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Genomic integration and expression of the *Aggregatibacter actinomycetemcomitans* catalase gene in *Aggregatibacter aphrophilus*

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Abstract

Objective—To test the hypothesis that virulence genes of *Aggregatibacter actinomycetemcomitans* can be expressed and confer fitness advantages in the closely related *Aggregatibacter aphrophilus*.

Design—Clinical isolates of *A. aphrophilus* were screened for natural competence with marked genomic DNA from *A. actinomycetemcomitans* and *A. aphrophilus*. The gene *katA* of *A. actinomycetemcomitans* D7S-1 and its flanking regions were constructed and inserted into a comparable locus in the genome of naturally competent *A. aphrophilus* strains by a markerless protocol via natural transformation. Mutants of *A. actinomycetemcomitans* with or without *katA* were also constructed by a similar protocol. Discs soaked with either 0.03% hydrogen peroxide or broth culture of *Streptococcus gordonii* Challis were placed on the agar with cultures of *A. actinomycetemcomitans* or *A. aphrophilus*. The size of the growth inhibition zone associated with the disc was measured after 2-day culture.

Results—Five of the 13 *A. aphrophilus* strains exhibited a transformation frequency of 10^{-6} or higher. The intra- and inter-species transformation frequencies were comparable. The inhibition zones for *katA*-negative strains of *A. actinomycetemcomitans* or *A. aphrophilus* were 3- to 7-fold larger than those associated with *katA*-positive strains ($p < 0.05$).

Conclusions—There was no apparent species barrier for the transfer and expression of *A. actinomycetemcomitans* *katA* in *A. aphrophilus*. The inserted *A. actinomycetemcomitans*-specific *katA* gene in *A. aphrophilus* strain NJ8700 conferred resistance to inhibition by hydrogen peroxide or *S. gordonii*. The potential to swap genes between these two closely related oral species may be an alternative approach for investigating the virulence determinants of *A. actinomycetemcomitans*.

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Keyword Index

Catalase; Genomic Islands; Gene Transfer; Hydrogen Peroxide; Mutagenesis; Virulence

INTRODUCTION

A. actinomycetemcomitans and *A. aphrophilus* are Gram-negative, non-motile, facultatively anaerobic rods. These two species are phylogenetically close and also share similar oral niches. However, *A. actinomycetemcomitans* is recognized as an etiology of aggressive periodontitis (Fine et al., 2007), while *A. aphrophilus* is considered an oral commensal compatible with periodontal health (Tempro & Slots, 1986). Interestingly, these two species share more than 70% of gene content in their genomes (Kittichotirat, Bumgarner, & Chen, 2016). The differences in the pathogenic potentials between these two species appear to be attributed to the presence of a number of virulence factors, such as catalase, leukotoxin and cytolethal distending toxins that are specific to *A. actinomycetemcomitans*.

Based on comparative genomic analysis by whole genome sequencing, we hypothesize that selective acquisition of genes via horizontal gene transfer drives the divergence of these two species, leading to distinct oral adaptation strategies and virulence potential between these two species. Testing this hypothesis necessitates the construction of mutants of *A. aphrophilus* that express *A. actinomycetemcomitans*-specific virulence genes inserted in the comparable loci in *A. aphrophilus*. In this study, we demonstrated the feasibility of this approach with the catalase gene, a virulence determinant specific to *A. actinomycetemcomitans*. This approach permits the isolation and characterization of one or multiple virulence genes of *A. actinomycetemcomitans* in phylogenetically closely related *A. aphrophilus*, and could be adapted for other experimental strategies.

MATERIALS & METHODS

Bacterial strains and plasmids

Table 1 lists the bacterial strains and plasmids used in this study. *A. aphrophilus* was grown with 5% CO₂ at 37°C on either chocolate agar (Slots, 1981) or in *Haemophilus* Test Media (HTM) containing 0.015% hematin, 0.021% Mueller Hinton broth (BD Bioscience, Sparks, MD, USA), 0.015% nicotinamide adenine dinucleotide (NAD), and 0.005% yeast extract (Dogan, Asikainen, & Jousimies-Somer, 1999). *A. actinomycetemcomitans* was routinely grown at 37°C in air supplemented with 5% CO₂ either in trypticase soy broth (TSB) agar (sTSB agar) containing 3% TSB, 0.3% yeast extract, 5% horse serum and 1.5% agar, or in modified TSB (mTSB) composed of 3% TSB and 0.6% yeast extract. *Streptococcus gordonii* Challis was grown on blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA) that contained 5% sheep blood in trypticase soy agar. As needed, spectinomycin (Sigma-Aldrich, St. Louis, MO, USA), rifampicin (Sigma-Aldrich) or ampicillin (Sigma-Aldrich) were added to the media at concentrations of either 50 µg/ml or 100 µg/ml.

The spontaneous rifampicin-resistant mutants *A. actinomycetemcomitans* D7S-Rif^r and *A. aphrophilus* NJ8700-Rif^r were generated by plating the *A. actinomycetemcomitans* D7S-1

and *A. aphrophilus* NJ8700 wildtype strains on mTSB and chocolate agar, respectively, supplemented with 50 µg/ml rifampicin. After 3 days of incubation with 5% CO₂ at 37 C, single colonies of the rifampicin-resistant mutants were selected, subcultured to assure purity, and stored at –80°C until use.

