae*. To investigate this possibility, we cultured *G. anatis* 12656-12 under standard conditions in BHI, taking aliquots at regular intervals during growth and after growth had stopped and incubating these with 12656-12 Nal^r chromosomal DNA. Although *H. influenzae* develops moderate levels of competence when growth slows (10^{-5} to 10^{-4} transformants CFU^{-1}), no *G. anatis* transformants were obtained at any point. The limit of transformation detection in these experiments varied between 6×10^{-7} transformants CFU^{-1} (at the initial time points when the total number of cells was relatively low) and 7×10^{-9} transformants CFU^{-1} (at the later time points when the cells were in stationary phase). Thus, *G. anatis* 12656-12 appears to differ from *H. influenzae* in that it does not develop detectable levels of competence during growth in rich medium.

In *H. influenzae*, maximum levels of competence are induced when exponentially growing cells are transferred from rich medium to the starvation medium M-IV (15). To test whether M-IV medium induces natural competence in *G. anatis* 12656-12, we grew cells in BHI to an OD_{600} of 0.2, incubated them in M-IV medium for 100 min, and then transformed aliquots with 12656-12 Nal^r chromosomal DNA. This procedure gave abundant resistant colonies, with a transformation frequency of $6 \times 10^{-4} \pm 3 \times 10^{-4}$ Nal^r colonies CFU^{-1} ($n = 5$). No resistant colonies appeared from cells without added DNA.

The competent cells prepared from cultures incubated in M-IV medium could be frozen (−80°C) as described previously (30), with thawed cells showing a slight (~10%) reduction in total viability but no loss in transformation frequency (data not shown). Competence could also be induced following transfer of cells to M-IV medium from colonies on a blood agar plate rather than from a BHI broth culture; this convenient procedure gave transformation frequencies that were much lower but still high enough to be useful for strain construction (3×10^{-6} transformants CFU^{-1}). Transfer from blood agar plates to phosphate-buffered saline (PBS) or BHI gave no transformants.

Effect of DNA concentration on transformation frequency. To study the effect of DNA concentration on transformation frequency, we transformed *G. anatis* with various amounts of DNA. With competent cell preparations containing 10^8 CFU ml^{-1}, the minimum concentration of chromosomal DNA required to obtain detectable levels of transformants was 0.1 to 1 ng ml^{-1} (Fig. 2). For DNA concentrations between 1 and 100 ng ml^{-1}, there was an approximate linear relationship between DNA concentration and transformation frequency; over this range, the average transformation efficiency was 3×10^5 transformants/μg DNA. The highest transformation frequencies were obtained at saturating concentrations of chromosomal DNA, ≥0.5 μg ml^{-1} (Fig. 2).

Efficient targeted mutagenesis of *G. anatis* by natural transformation. Having established that *G. anatis* can take up linear DNA and integrate it into its chromosome, we next tested whether this trait could be used for targeted mutagenesis. We tested two gene disruption constructs, *gtxA*::Km^r and *gtxBD*::Km^r. Both contained the kanamycin resistance cassette from pUC4KISS (Tn*903*) flanked by *G. anatis* sequences; for *gtxA*::Km^r the flanking sequences were 1,509 and 1,648 bp, and for *gtxBD*::Km^r they were 951 and 1,233 bp. The constructs were excised from the plasmids pDO6 and pT2 before use in transformation. Both constructs gave Km^r transformants at very high frequencies (2×10^{-4} and 2×10^{-3} transformants CFU^{-1}, respectively), with transformation efficiencies of 1×10^6 and 1×10^7 transformants/μg DNA, respectively.

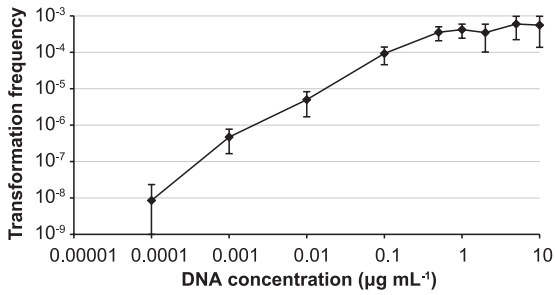

FIG 2 Effect of DNA concentration on transformation frequency. *G. anatis* 12656-12 was transformed with various concentrations of chromosomal Nal^r DNA, and the transformation frequency (transformants CFU^{-1}) was calculated. The averages and standard deviations of three independent experiments are shown.

