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Streptococcus gordonii pheromone s.g.cAM373 may influence the reservoir of antibiotic resistance determinants of *Enterococcus faecalis* origin in the oral metagenome

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Abstract

Streptococcus gordonii produces a pheromone heptapeptide, s.g.cAM373, which induces a conjugative mating response in *Enterococcus faecalis* cells carrying the responsive plasmid, pAM373. We investigated the extent of this intergeneric signaling on DNA acquisition by streptococcal species likely to cohabit oral biofilms. *E. faecalis*/pAM373/pAMS470 cells were incubated with synthetic s.g.cAM373, reverse peptide s.g.cAM373-R, or peptide-free medium and examined for their abilities to transfer plasmid DNA to streptococcal species in the presence of DNase. Preinduction of *E. faecalis* donors with s.g.cAM373 resulted in transconjugation frequencies in non-pheromone producing strains of *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus anginosus*, and *Streptococcus suis* that were significantly higher than frequencies when donors were preincubated with s.g.cAM373-R or medium alone. Peptide-mediated communication between commensal streptococci and *E. faecalis* carrying pheromone-responsive plasmids may facilitate conjugative DNA transfer to bystander species, and influence the reservoir of antibiotic resistance determinants of enterococcal origin in the oral metagenome.

Streptococcus gordonii are commensal members of the oral microbiota that play essential ecological roles in maintaining the community balance of bacterial species associated with health versus disease in the oral cavity. *Enterococcus faecalis*, although traditionally considered members of the gut microbiota, are also found in the oral cavities of healthy humans [1, 2]. Studies of oral *E. faecalis* have primarily focused on their role in persistent endodontic infections since these bacteria are consistently recovered from teeth with failed root canal treatment [3, 4]. However, enterococci are found on other oral surfaces [5] with higher carriage rates in medically compromised patients [6]. *E. faecalis* is widely-recognised as a medically-important opportunistic pathogen, particularly in nosocomial infections [7]. Its high degree of pathogenicity is attributed to the presence of multiple plasmids and mobile genetic elements that can carry genes conferring advantages for fitness, virulence, and antibiotic resistance within a single cell [8]. Representative species of *Enterococcus* are known to express resistance to virtually all known antibiotics [7]. Many *E. faecalis* conjugative plasmids, which are highly adapted for efficient horizontal gene transfer, respond to enterococcal pheromone peptides. These 7- or 8-amino acid active pheromones are specific for different families of conjugative plasmids and are processed from signal sequences of chromosomally-encoded lipoproteins [9]. In response to extracellular pheromone, *E. faecalis* cells carrying the cognate responsive plasmids (donors), exhibit a mating response that includes expression of genes encoding a surface aggregation substance (AS) protein that facilitates intercellular adhesion and conjugative transfer to pheromone-responsive-plasmid-free cells (recipients) [10, 11].

Members of the conjugative plasmid pAM373 family, some of which are known to carry vancomycin resistance determinants [12], are noteworthy because in addition to responding to *E. faecalis* pheromone cAM373 (e.f.cAM373), they respond to pheromone-like signals from at least three other species: *Staphylococcus aureus* [13], *S. gordonii* [14], and *Enterococcus hirae* [15]. We have previously identified the *S. gordonii* pheromone, *gordonii*-cAM373 (s.g.cAM373), a heptapeptide processed by lipoprotein signal peptidase II (LspA) and a zinc metalloprotease Eep (enhanced expression of pheromone) from the signal sequence of the lipoprotein, CamG [16]. *E. faecalis* cells carrying pAM373 or its derivatives express an aggregation substance (AS-373)-mediated clumping response to pheromone-containing

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Abbreviations: AS, aggregation substance; CSP, Competence Stimulating Peptide; Em, erythromycin; Fa, fusidic acid; Rf, rifampicin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; TH, Todd Hewitt.

One supplementary table and two supplementary figures are available with the online Supplementary Material.

filtrates of *S. gordonii* strains CH1 and G9B. Synthetic s.g. cAM373 induces conjugative plasmid transfer from *E. faecalis* cells carrying both pAM373 and a co-resident plasmid to *S. gordonii* recipients in filter matings [16]. Genetic analyses of *E. faecalis* clinical isolates recovered from patients with periodontal [17] and endodontic [18] diseases have identified determinants specific for pAM373-family plasmids. Recent studies of the oral metagenome support the concept that commensal oral bacteria may act as silent reservoirs for resistance-encoding genes [19]. Pheromone-mediated conjugative plasmid transfer is considered a critical mechanism for antibiotic resistance dissemination among enterococci [20] and potentially other members of the human gut microbiome [21]. The present studies were done to investigate the feasibility of a role for streptococcal-enterococcal pheromone-mediated communication in conjugative dissemination of DNA among members of the oral microbiome.