Plasmids were maintained in *Escherichia coli* strain DH5α by standard methods (Sambrook, Fritsch, & Maniatis, 1989).

Natural transformation assays

The assays were performed by a modification of the protocol described previously (Cheng et al., 2014; Wang, Goodman, Redfield, & Chen, 2002). Competent bacteria were prepared by growing on sTSB agar (for *A. actinomycetemcomitans*) or chocolate agar (for *A. aphrophilus*) in air with 5% CO₂ at 37 C for 2 days. Cells were collected from the agars and resuspended in mTSB (for *A. actinomycetemcomitans*) or HTM (for *A. aphrophilus*). Approximately 10⁸ cells (based on OD₆₀₀) in 50–100 µl of mTSB were then spotted onto the surface of pre-warmed sTSB agar or chocolate agar. Ten microliters of donor DNA (0.1 µg/µl) extracted from *A. actinomycetemcomitans* D7S-Rif^r or *A. aphrophilus* NJ8700-Rif^r were then applied onto the recipient cells. After incubating the plate with 5% CO₂ at 37°C overnight, the cells were collected and resuspended in 150 µl of suitable liquid media, i.e. mTSB or HTM. An aliquot of 10 µl of the suspension was serially diluted and plated on agar to enumerate the total colony-forming units (CFU). The remaining 140 µl of the suspension were plated onto suitable selective plates containing 50 µg/ml rifampicin and incubated for 3–5 days to enumerate the transformants. Recipient cells without donor DNA were used as the control to enumerate the numbers of spontaneous antibiotic-resistant mutants. Three biological replicates were performed for each experiment. In the competition assays of transformation, unmarked DNAs (genomic DNAs from D7S-1 of *A. actinomycetemcomitans*, NJ8700 of *A. aphrophilus* or plasmid DNA from *E. coli*) were mixed with marked DNAs from antibiotic-resistant strains D7S-Rif^r or NJ8700-Rif^r, at a ratio of 10:1 or 30:1 respectively and used for transformation assays. The transformation frequency was determined as: (CFU of transformants - CFU of spontaneous antibiotic-resistant mutants)/total CFU.

PCR

PCR was used for several different purposes. The PCR primers and their purposes are provided in Supplementary Tabel S1 (see Appendix). In a typical PCR, 25 µl of the reaction mixture contained 1 µl each of 10µM forward and reverse primers, 1 µl of 500 ng/µl template DNA, 125. µl of LongAmp *Taq* 2X Master Mix (New England Biolabs, Ipswich, MA, USA) and 95. µl of nuclease-free water. The thermo-cycler profile was set as follows: an initial denaturation step at 94 C for 30 seconds, followed by 35 cycles of denaturation (94 C/30 seconds), annealing (45–65°C/45 seconds based on the lengths and sequences of the primers), and extension at 65 C/10 minutes. The resultant amplicons were analyzed by electrophoresis in a 1% agarose gel. As needed, the amplicons were extracted using the QIAquick Gel Extraction kit (Qiagen Inc., Germantown, MD, USA) and submitted to Eton Bioscience, Inc. (San Diego, CA, USA) for sequencing.

Construction of the mutants

The markerless gene deletion and restoration protocols developed by our laboratory have been described in detail previously (Cheng et al., 2014; Fujise, Wang, Chen, & Chen, 2008). Briefly, the gene deletion protocol comprises three steps: (i) construction of the donor DNA with the *loxP*Sp^r/*loxP* cassette flanked by homologous regions upstream and downstream of the target gene to be deleted via cloning or by PCR amplification, (ii) allelic replacement of the target gene in *A. actinomycetemcomitans* by natural transformation with the donor DNA, and (iii) deletion of the *loxP*Sp^r/*loxP* cassette by introducing a Cre recombinase-expressing plasmid into the mutant leaving a *loxP* scar at the deletion site. For gene restoration, the protocol is essentially the same as the gene deletion protocol. However, one of the homologous regions of the donor DNA contains a full-length gene to be restored in the mutant.

Gene deletion and insertion experiments were designed in consideration of the similarities of the target loci between *A. actinomycetemcomitans* and *A. aphrophilus* (Figure 1). The donor DNA for the construction of the catalase expressing *A. aphrophilus* mutant NJ-kat⁺ was generated by PCR and cloning as depicted in Figure 2. Briefly, a 1,002 bp fragment that included the 828 bp *gatA* (NJ8700_00310) from *A. aphrophilus* NJ8700 was PCR amplified using genomic DNA from NJ8700 as the template and primers that contained a restriction site for *NotI* or *SpeI*. The PCR products were purified with QIAquick PCR purification kit and QIAquick Gel Extraction kit (both from Qiagen), and cloned into a previously constructed pLox-Sp^r plasmid (Cheng et al., 2014), containing a *loxP*Sp^r/*loxP* cassette, via *NotI* and *SpeI* sites to generate the plasmid pCAY-1. Next, a 37, 51 bp fragment encompassing glutamate dehydrogenase *gdhA* (D7S_00738) and catalase *katA* (D7S_00739) from *A. actinomycetemcomitans* strain D7S-1 was PCR amplified using D7S-1 genomic DNA as the template and primers that contained a restriction site for *XhoI* or *ApaI*. The PCR products were purified and digested as described above and cloned into pCAY-1 via *XhoI* and *ApaI* to generate the plasmid pCAY-2. A single fragment that included the 4 cloned genes (*gdhA*, *katA*, *loxP*Sp^r/*loxP*, and *gatA*) was released from pCAY-2 by digestion with *EagI* and *ApaI*, purified and used as the donor DNA, which was used directly for transformation to construct *katA*-expressing NJ-kat⁺ mutant of *A. aphrophilus* (also see Figure 3a).