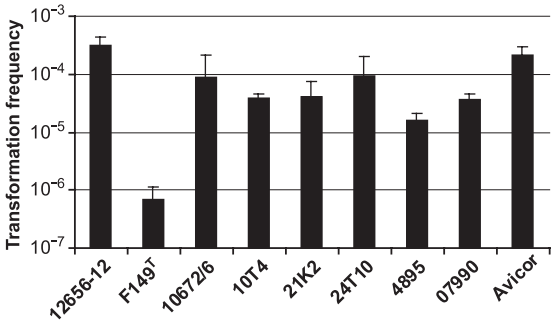

FIG 3 Transformation frequencies in various *G. anatis* strains. Competence-induced cells were transformed with 0.5 μg ml^{-1} chromosomal DNA from strain 12656-12; Nal^r DNA was used except for strains 4895 and 07990. These strains have reduced sensitivity to nalidixic acid and were transformed with chromosomal DNA from 12656-12 Δ*gtxA* (Km^r). Transformation frequency (transformants CFU^{-1}) was calculated, and the averages and standard deviations of a minimum of three independent experiments are shown.

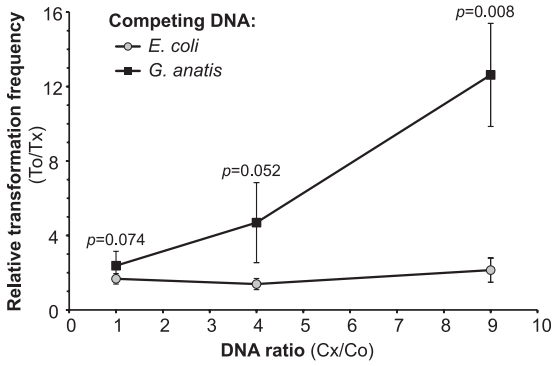

FIG 4 Transformation competition experiments. Competence-induced *G. anatis* 12656-12 was transformed with chromosomal DNA from 12656-12 Nal^r alone or mixed with various concentrations of competing DNA: chromosomal DNA from *E. coli* DH5α or unmarked chromosomal DNA from *G. anatis* 12656-12 as indicated. C_x/C_o, ratio of competing DNA to *G. anatis* 12656-12 Nal^r DNA; T_o/T_x, ratio of transformation frequency in the absence of competing DNA to transformation frequency in the presence of competing DNA. The averages and standard deviations of three independent experiments are shown. Numbers above each data point represent *P* values from comparisons (paired one-tailed Student's *t* test) of the average relative transformation frequencies with *E. coli* DNA as competing DNA and *G. anatis* DNA as competing DNA.

G. anatis 12656-12 is beta-hemolytic, and mutations in *gtxA* and *gtxBD* are known to reduce hemolytic activity (20, 21). Because the transformants were selected on blood agar plates, the beta-hemolytic phenotype of the transformants could be visually scored. All *gtxA*::Km^r transformants were nonhemolytic, and all *gtxBD*::Km^r transformants had highly reduced beta-hemolytic zones. PCR analysis of the transformants showed that 5/5 *gtxA*::Km^r transformants and 5/6 *gtxBD*::Km^r transformants were the result of homologous recombination between the recombinant DNA fragments and the host chromosome, resulting in gene disruptions of *gtxA* and *gtxBD*, respectively. Therefore, these data show that natural transformation can be used to efficiently generate targeted gene disruptions in *G. anatis*.

