An Evolutionary Link between Natural Transformation and CRISPR Adaptive Immunity

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ABSTRACT Natural transformation by competent bacteria is a primary means of horizontal gene transfer; however, evidence that competence drives bacterial diversity and evolution has remained elusive. To test this theory, we used a retrospective comparative genomic approach to analyze the evolutionary history of Aggregatibacter actinomycetemcomitans, a bacterial species with both competent and noncompetent sister strains. Through comparative genomic analyses, we reveal that competence is evolutionarily linked to genomic diversity and speciation. Competence loss occurs frequently during evolution and is followed by the loss of clustered regularly interspaced short palindromic repeats (CRISPRs), bacterial adaptive immune systems that protect against parasitic DNA. Relative to noncompetent strains, competent bacteria have larger genomes containing multiple rearrangements. In contrast, noncompetent bacterial genomes are extremely stable but paradoxically susceptible to infective DNA elements, which contribute to noncompetent strain genetic diversity. Moreover, incomplete noncompetent strain CRISPR immune systems are enriched for self-targeting elements, which suggests that the CRISPRs have been co-opted for bacterial gene regulation, similar to eukaryotic microRNAs derived from the antiviral RNA interference pathway.

IMPORTANCE The human microbiome is rich with thousands of diverse bacterial species. One mechanism driving this diversity is horizontal gene transfer by natural transformation, whereby naturally competent bacteria take up environmental DNA and incorporate new genes into their genomes. Competence is theorized to accelerate evolution; however, attempts to test this theory have proved difficult. Through genetic analyses of the human periodontal pathogen Aggregatibacter actinomycetemcomitans, we have discovered an evolutionary connection between competence systems promoting gene acquisition and CRISPRs (clustered regularly interspaced short palindromic repeats), adaptive immune systems that protect bacteria against genetic parasites. We show that competent A. actinomycetemcomitans strains have numerous redundant CRISPR immune systems, while noncompetent bacteria have lost their CRISPR immune systems because of inactivating mutations. Together, the evolutionary data linking the evolution of competence and CRISPRs reveals unique mechanisms promoting genetic heterogeneity and the rise of new bacterial species, providing insight into complex mechanisms underlying bacterial diversity in the human body.

Natural transformation is a primary means of bacterial horizontal gene transfer (HGT). Natural transformation occurs when environmental cues trigger the expression of competence genes, which allow the bacterium to take up DNA from its environment and incorporate new genes into its genome. Bacterial competence systems can vary among species, but all systems involve DNA transport proteins and machinery that incorporates the new DNA into the chromosome via homologous recombination (1–10). Because of their genetic complexities, competence systems are thought to be ancestral traits in many bacterial lineages; however, competence loss within bacterial families is common (9, 10). Evolutionary models predict that HGT and homologous recombination by natural transformation can accelerate adaptation to new environments and allow organisms to more rapidly reach fitness equilibria (11, 12). Despite predictions that competence is a beneficial trait, many bacteria are noncompetent and, among competent bacteria, competence genes are sometimes lost by strains within a species (13, 9). This leads to the hypothesis that, throughout evolution, naturally competent bacterial populations are constantly giving rise to noncompetent siblings, and at certain points, noncompetent genomes are selected for over the dynamic genomes of competent bacteria.