The second donor DNA for the construction of the catalase-deletion mutant D7S-dkat was generated using PCR according to a protocol described previously (Cheng et al., 2014) (also see Figure 3b). Briefly, two genes in the flanking regions of *katA* (D7S_00738 [encoding *gdhA*] and D7S_00740 [annotated as signal peptide protein]) of *A. actinomycetemcomitans* strain D7S-1 were PCR amplified to generate two DNA fragments each with a *DraIII* site near the target deletion site. Approximately 1,600 ng of the PCR products, purified by the QIAquick PCR purification kit (Qiagen), were mixed with approximately 800 ng of recombinant plasmid pLox-Sp^r that contained a cloned *loxP*Sp^r/*loxP* cassette flanked by *DraIII* sites. The mixture was digested with *DraIII* and ligated at 16°C overnight. After heat inactivation of the ligation reaction, the ligation mixture was gel purified with the QIAquick PCR Purification Kit (Qiagen) and used directly as donor DNA for transformation.

The same protocol was used to generate the third donor DNA of ligated *gdhA-katA* and the gene for signal peptide protein for the construction of the catalase-restored D7S-dkatC mutant (also see Figure 3c).

Pure cultures of transformants were first isolated from selective agar, presumptively identified by applying 3% hydrogen peroxide to test the expression of catalase, and confirmed by PCR and sequencing. The *loxP/Sp^r/loxP* cassette was then deleted via expression of Cre recombinase to generate NJ-kat⁺, D7S-dkat, and D7S-dkatC. The mutants were confirmed by PCR and sequencing as described previously (Cheng et al., 2014). Growth analysis of the wildtype and mutants was performed with Bioscreen. Briefly, the bacteria were inoculated into mTSB at OD₆₅₀=0.05 and incubated for 48 hours (the early stationary phase). An OD₆₅₀ of 0.4 was found for both the wild type *A. actinomycetemcomitans* D7S-1 and the corresponding *katA*-deleted mutant. An OD₆₅₀ of 0.3 was found for both the wild type *A. aphrophilus* NJ8700 and the corresponding *katA*-expressing mutant. Also, we did not notice any growth defects when these strains were plated and cultured on agar.

H₂O₂ inhibition assays in liquid media

Overnight cultures of *A. actinomycetemcomitans* in mTSB and *A. aphrophilus* in HTM at 37°C with 5% CO₂, adjusted to OD₆₀₀=0.1, were incubated for an additional 6 hours under the same conditions (37°C with 5% CO₂). After the final OD of the culture was determined, the culture was adjusted to OD₆₀₀=0.8 with the addition of fresh medium, and then 0.000015% or 0.00006% hydrogen peroxide was added to the culture respectively. At times 0 and 30 minutes, aliquots of 50 µl were removed, serially diluted with fresh media, and plated onto agar and incubated for 3 days to enumerate CFU. Three biological replicates were performed.

H₂O₂ inhibition assays by disc

Overnight cultures of bacteria in either mTSB for *A. actinomycetemcomitans* or HTM for *A. aphrophilus* were adjusted to 5×10⁸ CFU/ml with fresh media. Fifty microliters of the bacterial culture were plated on mTSB plates. Sterile discs (6-mm Sensi-Discs, BD Bioscience, Sparks, MD, USA) were placed into wells of a microtiter plate and soaked with 0.03% hydrogen peroxide for 30 seconds. The soaked discs were then placed on top of each agar plate and incubated for 2 days at 37°C with 5% CO₂. Three biological replicates were performed. The area of the zone of inhibition was calculated from images using the SketchAndCalc online area calculator (<https://www.sketchandcalc.com>).

Assays for growth inhibition by *S. gordonii*

Wild type *A. actinomycetemcomitans* and *A. aphrophilus* and their mutants were grown in the liquid media specified above overnight at 37°C with 5% CO₂ on agars. The bacteria were harvested by centrifugation and resuspended and adjusted to 5×10⁸ CFU/ml in mTSB. *S. gordonii* was grown on blood agar plates (5% sheep blood in trypticase soy agar, Hardy Diagnostics) overnight at 37°C with 5% CO₂ and resuspended and adjusted to 5×10⁸ CFU/ml with mTSB. Fifty microliters of the cultures of *A. actinomycetemcomitans* or *A. aphrophilus* were plated onto sTSB agar plates. Sensi-Discs (BD Diagnostic Systems)

soaked with 15 μ l of *S. gordonii* were then placed individually on the agar surface and incubated at 37°C with 5% CO₂ for 2 days. Three biological replicates were performed. The areas of the inhibition zone and the zone with biofilm formed surrounding the discs were calculated as described above.