Transformation with circular plasmid DNA. Transformation with circular plasmid DNA containing no *G. anatis* sequences was also tested, using the *H. influenzae* procedure of incubating competent cells in 30% (vol/vol) glycerol to transiently permeabilize the inner membrane (36). We used plasmid pBA1100, an *E. coli-P. multocida* shuttle plasmid containing the kanamycin resistance gene from Tn*5* (16). Km^r transformants were obtained with a transformation frequency of 2×10^{-5} transformants CFU^{-1} and a transformation efficiency of 7×10^3 transformants per μg DNA. The presence of pBA1100 plasmid DNA was verified by plasmid purification and restriction digestion. Thus, pBA1100 can be used as an *E. coli-G. anatis* shuttle vector.

Natural competence among *G. anatis* strains. The distribution of competence within *Pasteurellaceae* species is sporadic: 1 out of 17 strains of *Aggregatibacter actinomycetemcomitans*, 6 out of 16 strains of *Actinobacillus pleuropneumoniae*, and 30 out of 34 strains of *H. influenzae* could be transformed by natural transformation (5, 25, 40). We tested whether eight additional *G. anatis* strains of different origins could be naturally transformed by the M-IV procedure. All strains were transformable with chromosomal DNA (Nal^r or Km^r) (Fig. 3). The *G. anatis* type strain, $F149^T$, had the lowest transformation frequency, almost 3 orders of magnitude lower than that of 12656-12, but the other strains all had transformation frequencies within 20-fold that of *G. anatis* 12656-12. All strains were also transformable with linearized gene disruption constructs (data not shown), showing that the method described here is broadly applicable to *G. anatis* strains. However, only five of the eight strains (10672/6, 10T4, 21K2, 24T10, and Avicor) could be transformed with plasmid pBA1100 (data not shown).

***G. anatis* takes up *G. anatis* DNA preferentially in comparison to *E. coli* DNA.** The naturally competent species within the *Pasteurellaceae* do not take up DNA indiscriminately but take up their own DNA in preference to foreign DNA (33, 35). DNA fragments containing short motifs known as uptake signal sequences (USSs) are preferred substrates for uptake; these motifs are overrepresented in the genomes of species from within the *Pasteurellaceae* and mediate the uptake specificity. We examined whether *G. anatis* also preferentially takes up its own DNA using transformation competition experiments, in which competent *G. anatis* cells were incubated with a standard concentration of *G. anatis* Nal^r chromosomal DNA mixed with various concentrations of either its own unmarked DNA or unmarked *E. coli* DNA. The transformation frequency was compared to that obtained using only *G. anatis* Nal^r DNA. The use of unmarked *G. anatis* DNA as competing DNA served as a control; as expected, it resulted in an almost linear reduction in transformation frequency across the tested range (upper line in Fig. 4), which confirms that unmarked *G. anatis* DNA does compete with *G. anatis* Nal^r DNA for uptake. In contrast, the addition of unmarked *E. coli* DNA altered the transformation frequency only slightly. Therefore, *E. coli* DNA competes poorly with *G. anatis* Nal^r DNA for uptake, and *G. anatis* preferentially takes up its own DNA rather than *E. coli* DNA (Fig. 4). The slope of the *E. coli* line is slightly positive, and the relative transformation frequency at DNA ratio 9 differs significantly from that at DNA ratio 1 ($P = 0.047$, one-tailed one-sample *t* test), which indicates that *E. coli* DNA does compete weakly and therefore suggests that *G. anatis* may be less selective in its DNA uptake than other members of the *Pasteurellaceae*.

Absence of overrepresented motifs in the *G. anatis* genome sequence. Phylogenetic analyses have shown that the species of *Pasteurellaceae* divide into two main subclades, the *Hin* subclade and the *Apl* subclade (3, 33). Each of the two subclades has its own

TABLE 3 Pasteurellacean uptake signal sequences in bacterial genome sequences

Species	GC content (%)	*Hin*-type USS core (AAGTGCGGT)		*Apl*-type USS core (ACAAGCGGT)	
		No. present (no. expected)	Fold enrichment	No. present (no. expected)	Fold enrichment
G. anatis	39.4	120 (13.8)	8.7	47 (13.8)	3.4
H. influenzae	38.1	1,471 (8.5)	174	56 (8.5)	5.7
A. pleuropneumoniae	41.2	74 (12.9)	5.7	781 (12.9)	60.4
E. coli	50.8	22 (36.0)	0.61	31 (36.0)	0.86

distinct USS, the *Hin* type and *Apl* type, respectively, and the species of each subclade preferentially take up DNA containing the corresponding USS (33). *Gallibacterium* is positioned as the deepest branch in *Pasteurellaceae* phylogenies and thus is a sister group to the rest of the *Pasteurellaceae* and not a member of either subclade (22, 23).