S. GORDONII S.G.CAM373-INDUCED CONJUGATIVE DNA TRANSFER IS INDEPENDENT OF RECIPIENT CELL COMPETENCE.

In addition to their ability to take up naked DNA by transformation via the competence response, *S. gordonii* cells can acquire plasmids and mobile genetic elements through conjugation (not necessarily requiring pheromone induction). Previous transcriptome analysis showed that neither the structural gene, *camG,* nor *eep* and *lspA,* essential for processing the pheromone precursor to active s.g.cAM373 [16], were differentially expressed in response to Competence Stimulating Peptide (CSP) [22], suggesting that upregulation of genes that facilitate pheromone-induced conjugation is independent of the competence response. We further investigated the independence of these two genetic mechanisms in *S. gordonii* CH1 by examining isogenic mutants with either a deletion of the DNA encoding s.g.cAM373 (strain CH1811 [16]; Table S1, available in the online Supplemental Material) or allelic replacement of the *comCDE* region required for competence with the *aad9* determinant encoding spectinomycin resistance (Sp^R) (strain CH9278; see Supplemental Material).

Strains CH1, CH1811 and CH9287 were incubated with heat-fixed horse serum or CSP in Todd Hewitt (TH) medium as previously described [22] and transformed with pVA749 [23], a replicative plasmid that carries an erythromycin resistance (Em^R) determinant. There were no significant differences in transformation frequencies between *comCDE*-positive strains CH1 and CH1811 (Table 1) indicating similar competence levels of the s.g.cAM373-positive and -negative strains, respectively.

A microtiter plate clumping assay [16, 24] was used to determine the AS-373-mediated clumping of *E. faecalis* cells carrying pAM373 or its derivatives, pAM4020 or pAM378 (Table S1) that was induced by two-fold dilutions of cell-free filtrates from CH1, CH9287 and CH1811 cultures. The intra-experiment equivalent titers (the most dilute filtrate that yielded macroscopically visible cell clumping) of strains CH1 and CH9278 (ranging from 16 to 32 for both *S. gordonii* strains) and the negative titers (less than 2) of control strain CH1811 [16] with all *E. faecalis* responders supported the independence of s.g.cAM373 expression and the competence response.

The *in vivo* function of s.g.cAM373 in facilitating conjugative transfer was examined in overnight filter matings using *E. faecalis* donors with the s.g.cAM373-positive strains CH1-S [spontaneous streptomycin resistant (Sm^R) derivative of CH1 with an identical titer in the clumping assay] and CH9287, and with the s.g.cAM373-negative strain CH1811-S (spontaneous Sm^R derivative of CH1811) recipients. An inherent limitation in measuring conjugative transfer of pAM373 to non-enterococcal recipients is that the pAM373 replicon is not known to function in non-enterococcal recipients; evidence indicates that pAM373 can transfer itself and carry additional DNA, but may not be maintained [14]. This limitation has been overcome by either the use of chimeric plasmids that carry a copy of the recipient replicon so that transferred plasmids were detectable in *S. aureus* transconjugants [15], or alternatively, by detecting transfer of a co-mobilisable plasmid as was done in *S. gordonii* [16]. We used the latter approach with pAMS470 [16], a mobilisable derivative of pVA749, known to be stable in a broad range of streptococcal species, to demonstrate pheromone-induced DNA transfer by using *E. faecalis* JH2-2/pAM378/pAMS470 as donors in filter mating experiments. Donors and recipients were grown to OD_{600} 1.0 (Spectronic 20D+spectrophotometer), mixed at a ratio of 1 : 10 and then pelleted by centrifugation (3000 ***g***). Donor and recipient cell mixtures were resuspended in 50 µl of fresh TH containing 10 units DNase (Thermo Fisher Scientific) to control for DNA acquisition by transformation [22]. The mating mixture was placed on a sterile 0.45 µm filter (Millipore Sigma) on a TH agar surface and incubated at 36 °C in 5 % CO_2 for 24 h. Cells were resuspended by vortexing the filter in TH, diluted, and plated on selective antibiotic agar to determine the c.f.u. of donors [chromosomal resistance to rifampicin (Rf^R) and fusidic acid (Fa^R) for strain JH2-2], recipients (Sm^R for strains CH1-S and CH1811-S or Sp^R for strain CH9287), and putative streptococcal transconjugants that had acquired pAMS470 (Sm^R+Em^R or Sp^R+Em^R). The mating results (Table 1) suggest that endogenous s.g.cAM373 from the pheromone-positive strains CH1-S and CH9278 induced co-resident plasmid transfer from *E. faecalis* donors, whereas frequencies were significantly lower with the s.g.cAM373-negative strain CH1811-S. The similar transconjugation frequencies for *S. gordonii* CH1-S and the competence-deficient strain CH9278 (P=0.35 Student's t-test) in the presence of DNase supported the independence of these conjugation and transformation mechanisms.