Statistical analysis

The paired Student's t-test (significance level $p < 0.05$) was used to test the statistical significance in the differences if the size of the inhibition zone associated with hydrogen peroxide or *S. gordonii* between the catalase-positive bacteria (wild type *A. actinomycetemcomitans* D7S-1 and mutant D7S-dkatC, and *A. aphrophilus* NJ-kat⁺) and the catalase-negative bacteria (wild type *A. aphrophilus* NJ8700 and *A. actinomycetemcomitans* D7S-dkat).

RESULTS

Natural competence of *A. aphrophilus*

Genomic DNA from rifampicin-resistant *A. aphrophilus* NJ8700-Rif^r was used as the donor DNA to screen for naturally competent *A. aphrophilus* strains. Five of the 13 *A. aphrophilus* strains exhibited a transformation frequency higher than 10⁻⁶ and at least 100-fold higher than the corresponding spontaneous mutation rate (Supplementary Table S2, see Appendix). Two strains each of *A. actinomycetemcomitans* and *A. aphrophilus* were subsequently selected in experiments to determine the specificity of the donor DNA for transformation. The results showed that the transformation frequency was very close between the 2 species. However, a slightly higher transformation frequency was noted for intra-species compared to inter-species transformations (Supplementary Table S3, see Appendix). The transformation frequencies of D7S-1 were reduced up to 500-fold in the presence of the genomic DNA from either *A. actinomycetemcomitans* or *A. aphrophilus*, but showed no apparent reduction in the presence of genomic DNA from *E. coli* (Supplementary Table S4, see Appendix). Similarly, the transformation frequencies of *A. aphrophilus* NJ8700 were reduced up to 35-fold in the presence of competing genomic DNA from D7S-1 or NJ8700, but not in the presence of genomic DNA from *E. coli*.

Hydrogen peroxide (H₂O₂) growth inhibition assays

NJ8700 of *A. aphrophilus* was selected for subsequent experiments because of its relatively high transformation frequency and the availability of the full genome sequence information (Di Bonaventura et al., 2009). The genetic constructs of the *katA*-expressing mutant (strain NJ-kat⁺) of *A. aphrophilus* NJ8700, the *katA*-deleted mutant (strain D7S-dkat) of *A. actinomycetemcomitans* D7S-1 and the *katA*-restored mutant (strain D7S-dkatC) of *A. actinomycetemcomitans* D7S-1 were verified by sequencing and exhibited the expected phenotype of catalase expression, and did not exhibit growth defects (See Materials and Methods).

The sensitivity of the bacteria to inhibition by hydrogen peroxide was assessed by two different methods. Bacteria without *katA* and incubated with hydrogen peroxide exhibited a greater reduction of viability than bacteria with *katA* (Supplementary Table S5, see

Appendix). Consistent with these results, bacteria without a functional *katA* showed a 3-fold wider zone of inhibition than strains with *katA* ($p < 0.05$) using the inhibition assay by discs containing 0.03% hydrogen peroxide (Table 2).

Growth inhibition by *S. gordonii*

Catalase has been shown to mediate the resistance of *A. actinomycetemcomitans* to inhibition by hydrogen peroxide producing species such as *S. gordonii* (Stacy et al., 2014). In this assay, we observed a ring of bacterial growth surrounding the disc, and an area devoid of visible bacterial colonies that represents the zone of inhibition. Strains lacking a functional *katA* showed 5- to 7-fold differences in the zone of inhibition in comparison to *katA*-positive bacteria (Figure 4 and Table 2). Gram staining of the bacteria in the thick rings suggested that the rings were comprised of *S. gordonii* with a few *A. actinomycetemcomitans* or *A. aphrophilus* cells (data not shown).

DISCUSSION

Horizontal gene transfer (HGT) has been shown to be a major driving force in the evolution of bacteria, resulting in a mosaic genomic structure consisting of discrete blocks of DNA from different sources (Lawrence & Ochman, 1998; Martin, 1999; Ochman, Lawrence, & Groisman, 2000; Whittam & Bumbaugh, 2002). In some instances, the distinctions between pathogenic and non-pathogenic bacteria have been explained by the acquisition of genomic islands by pathogenic bacteria. In this regard, the comparison between *A. actinomycetemcomitans* and *A. aphrophilus* serves as an example of two closely related oral species that evolved to use different strategies of oral adaptations. These two species are found in the same oral niches and share 65–80% of their gene content, which nearly overlaps with the range of 82–100% shared gene content among strains of *A. actinomycetemcomitans* (Kittichotirat et al., 2016). They also show collinearity in gene orders and readily identifiable insertion/deletion relationships in some genes that are found in one but not the other species. Interestingly, genes encoding leukotoxin and cytolethal distending toxins are carried on genomic islands specific to *A. actinomycetemcomitans*. Based on these observations, we hypothesized that the pathogenesis of *A. actinomycetemcomitans* can be attributed to the functions of a limited number of virulence genes that were acquired by the species via horizontal gene transfer. This study tested the feasibility of inserting and expressing an *A. actinomycetemcomitans*-specific *katA* in *A. aphrophilus*, which conferred the phenotype to the bacteria of resistance to hydrogen peroxide and growth inhibition by *S. gordonii*.