We first determined the number of *Hin*-type and *Apl*-type core USSs in the complete *G. anatis* UMN179 genome sequence (Table 3). Both clade-specific USS cores were enriched in *G. anatis* relative to random expectations, while counts in the *E. coli* genome were no higher than the random expectation. However, the 8.7-fold and 3.4-fold enrichments in *G. anatis* were very low relative to the 174-fold and 60-fold enrichments of the species-specific USS cores in *H. influenzae* and *A. pleuropneumoniae*, and more similar to the 5.9-fold and 5.7-fold enrichments of *Apl*-type cores in *H. influenzae* and *Hin*-type cores in *A. pleuropneumoniae*. These results indicate that *G. anatis* competent cells may have a weak preference for DNA molecules containing *Hin*-type or possibly *Apl*-type USSs. Alignment of the 120 *Hin*-type USS cores revealed two flanking AT-rich motifs like those flanking the *Hin*-type USS cores in the *H. influenzae* genome, suggesting that these sequences do function in DNA uptake by *G. anatis*. The 47 *Apl*-type USSs showed no such consensus (Fig. 5).

We also investigated the possibility that *Gallibacterium* might have a distinct type of USS. Although an unbiased search using the Gibbs motif sampler (37) found no USS-like motif, these searches are insensitive to low-frequency motifs. Thus, we also analyzed the frequencies of all 9-mers in the *G. anatis* genome. Figure 6a to c show genomic 9-mer spectra, i.e., the number of 9-mers (y) that occurs x number of times. In the *H. influenzae* and *A. pleuropneumoniae* spectra, their respective USS cores clearly stand out as the most overabundant 9-mers. In contrast, in *G. anatis*, no strongly overrepresented repeats were found, consistent with *G. anatis* having weaker uptake specificity than either *H. influenzae* or *A. pleuropneumoniae*. However, this specificity does not create a practical barrier to efficient transformation, as the plasmids and mutant constructs described above did not contain USS sequences of either type. One possible explanation for the preference of *G. anatis* DNA over that of *E. coli* is that AT-rich *k*-mers are significantly more abundant in *G. anatis* (Fig. 6d) than in *E. coli*, similar to the other *Pasteurellaceae* and the plasmids used that were also efficiently taken up.

Transformation of *G. anatis* by electroporation. We tested whether *G. anatis* 12656-12 could be transformed by electroporation, using plasmid pBA1100 as donor DNA. Transformants were obtained with transformation frequencies of 10^2 to 10^4 transformants per μg DNA. The presence of pBA1100 plasmid DNA in the transformants was verified by plasmid purification and restriction digestion.

Growth phase affected transformation efficiency, with cells harvested at the transition to stationary phase ($OD_{600} \approx 1.5$) and in stationary phase ($OD_{600} \approx 3$) yielding 1,000-fold-fewer transformants than cells harvested at an OD_{600} of 0.7. Large (100-fold) day-to-day variations in transformation efficiency were also observed; the maximum transformation efficiency obtained was 2×10^4 transformants/μg DNA.

The pBA1100 plasmid used for the electroporation experiments contained no *G. anatis* sequences. However, transformation with circular or linearized plasmid pDO6, which carries an insertionally inactivated *gtxA* gene (*gtxA*::Km^r), and a pSC101 origin of replication which is not expected to replicate in *G. anatis*, also gave no Km^r transformants under the optimal conditions for electroporation. As the *gtxA*::Km^r construct did not integrate into the chromosome, electroporation may not be a suitable method for targeted mutagenesis in *G. anatis*. However, for simply introducing plasmids into cells, electroporation may sometimes be more efficient or convenient than natural transformation.