To test this hypothesis, we focused on the evolutionary history of the opportunistic human periodontal pathogen Aggregatibacter actinomycetemcomitans, of which ~30% of the isolated strains are naturally competent (13). A. actinomycetemcomitans is a member of Pasteurellaceae, a family of bacteria predicted to have descended from a competent ancestor (10). An experimental survey testing natural transformation demonstrated that A. actinomycetemcomitans competence loss is clonal (13), suggesting that noncompetent strains arose and radiated into noncompetent lineages. The persistence of noncompetent A. actinomycetemcomitans strains during evolution allows us to utilize genome sequence information to delineate the evolutionary history of competence loss and genome stability. Using comparative genomics, we have found that competence loss has occurred multiple times during A. actinomyce-
A. actinomycetemcomitans evolution in parallel. Compared to competent strains, noncompetent A. actinomycetemcomitans strains have smaller genomes with fewer rearrangements, as demonstrated by whole-genome alignments. Pairwise strain average nucleotide identity (ANI) calculations reveal that competence loss is tied to evolutionary divergence among A. actinomycetemcomitans strains. Moreover, the loss of competence is directly correlated with the loss of A. actinomycetemcomitans CRISPR-cas adaptive immune systems, resulting in noncompetent strains that have more parasitic genetic elements than competent siblings do. In noncompetent strains, remnants of CRISPR-cas systems that remain appear to have been co-opted for A. actinomycetemcomitans self gene regulation, similar to eukaryotic microRNAs. Together, these results support a model of A. actinomycetemcomitans evolution whereby competence and noncompetence are means to genetic diversity and stability, respectively.

RESULTS AND DISCUSSION
Parallel loss of competence by common mechanisms. Because the Pasteurellaceae common ancestor is predicted to be competent, we sought to determine the genetic nature of competence loss throughout A. actinomycetemcomitans evolution. Therefore, it was important to generate an accurate evolutionary history of the species and map competence loss to this phylogeny. The alignment of concatenated core genes can be used to accurately determine phylogenetic relationships among bacteria (14). We aligned core genes from 17 sequenced A. actinomycetemcomitans genomes, including 14 previously sequenced strains (15–18) and 3 genomes sequenced in this study (see Table S1 in the supplemental material). The concatenated core gene superalignment was subjected to maximum-likelihood phylogenetic analysis to determine evolutionary relationships between strains, revealing the presence of three major A. actinomycetemcomitans lineages (Fig. 1). Of these 17 strains, 7 have previously been subjected to natural transformation assays and 2 were found to be competent (13, 19). Subsequent alignment of multiple individual competence genes from the 17 sequenced A. actinomycetemcomitans genomes reveals that this bacterial lineage derived from a competent ancestor, and throughout evolutionary history, several noncompetent lineages have arisen via inactivation or deletion of genes critical for DNA uptake and incorporation into the chromosome (Fig. 1; see Fig. S1 in the supplemental material). The results indicate the presence of 3 competent and 14 noncompetent sequenced strains, which reflects the prevalence of noncompetent strains in larger A. actinomycetemcomitans populations (13). Importantly, the bioinformatic predictions for competent and noncompetent strains correlate with results of previously published natural transformation assays. Overall, noncompetence has arisen on at least six independent occasions (Fig. 1), with the most common mutation (three occasions) occurring via the insertion of a site-specific mobile DNA element into comM (see Fig. S1), whose gene product is important for efficient incorporation of DNA onto the chromosome following uptake (3). This pattern of A. actinomycetemcomitans competence loss contrasts with that of other naturally competent bacteria, like Haemophilus influenzae, in which competence loss is common but occurs randomly throughout evolution and typically by many unique mechanisms (9, 20). Because A. actinomycetemcomitans competence loss occurred multiple times during evolution in more than one lineage, these data support a model of the parallel evolution of competence loss.

While parallel evolution is indicative of natural selection, this does not signify that competence loss is adaptive; in fact, it may be neutral, but the genome content of the stably maintained non-competent strains is presumably beneficial.