The selection of catalase for this study was based on the following considerations. Verification of the expression of catalase is relatively simple. The homologues of the neighboring genes of the catalase of *A. actinomycetemcomitans* and their gene order were readily identifiable in the genome of *A. aphrophilus*. Finally, catalase is a known virulence determinant of *A. actinomycetemcomitans* in the polymicrobial synergy of the organism and *S. gordonii* (Stacy, Abraham, Jorth, & Whiteley, 2016). Here we showed that wildtype *A. aphrophilus* was inhibited while the catalase-expressing *A. aphrophilus* mutant, similar to *A. actinomycetemcomitans*, demonstrated a greater resistance to *S. gordonii*. In our

experimental approach, the relationship and the noncoding region between the *gdhA* and *katA* in *A. actinomycetemcomitans* is reproduced in the genome of *A. aphrophilus*. Therefore there is no concern about the effect of the insertion of *katA* to the expression of *gdhA* in *A. aphrophilus*. The gene *gatA* (downstream of the inserted *katA*) encodes a homolog of glutamine amido-transferase, which does not appear to play a role in bacterial resistance to killing by hydrogen peroxide. Moreover, the resistance to hydrogen peroxide killing was determined based on the comparison between strains with and without catalase. Therefore, we concluded that the observed resistance to killing by hydrogen peroxide was attributed to catalase. Additional experiments are underway in an animal model to test whether catalase also enhances the virulence of *A. aphrophilus in vivo* in co-culture with *S. gordonii* similar to what has been observed for *A. actinomycetemcomitans* (Stacy et al., 2014).

The studies of *A. actinomycetemcomitans* virulence determinants often entail the cloning of a virulence gene in a plasmid and expressing the gene in *E. coli*. The drawbacks of such approaches include the influence of copy number of the plasmid vector to the expression of the virulence gene and the necessity of using antibiotics to maintain the plasmid. Moreover, *E. coli* and *A. actinomycetemcomitans* have evolved to adapt to vastly different environments. It may not be possible to use an *E. coli* that expresses a virulence determinant of *A. actinomycetemcomitans* in experiments that involve a polymicrobial setting, *in vitro* cell culture systems or *in vivo* animal models. In our approach, the catalase gene is stably integrated, does not require antibiotics to maintain its presence, and is inserted in a comparable locus to avoid the potential global effect to gene expression associated with gene location. Moreover, since *A. aphrophilus* and *A. actinomycetemcomitans* share similar oral ecological niches, the recombinant *A. aphrophilus* strains are expected to colonize and persist in the same oral sites. Single or multiple virulence determinants of the pathogenic *A. actinomycetemcomitans* may be transferred into the genome of *A. aphrophilus* creating double, triple, or quadruple mutants to examine how they affect virulence potentials *in vitro* and *in vivo*, of the recombinant strains.

The results of the transformation assays indicated a high degree of similarity in the characteristics of natural transformation between *A. actinomycetemcomitans* and *A. aphrophilus*. A number of species in the family *Pasteurellaceae*, including the two species in this study, are known to be naturally competent for DNA uptake (Bosse et al., 2009; Fujise, Lakio, Wang, Asikainen, & Chen, 2004; Maughan & Redfield, 2009a; Mell & Redfield, 2014; Redfield et al., 2006; Sinha, Mell, & Redfield, 2013). These species preferentially take up DNA that contains specific short sequences. Two distinct clades were identified within *Pasteurellaceae* based on protein coding genes (Redfield et al., 2006). Each clade has its own distinct consensus uptake signal sequences (USSs) represented by *H. influenza* (AAGTGCGGT) and *A. pleuropneumoniae* (ACAAGCGGT). A quick search confirmed that the NJ8700 strain has 1859 occurrences of AAGTGCGGT and its complementary sequence, but only 37 occurrences of ACAAGCGGT and its complementary sequence (unpublished observations). The same USS was identified and has been shown to mediate DNA uptake in *A. actinomycetemcomitans* (Wang et al., 2002). In this study, the preferential uptake of DNA with specific short sequences for *A. actinomycetemcomitans* and *A. aphrophilus* was verified by competition assays, in which the unmarked genomic DNA from

A. aphrophilus interfered with the DNA uptake of *A. actinomycetemcomitans*, and vice versa.

The transformability of *A. aphrophilus* strains varied in this study. Transformation is a complex process that requires the participation of 30 or more genes (Maughan & Redfield, 2009b). Strains of the same species may exhibit distinct transformation efficiencies (Maughan & Redfield, 2009a). It is not uncommon to find the poorly transformable strains to have genetic defects of competence genes (Maughan & Redfield, 2009a). We have previously found that serotype b and c strains of *A. actinomycetemcomitans* were non-competent for transformation (Fujise et al., 2004). These noncompetent serotype b and c strains shared a common feature of having an insertional inactivation of a competence gene *comM* by a genomic island (Kittichotirat, Bumgarner, Asikainen, & Chen, 2011). It is likely that poorly transformable *A. aphrophilus* strains may also have genetic defects in one or more of the competence genes.