To test if the method we established could be used for electroporation of other *G. anatis* strains, we electroporated five *G. anatis* strains—$F149^T$, Avicor, 10672/6, 4895, and 07990—with plasmid pBA1100. No transformants were obtained with $F149^T$, 10672/6, 4895, or 07990, but Avicor had a transformation efficiency that was 100 times higher than that of 12656-12. This shows that there is large strain-to-strain variation in electroporation efficiency and that some *G. anatis* strains cannot be transformed with pBA1100 by the electroporation procedure described here. Strains $F149^T$, 4895, and 07990 were also not transformable with the plasmid pBA1100 following M-IV-induced natural competence, indicating that plasmid incompatibility or DNA restriction systems might hinder the establishment of this plasmid in these strains.

DISCUSSION

Our discovery and characterization of natural transformation in *G. anatis* are of great value for future genetic analysis of this species. We have established a procedure for efficient construction of targeted mutants, which will facilitate the identification and characterization of *G. anatis* virulence factors and will eventually contribute to a better understanding of the pathogenicity of *G. anatis*. In addition, the procedure can be refined for the construction of nonpolar mutants without resistance marker genes (39).

We found competence of *G. anatis* to be induced under the same conditions as *H. influenzae*, by transfer to the starvation medium M-IV, indicating that the regulation of natural competence shares features with *H. influenzae*. *G. anatis*'s M-IV-induced transformation frequency is high enough that the procedure is likely to be readily reproducible in other laboratories. However, in