Divergence and speciation of noncompetent strains. Competence is a primary mechanism of HGT and DNA acquisition in bacteria (21), and as expected, the genomes of competent A. actinomycetemcomitans strains are, on average, 200,000 bp larger than those of noncompetent strains (2.3 Mb versus 2.1 Mb, respectively; P = 9 × 10^-3, two-tailed unpaired t test). A major consequence of competence loss is the inability to acquire new traits via genomic exchange with related species, leading to the hypothesis that competence loss leads to genetic isolation, which could ultimately result in speciation. To test this hypothesis, we analyzed noncompetent and competent genomes for genomic rearrangements and ANI across whole genomes. Our results revealed that noncompetent strains have similar syntenic genomes (i.e., similar gene arrangements), while competent strains have dynamic genomes with multiple rearrangements (Fig. 2; see Fig. S2 in the supplemental material). Further differences were revealed by ANI. On the basis of a proposed modern species definition (ANI of >95%) (22), the noncompetent strain with the most ancient branch point (lineage 1, Fig. 1) is a different species than strains within lineage 2 and lineage 3 (Fig. 3; see Fig. S3). Interestingly, ANI comparisons of strains within lineage 2 and within lineage 3 show that strains within these lineages are ~99% identical; however, comparisons of strains in lineages 2 and 3 reveal that they differ significantly (ANI of ~97%; P < 0.001). Thus, lineage 2 and 3 strains have clearly begun to diverge into distinct populations. This supports a model in which competence loss leads to genetic isolation and ultimately divergence in A. actinomycetemcomitans.
Noncompetent strain diversity due to mobile genetic elements. Despite the fact that noncompetent genomes had high synteny and ANI, some differences among noncompetent genomes were observed. We hypothesized that noncompetent strains of *A. actinomycetemcomitans*, having eliminated a predominant pathway for HGT, would utilize alternative mechanisms for HGT to maintain evolutionary fitness. Indeed, mathematical modeling suggests that in the absence of HGT and homologous recombination, genetic drift resulting from the accumulation of random neutral mutations will ultimately lead to decreased fitness (12), indicating that at least some nominal level of HGT is required for a strain to remain fit. Transducing phage and conjugal plasmids are also sources of HGT, and on average, there were about five unique plasmid- and phage-related DNA elements in each noncompetent genome, significantly more than were observed in competent lineages, which had about two (two-tailed unpaired *t* test, *P* < 0.02). The unique DNA elements in the non-competent strains include two self-replicating plasmids, 14 integrated plasmid elements, and 22 prophage elements. Thus, while noncompetent strains possess increased synteny and smaller genomes than competent strains (Fig. 2; see Fig. S2 in the supplemental material), the observed differences between the DNA contents of these strains are almost entirely due to the incorporation of plasmid and prophage DNA.

Noncompetent strains have compromised CRISPR adaptive immune systems. The presence of plasmids and prophage in the noncompetent strains suggests that their defenses against foreign nucleic acids were potentially compromised. Bacterial adaptive immune systems called CRISPRs have evolved to protect against phage- and plasmid-mediated HGT (23–25). Many variations of CRISPRs exist, and they have been extensively reviewed (26, 27). Briefly, CRISPRs are genetic loci consisting of direct repeats and spacers (28). Spacers are often derived from phage and plasmid DNA (29). During an infection, short CRISPR RNAs (crRNAs) are transcribed from the CRISPR loci and base pair with foreign (plasmid and bacteriophage) DNA via spacer-encoded sequences (24). Both the invading DNA and crRNA are cleaved by proteins encoded by CRISPR-associated cas genes, thereby eliminating the phage and plasmid DNA (23, 25, 30, 31). Recent work has shown that bacteria readily lose CRISPRs under certain selective conditions. Specifically, enterococci lost CRISPRs allowing the acquisition of parasite-encoded antibiotic resistance genes and *Mycoplasma gallisepticum* lost CRISPRs following the shift to a new avian host (32, 33). Because CRISPR loss is common in other organisms, we predicted that noncompetent *A. actinomycetemcomitans* strains were more susceptible to phage and plasmids because of CRISPR-mediated compromised immunity. To test this prediction, we examined the CRISPR-cas contents of competent and noncompetent *A. actinomycetemcomitans* strains. On the basis of phylogeny, the common ancestor of *A. actinomycetemcomitans* possessed two CRISPR-cas systems, CRISPR1-cas and...
CRISPR3-cas, duplicated from a single system in a more ancient ancestor (Fig. 4). Indeed, Aggregatibacter aphrophilus has only one CRISPR-cas system related to A. actinomycetemcomitans CRISPR1-cas and CRISPR3-cas (see Fig. S4 in the supplemental material), supporting the hypothesis that they were duplicated from an ancient system. The competent A. actinomycetemcomitans strains in our study contain both of these ancient CRISPR-cas systems and have acquired a third intact CRISPR-cas system (Fig. 4). In contrast, the CRISPR-cas systems in ancient noncompetent lineage 1 and 2 strains have lost cas genes to deletion and/or acquired nonsense mutations in cas genes (Fig 5A; see Fig. S5 in the supplemental material). On the basis of these mutations, CRISPR elements in noncompetent lineage 1 and 2 strains are predicted to be nonfunctional. CRISPR-cas loss was evident even in the most recently evolved (lineage 3) noncompetent strains, indicating that loss of CRISPR-cas occurs quickly within evolutionary time. The most parsimonious evolutionary history is that competence loss preceded CRISPR-cas loss among A. actinomycetemcomitans strains. This inference can be drawn because lineage 2 strains have the same disruption of the comM competence gene; however, they have many different CRISPR-cas mutations and deletions. These data indicate that the emergence of noncompetence in A. actinomycetemcomitans is correlated with the loss of CRISPR-based adaptive immunity.