Horizontal gene transfer may occur between strains of the same species, or between species. Kroll et al. (Kroll, Wilks, Farrant, & Langford, 1998) found two *H. influenzae* USSs 3' to the stop codon of *sodC* in *N. meningitidis*. The protein sequences of SodC in *N. meningitidis* and *H. influenzae* were more similar to each other than expected based on the phylogenetic relationship between these species. This was taken to suggest that this virulence gene, *sodC*, arose through horizontal transfer from *H. influenzae* to *N. meningitidis*. While in principle *A. actinomycetemcomitans* and *A. aphrophilus* may similarly engage in genetic exchange via natural transformation, such exchange is expected to be rare. For genetic exchange to succeed, the species must be in close proximity *in vivo*, the exchanged genes must not be deleterious to the recipient bacteria, and must confer critical advantages *in vivo* to become fixed in the genome and propagate in the population. To the best of our knowledge, there is no evidence for acquisition of genes from *A. actinomycetemcomitans* by *A. aphrophilus* by horizontal gene transfer, and vice versa.

In conclusion, this study demonstrates the feasibility of inserting genes of *A. actinomycetemcomitans* into the comparable loci in the genome of *A. aphrophilus*. The inserted catalase gene was expressed and conferred a new phenotype of resistance to *S. gordonii* in co-cultures. This novel genetic approach has several advantages over conventional methods for studies of the functions of *A. actinomycetemcomitans* virulence determinants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Bosse JT, Sinha S, Schippers T, Kroll JS, Redfield RJ, Langford PR. Natural competence in strains of *Actinobacillus pleuropneumoniae*. *FEMS Microbiol Lett*. 2009; 298(1):124–130. [PubMed: 19659732]
- Cheng YA, Jee J, Hsu G, Huang Y, Chen C, Lin CP. A markerless protocol for genetic analysis of *Aggregatibacter actinomycetemcomitans*. *J Formos Med Assoc*. 2014; 113(2):114–123. [PubMed: 24530245]
- Di Bonaventura MP, DeSalle R, Pop M, Nagarajan N, Figurski DH, Fine DH, et al. Complete genome sequence of *Aggregatibacter (Haemophilus) aphrophilus* NJ8700. *J Bacteriol*. 2009; 191(14):4693–4694. [PubMed: 19447908]
- Dogan B, Asikainen S, Jousimies-Somer H. Evaluation of two commercial kits and arbitrarily primed PCR for identification and differentiation of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus*. *J Clin Microbiol*. 1999; 37(3):742–747. [PubMed: 9986843]
- Fine DH, Markowitz K, Furgang D, Fairlie K, Ferrandiz J, Nasri C, et al. *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. *J Clin Microbiol*. 2007; 45(12):3859–3869. [PubMed: 17942658]
- Fujise O, Lakio L, Wang Y, Asikainen S, Chen C. Clonal distribution of natural competence in *Actinobacillus actinomycetemcomitans*. *Oral Microbiol Immunol*. 2004; 19(5):340–342. [PubMed: 15327649]
- Fujise O, Wang Y, Chen W, Chen C. Adherence of *Aggregatibacter actinomycetemcomitans* via serotype-specific polysaccharide antigens in lipopolysaccharides. *Oral Microbiol Immunol*. 2008; 23(3):226–233. [PubMed: 18402609]
- Kittichotirat W, Bumgarner RE, Asikainen S, Chen C. Identification of the pangenome and its components in 14 distinct *Aggregatibacter actinomycetemcomitans* strains by comparative genomic analysis. *PLoS One*. 2011; 6(7):e22420. [PubMed: 21811606]
- Kittichotirat W, Bumgarner RE, Chen C. Evolutionary Divergence of *Aggregatibacter actinomycetemcomitans*. *J Dent Res*. 2016; 95(1):94–101. [PubMed: 26420795]
- Kroll JS, Wilks KE, Farrant JL, Langford PR. Natural genetic exchange between *Haemophilus* and *Neisseria*: intergeneric transfer of chromosomal genes between major human pathogens. *Proc Natl Acad Sci U S A*. 1998; 95(21):12381–12385. [PubMed: 9770495]
- Lawrence JG, Ochman H. Molecular archaeology of the *Escherichia coli* genome. *Proc Natl Acad Sci U S A*. 1998; 95(16):9413–9417. [PubMed: 9689094]
- Martin W. Mosaic bacterial chromosomes: a challenge en route to a tree of genomes. *Bioessays*. 1999; 21(2):99–104. [PubMed: 10193183]
- Maughan H, Redfield RJ. Extensive variation in natural competence in *Haemophilus influenzae*. *Evolution*. 2009a; 63(7):1852–1866. [PubMed: 19239488]
- Maughan H, Redfield RJ. Tracing the evolution of competence in *Haemophilus influenzae*. *PLoS One*. 2009b; 4(6):e5854. [PubMed: 19516897]
- Mell JC, Redfield RJ. Natural competence and the evolution of DNA uptake specificity. *J Bacteriol*. 2014; 196(8):1471–1483. [PubMed: 24488316]
- Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. *Nature*. 2000; 405(6784):299–304. [PubMed: 10830951]
- Redfield RJ, Findlay WA, Bossé J, Kroll JS, Cameron ADS, Nash JHE. Evolution of competence and DNA uptake specificity in the Pasteurellaceae. *BMC Evolutionary Biology*. 2006; 6:82–82. [PubMed: 17038178]
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. 2. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press; 1989.
- Sinha S, Mell J, Redfield R. The availability of purine nucleotides regulates natural competence by controlling translation of the competence activator Sxy. *Mol Microbiol*. 2013; 88(6):1106–1119. [PubMed: 23663205]