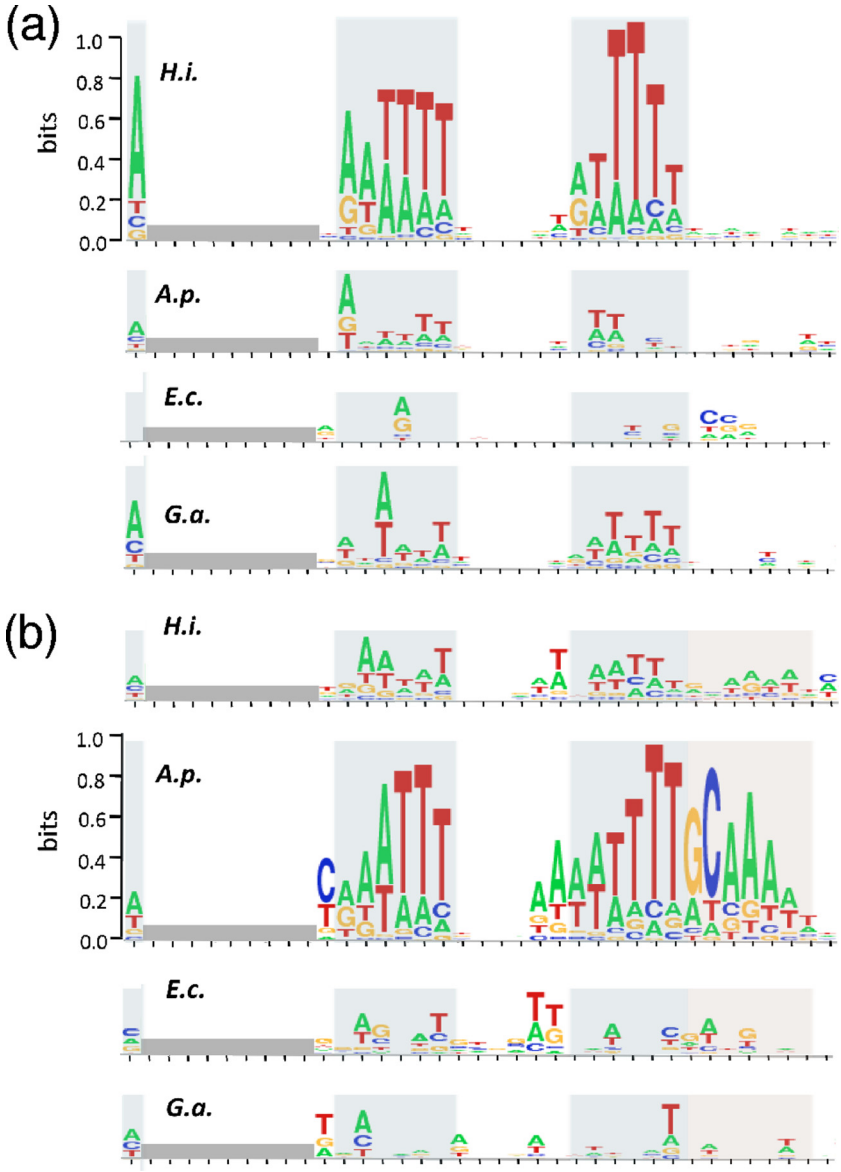

FIG 5 Sequence logos of positions flanking *Hin*-type USS cores (a) or *Apl*-type USS cores (b). The height of the base at each position is a relative indicator of consensus strength at that position. The gray rectangles show the position of the USS cores. The light blue shading highlights known informative positions flanking USS cores, and the grey shading shows the *Apl*-specific extended USS. *H.i.*, *H. influenzae*; *A.p.*, *A. pleuropneumoniae*; *E.c.*, *E. coli*; *G.a.*, *G. anatis*.

contrast to the case with *H. influenzae*, no transformants were detected in dense cultures in rich medium.

The absence of *comD* from the genome of this highly transformable species is surprising. In *H. influenzae*, *comD* is essential for DNA uptake (S. Sinha, unpublished). In the homologous *pilMNOPQ* operons of *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, the *comD* homologs (*pilP* genes) encode predicted inner membrane lipoproteins required for assembly of the pilus fiber. This fiber is thought to pull DNA through the outer membrane, and a knockout of the *Neisseria* gene reduces transformation at least 1,000-fold (7, 12, 24). If ComD is essential for natural transformation in *G. anatis*, this function must be encoded elsewhere on the genome. The amino acid sequences of ComABCD proteins are not well conserved across taxa (13), so it is possible that *G. anatis* encodes an unusually divergent ComD homolog. Alternatively, this function might be provided by an unrelated protein.

Analysis of DNA uptake specificity showed that *G. anatis*, like other competent *Pasteurellaceae*, preferentially takes up its own DNA. However, this preference may be weaker than in other species, as some competition was seen with *E. coli* DNA, and the density of uptake sequences in its genome is substantially lower than in other species in the family. This shows that the presence of overabundant *k*-mers is not always a good indicator of whether an organism will show uptake specificity. This was previously observed for *Helicobacter pylori*, which shows uptake specificity but has no overabundant *k*-mers (17, 34). Because neither pBA1100 nor the linear constructs used for targeted mutagenesis of *G. anatis* contained detectable *Hin*- or *Apl*-type uptake sequences, uptake specificity is unlikely to present any practical problems for strain construction.