Evolution of CRISPR self gene regulation in noncompetent strains. Despite the fact that noncompetent strains have lost many cas genes required for immunity, most of these strains maintain one CRISPR with a small number of spacers. A current hypothesis is that “broken” CRISPR-cas loci tend toward autoimmunity and acquire self-targeting spacers (34) that are used to control the expression of chromosomal genes. To test this hypothesis, we identified spacers in all of our strains and predicted their targets (35, 36). Most spacers target unknown sequences (Fig 5B; see Fig S6 in the supplemental material). However, identifiable targets in competent strains were predominantly phage and plasmids, while strains in noncompetent lineages were enriched for spacers with specificity for A. actinomycetemcomitans genes (Fig 5B; see Fig S6 and Table S2 in the supplemental material). Some of these self-targeting spacers within the noncompetent strains are antisense to coding genes, indicating that they could regulate self gene expression similar to that observed for an orphan crRNA in Listeria monocytogenes (37). These data suggest that noncompetent A. actinomycetemcomitans strains are co-opting CRISPRs for A. actinomycetemcomitans chromosomal gene regulation, reminiscent of eukaryotic microRNAs that have been co-opted from the RNA interference immune surveillance system in higher eukaryotes (38). To test whether the self-targeting CRISPRs could be used for self gene regulation, we used Northern blot analysis to probe for the self-targeting crRNA in A. actinomycetemcomitans VT1169. Similar to the orphan CRISPR in L. monocytogenes, the A. actinomycetemcomitans self-targeting spacer is expressed primarily dur-
The concomitant loss of CRISPR in a relatively constant environment. One potential consequence selected for when the organisms have beneficial genome content noncompetent siblings constantly arising in the population are organism under changing environmental conditions. However, competent bacterial genomes quickly drive evolution, benefiting the motes genome stability. On the basis of this model, dynamic commensals genetic diversity while noncompetence promotes rapid evolution.