- Slots J. Enzymatic characterization of some oral and nonoral gram-negative bacteria with the API ZYM system. *J Clin Microbiol.* 1981; 14(3):288–294. [PubMed: 7026598]
- Stacy A, Abraham N, Jorth P, Whiteley M. Microbial Community Composition Impacts Pathogen Iron Availability during Polymicrobial Infection. *PLoS Pathog.* 2016; 12(12):e1006084. [PubMed: 27973608]
- Stacy A, Everett J, Jorth P, Trivedi U, Rumbaugh KP, Whiteley M. Bacterial fight-and-flight responses enhance virulence in a polymicrobial infection. *Proc Natl Acad Sci U S A.* 2014; 111(21):7819–7824. [PubMed: 24825893]
- Tempro PJ, Slots J. Selective medium for the isolation of *Haemophilus aphrophilus* from the human periodontium and other oral sites and the low proportion of the organism in the oral flora. *J Clin Microbiol.* 1986; 23(4):777–782. [PubMed: 3700628]
- Wang Y, Goodman SD, Redfield RJ, Chen C. Natural transformation and DNA uptake signal sequences in *Actinobacillus actinomycetemcomitans*. *J Bacteriol.* 2002; 184(13):3442–3449. [PubMed: 12057937]
- Whittam TS, Bumbaugh AC. Inferences from whole-genome sequences of bacterial pathogens. *Curr Opin Genet Dev.* 2002; 12(6):719–725. [PubMed: 12433587]

APPENDIX

Supplementary information is included in Supplementary Tables S1–S5.

Highlights

- *A. actinomycetemcomitans* *katA* gene is functional in *A. aphrophilus*.
- The *katA* gene confers resistance to hydrogen peroxide and *S. gordonii*.
- No barrier exists for gene transfer between *A. actinomycetemcomitans* and *A. aphrophilus*.

Figure 1. The genetic locus of *kat* in *A. actinomycetemcomitans* D7S-1 and the comparable locus in *A. aphrophilus* NJ8700

The genes and their directions are represented by arrows drawn to scale. The gene names or gene identifiers (IDs) are provided under the arrows. The loci of interest are flanked by *gdhA* and *dnaE*. The locus in *A. aphrophilus* NJ8700 has an *A. aphrophilus*-specific *gdhA* and *gataA* in place of *katA*. The locus in *A. actinomycetemcomitans* has three other D7S-1-specific genes.

Figure 2. Genetic maps of pLox-Sp^F, pCAY-1, and pCAY-2 plasmids used to generate donor DNA *gdhA-katA-LoxP/Sp^F/LoxP-gatA* for the *katA*-expressing mutant of *A. aphrophilus*

The gene *gatA* from *A. aphrophilus* NJ8700 was PCR amplified and cloned into pLox-Sp^F to generate pCAY-1. The fragment of *gdhA* and *katA* amplified from *A.*

actinomycetemcomitans D7S-1 was cloned into pCAY-1 to generate pCAY-2. The fragment of *gdhA-katA-loxP/Sp^F/loxP-gatA* in pCAY-2 was subsequently used to generate the catalase-expressing *A. aphrophilus* mutant NJ-kat⁺.

Figure 3. Construction of *katA*-expressing mutant NJ-kat⁺ of *A. aphrophilus*, *katA*-deletion mutant D7S-dkat, and *katA*-restored mutant D7S-dkatC of *A. actinomycetemcomitans*
 Details of the methods are provided in Materials & Methods. Briefly, a) The fragment *gdhA*-*katA*-*loxP*/*Sp^r*/*loxP*-*gatA* was released from pCAY-2 by digestion with *EagI* and *ApaI*, purified and used as donor DNA to insert into the genome of the wildtype *A. aphrophilus* strain NJ8700 via allelic exchange by natural transformation. b) Fragments D7S_0074 and *gdhA* were individually PCR-amplified from genomic DNA of *A. actinomycetemcomitans* strain D7S-1, ligated to the *loxP*-*Sp^r*-*loxP* cassette, and then the ligation mixture was used as the donor DNA to transform strain D7S-1 to generate D7S-dkat. c) Fragments D7S_0074 and *katA*-*gdhA* were individually PCR-amplified from genomic DNA of strain D7S-1, ligated to the *loxP*/*Sp^r*/*loxP* cassette, and then the ligation mixture was used as donor DNA

for transformation to generate D7S-dkatC. The final step of the construction of the mutants depicted in panels a-c consisted of removing the Sp^r gene by recombination via Cre recombinase.