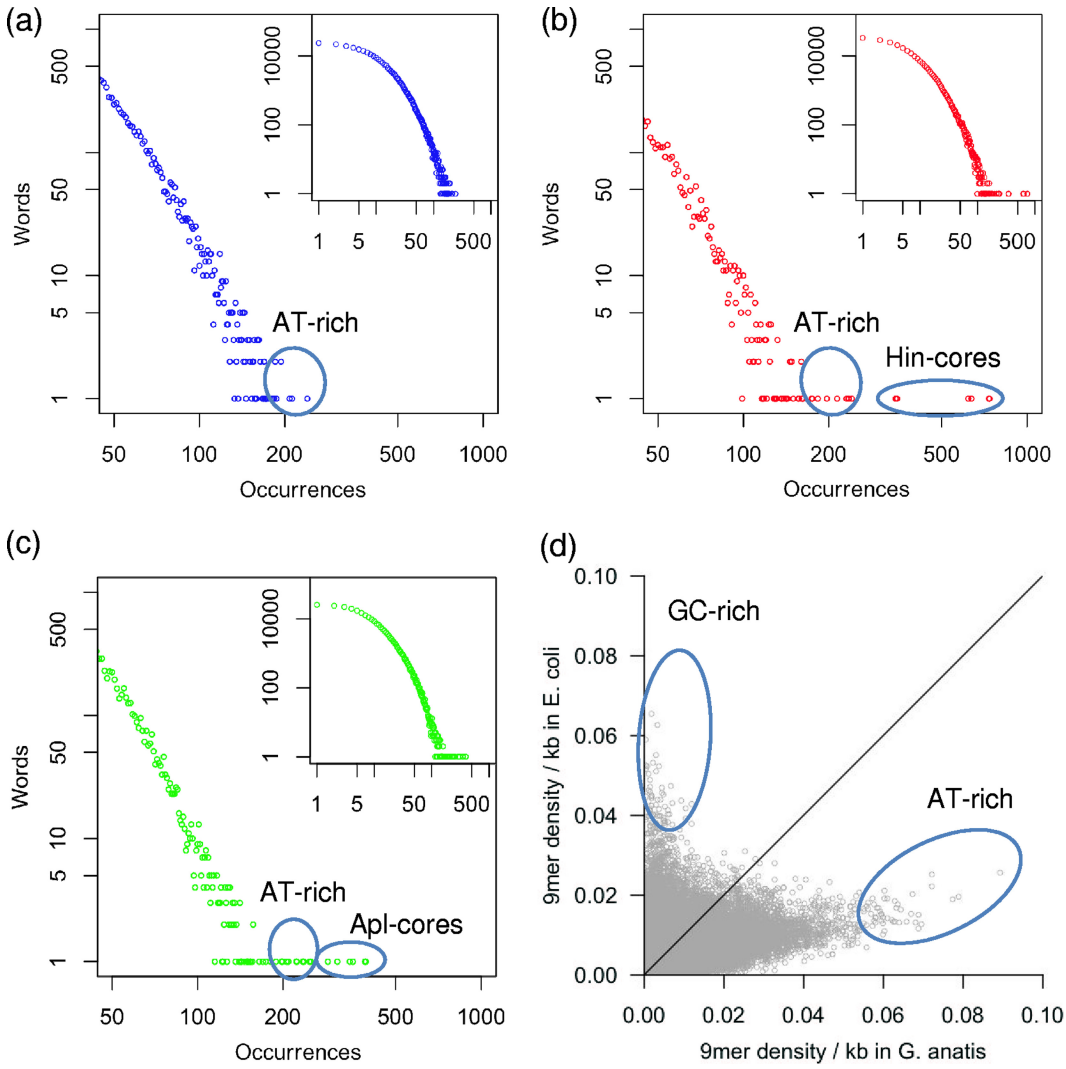

FIG 6 Genomic 9-mer spectra. (a to c) Genomic 9-mer spectra for *G. anatis* (a), *H. influenzae* (b), and *A. pleuropneumoniae* (c). Plots zoom in on the abundant 9-mers, while the insets show the full spectra. *x* axes indicate the number of occurrences of each specific 9-mer word, while the *y* axes indicate the number of words with that many occurrences. (d) Plot of the density per kb of each 9-mer in *G. anatis* against the density in *E. coli*. Blue ovals show 9-mers with the indicated properties. For USS cores, several 9-mers are circled, because of the reverse complements and those shifted by a single base.

All of the nine *G. anatis* strains examined in this study could be transformed with chromosomal DNA and insertionally inactivated gene constructs. However, three of the nine strains could not be naturally transformed with the plasmid pBA1100. These three strains also could not be transformed with this plasmid by electroporation. Many strains of *G. anatis* harbor one or more native plasmids (10), and some of these plasmids may belong to the same incompatibility group as pBA1100 and impair its establishment in the cell. Alternatively, since pBA1100 was purified from *E. coli*, incoming plasmid DNA in these strains may have been efficiently degraded by strain-specific restriction endonucleases.

In conclusion, natural competence is induced in *G. anatis* when cells are transferred from rich medium to a defined starvation medium, and *G. anatis* can be naturally transformed with various forms of DNA. Natural transformation is a valuable and efficient tool for targeted mutagenesis in *G. anatis*.

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