We have discovered an instance in which changes in the environment through phage- and plasmid-competent strains is that they can sample and adjust to small changes in the environment through phage- and plasmid-mediated HGT. The VT1169 crRNA is expressed primarily as an unprocessed transcript. Cells were grown to exponential phase (Exp.) and stationary phase (Stat.), and total RNA was harvested and subjected to Northern blot analysis with a probe for the self-targeting crRNA. The ethidium bromide-stained RNA (right panel, top two bands) demonstrates equal RNA loading and RNA integrity. The radiolabeled RNA Century Marker-plus (M) is overexposed at the left to serve as the molecular size standard. Molecular sizes in nucleotides are shown on the left. The Northern blot assay shown is representative of three biological replicates. (B) Predicted binding interaction between A. actinomycetemcomitans VT1169 crRNA and the glgP mRNA.
tencomitas 624, VT1169, and Y4 were aligned with the complete A. actinomycetemcomitans reference genomes (A. actinomycetemcomitans HK1651, D75-1, and D115-1) using CLC Genomics Workbench software (see Table S1). Supplemental sequencing of VT1169 CRISPR1 was carried out by capillary sequencing of a PCR product (obtained with primers C1-For [5’ AGCGAAATTCCACCCGAC 3’] and C1-Rev [5’ TTGATGGAATTGATGAC 3’]).

Multiple sequence alignments, ANI calculations, and phylogenetic tree construction Complete and draft genome sequences for all A. actinomycetemcomitans strains were generated in this study or downloaded from GenBank and Oralgen (http://www.oralgen.lanl.gov/index.html) (15–18). When complete gene sequences were not found in the de novo genome assembly of VT1169, consensus gene sequences were inferred from alignment with HK1651. The core gene phylogenetic tree was constructed from the sequence alignment of 30 concatenated core genes (pyrG, rplA, rplB, rplC, rplD, rplF, rplK, rplM, rplP, rpsL, rpsC, rpsE, rpsL, rpsM, rpsS, smBP, tfS, frr, rplE, rpoB, rpoS, dam, dnaN, dnaQ, holB, holD, recR, rpoA, and rpoH) by the maximum-likelihood method using the Tamura-Nei model in MEGA5 using MUSCLE with 100 bootstrap replicates (44–47). Whole-genome alignments were performed with progressive MAUVE to identify genome rearrangements, local collinear genomic blocks, and variable mobile elements (48). ANI values for pairwise genome comparison were calculated using JSpecies with BLAST (ANIB) and MUMMER (ANIm) (22). Individual competence genes were aligned with MUSCLE in MEGAS (44, 45, 47). Whole case operons were aligned with CLC Sequence Viewer. The consensus gene sequences from VT1169 sequencing read alignment with HK1651 were used when genes were unassembled de novo.

CRISPR detection and characterization. CRISPRs were identified with CRISPRFinder (36). CRISPR spacer sequences were subjected to BLASTn analysis against the nr/nt database, and hits with >94% identity were recorded (49). Hits were classified as directed toward plasmid, prophage, phage, or self, while negative results were classified as unknowns.

Northern blot analysis. A. actinomycetemcomitans VT1169 was grown to mid-exponential phase (optical density at 600 nm [OD 600], 0.80) and stationary phase (OD 600, 1.60) in TSBYE, and total RNA was harvested using RNA bee (Tel-Test) as described previously (50). Northern blotting was carried out by using previously described methods (50). Briefly, 10 μg RNA was separated on an 8% polyacrylamide–8 M urea denaturing gel, stained with ethidium bromide, transferred to nitrocellulose, UV cross-linked, blocked for 1 h at 55°C in UltraHyb buffer (Ambion), and probed for 16 h at 38.5°C with a radiolabeled DNA oligonucleotide anti-crRNA probe (5’ GGCGGTGGATTGGAAGGCCTCGCCGCCAC 3’). The radiolabeled RNA Century Marker-plus (Ambion) was synthesized according to the manufacturer’s protocol and served as a size standard. Prior to transfer, gels were stained with ethidium bromide and imaged with a G:BOX apparatus (Syngene) to visualize rRNA, which served as the loading control.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00309-12/-/DCSupplemental.

Figure S1. PDF file, 0.5 MB.
Figure S2. PDF file, 2.2 MB.
Figure S3. PDF file, 0.6 MB.
Figure S4. PDF file, 0.4 MB.
Figure S5. PDF file, 0.4 MB.
Figure S6. PDF file, 0.4 MB.
Table S1. DOCX file, 0.1 MB.
Table S2. DOCX file, 0.1 MB.

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