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Figure 4. Growth inhibition assays by *S. gordonii*

The 6-mm Sensi-Discs were soaked with *S. gordonii* and placed over plates for 1 day at 37°C in an aerobic chamber with 5% CO₂. Details of the methods are provided in Materials & Methods. Wildtype *A. actinomycetemcomitans*, *A. aphrophilus*, and *S. gordonii* were collected from agar, resuspended in fresh media and adjusted to 5×10^8 CFU/ml. Fifty microliters of the cultures of *A. actinomycetemcomitans* or *A. aphrophilus* were plated onto sTSB plates. Sterile discs soaked with 15 μ l of *S. gordonii* were then placed on the agar surface. The plates were incubated at 37°C with 5% CO₂ for 2–3 days. The results showed that *S. gordonii* inhibits the growth of the *A. actinomycetemcomitans* and *A. aphrophilus* strains without catalase, D7S-dkat and NJ8700, respectively. The size of the inhibition zone was larger for strains without *katA*.

Table 1

Bacterial strains and plasmids

Strains and plasmids	Property/relevant genotype	Source/reference
STRAINS		
<i>A. actinomycetemcomitans</i>		
ATCC29523	Laboratory strains	ATCC (Manassas, VA, USA) *
D7S-1	Wildtype <i>A. actinomycetemcomitans</i> , serotype a	Oral isolation (Chen et al., 2010)
D7S-Rif ^r	Rifampicin resistant	This study
D7S-dkat-Sp ^r	D7S-1 (<i>katA</i> ::Sp ^r)	This study
D7S-dkat	D7S-1 (<i>katA</i>)	This study
D7S-dkatC	D7S-1 (<i>katA</i> :: <i>katA</i>)	This study
<i>A. aphrophilus</i>		
NJ8700	Wildtype <i>A. aphrophilus</i>	Sequenced strain (Di Bonaventura et al., 2009)
NJ8700-Rif ^r	Rifampicin resistant	This study
NJ-kat ⁺ -Sp ^r	NJ8700:: <i>katA</i> -Sp ^r	This study
NJ-kat ⁺	NJ8700:: <i>katA</i>	This study
ATCC19415, ATCC29241, ATCC29242, ATCC33389, ATCC13252	Laboratory strains	ATCC
RMA1185, RMA4465, RMA7085, RMA9045, RMA12201, RMA12676, RMA12929	Clinical isolates	R. M. Alden Research Laboratory (Culver City, CA, USA)
<i>S. gordonii</i> Challis		
ATCC35105	Laboratory strains	Gift from Dr. Elaine Haase (SUNY Buffalo, Buffalo, NY, USA)
<i>E. Coli</i>		
DH5α	Host for cloning/expression vector pBluescript II KS ⁺	Promega BioSciences (San Luis Obispo, CA, USA)
PLASMIDS		
pBluescript II KS ⁺	Cloning vector, Amp ^r	Stratagene (San Diego, CA, USA)
pLox-Sp ^r	Amp ^r and Sp ^r in pBluescript II KS ⁺ , containing two <i>loxP</i> sites (spacer sequence: ATGGATGC) flanking the Sp ^r gene	Laboratory collection(Cheng et al., 2014)
PCAY-1	<i>gatA</i> (NJ8700_00310) from NJ8700 cloned into pLox-Sp ^r , Amp ^r and Sp ^r	This study
PCAY-2	<i>gdhA</i> (D7S_00738) and <i>katA</i> (D7S_00739) from D7S-1 cloned into pCAY-1, Amp ^r and Sp ^r	This study

* ATCC, American Type Culture Collection

Table 2

Growth inhibition assay with sterile discs (H₂O₂ and *S. gordonii*) by area

	Hydrogen Peroxide (H ₂ O ₂)			<i>S. gordonii</i>		
	Zone of inhibition (mm ²)	Zone of inhibition (mm ²)	p-value	Zone of inhibition (mm ²)	Zone of inhibition (mm ²)	p-value
Wildtype <i>A. actinomycetemcomitans</i> (D7S-1)	65.05	68.32		91.25	100.97	
<i>katA</i> -restored mutant of <i>A. actinomycetemcomitans</i> (D7S-dkatC)	78.39	76.25	0.159 ^a	162.17	151.28	0.107 ^a
<i>katA</i> -deletion mutant of <i>A. actinomycetemcomitans</i> (D7S-dkat)	219.96	230.33	0.001 ^{*a}	736.49	726.82	< 0.001 [*]
<i>katA</i> -expressing mutant of <i>A. aphrophilus</i> (NJ- <i>kat</i> ⁺)	75.19	72.23		126.2	138.20	
Wildtype <i>A. aphrophilus</i> (NJ8700)	221.92	233.15	0.029 ^{*b}	734.29	758.07	0.006 ^{*b}

* Denotes significant difference (p<.05);

^a compared with wildtype D7S-1.

Denotes significant difference (p<.05);

^b NJ-*kat*⁺ compared with wildtype NJ8700.