Table 1. Plasmid DNA acquisition by *S. gordonii* via transformation and transconjugation

S. gordonii strain	Transformation frequency*	Transconjugation frequency†
CH1	$2.0\times10^{-3}\pm2.1\times10^{-4}$	–
CH1-S	–	$6.3\times10^{-6}\pm5.7\times10^{-6}$
CH9278	$<9.0\times10^{-8}\pm1.8\times10^{-8}$‡	$9.3\times10^{-6}\pm1.1\times10^{-5}$
CH1811	$2.3\times10^{-3}\pm1.2\times10^{-3}$	–
CH1811-S	–	$4.2\times10^{-8}\pm7.0\times10^{-8}$§

*Number of c.f.u. of transformants ml^{-1} (Em^R) divided by the number of c.f.u. ml^{-1} of cells made competent with serum or CSP [22] and transformed with 3.5 µg pVA749 [23] DNA ml^{-1}. *N*>2 independent biological replicates. Similar frequencies were seen for both serum and CSP competence induction.

†24 h filter mating of JH2-2/pAM378/pAMS470 donors with *S. gordonii* recipients in the presence of DNase. Average (number of c.f.u. of transconjugants [Sm^R Em^R for CH1-S and CH811-S; Sp^R Em^R for CH2978] divided by the number of c.f.u. of donors)±S.D.; *N*=3 independent biological replicates.

‡Mean log values different from strains CH1 and CH1811 ($P\leq0.006$, Student's *t*-test).

§Mean log values different from strains CH1-S and CH9278 ($P\leq0.017$, Student's *t*-test).

THE S.G.CAM373 PEPTIDE INDUCES DNA TRANSFER FROM *E. FAECALIS* TO A RANGE OF NONPHEROMONE-PRODUCING STREPTOCOCCAL SPECIES

We have previously shown that synthetic s.g.cAM373 peptide induces co-resident plasmid transfer from *E. faecalis* to *S. gordonii* recipients in filter matings [16]. To investigate the potential role of s.g.cAM373 in horizontal DNA transfer in the oral microenvironment where *S. gordonii* and *E. faecalis* co-exist in a multispecies community, representative strains of the commensal species *Streptococcus sanguinis,* the pyogenic species *Streptococcus anginosus* [25], as well as the cariogenic species *Streptococcus mutans,* were selected to determine their abilities to act as recipients for conjugatively transferred DNA from s.g.cAM373-induced *E. faecalis* donors. To further examine the scope of these signaling interactions, the emerging zoonotic pathogen *Streptococcus suis* which also may acquire antibiotic resistance in multispecies oropharyngeal communities [26, 27] was included. Recipient strains were verified for an absence of endogenous cAM373-like activity as indicated by an inability of *E. faecalis* cells carrying pAM373, or its derivatives pAM378 or pAM4020 to respond to their filtrates in the clumping assay. Pheromone-free streptococcal strains were then sequentially passaged to acquire the Sm^R phenotype to facilitate their selection in filter matings. Donor *E. faecalis* JH2-2/pAM378/pAMS470, as well as control JH2-2/pAMS470 (without pheromone-responsive plasmid) were grown to OD_{600} 1.0, diluted to OD_{600} 0.6 and preincubated for 90 min at 36 °C 5 % CO_2 with 50 ng ml^{-1} of the synthetic peptides (>90 % purity; GenScript) s.g.cAM373 (SVFILAA) or reverse pheromone s.g.cAM373-R (AALIFVS), or an equivalent volume of TH broth. Recipient *S. gordonii* strains CH1-S and CH9278, *S. sanguinis* SK36-S, *S. anginosus* ATCC 33397-S, *S. mutans* ATCC 25175-S, and *S. suis* ATCC 43765-S were grown to OD_{600} 1.0 and mixed with the pre-induced *E. faecalis* cells in donor to recipient ratios of 1 : 10 or 1 : 100. Filter matings were performed as above for two hours to minimize potential effects of confounding bacterial interactions. Transconjugants were plated on Sm+Em or Sp+Em agar and co-resident plasmid transconjugation frequencies were calculated.

In matings with all recipient strains, *E. faecalis*/pAM378/pAMS470 donors that were preincubated with s.g.cAM373 peptide had transconjugation frequencies that were at least two to three orders of magnitude higher than when donors were pre-incubated with either the s.g.cAM373-R peptide or medium alone (Table 2). As expected, control experiments with *E. faecalis*/pAMS470 donors that did not carry a pAM373 derivative did not yield transconjugants. To confirm the presence of transferred plasmid, DNA was purified from donors and randomly selected transconjugants lysed with 20 mg lysozyme ml^{-1} and 25 units mutanolysin ml^{-1} using a plasmid purification kit (Qiagen). Purified plasmids were digested with *Hin*dIII and visualized by agarose gel electrophoresis and ethidium bromide staining (Fig. S1). All selected Em^R transconjugants carried stably-maintained pAMS470 DNA indicating that the co-resident plasmid was successfully transferred in the filter matings in the presence of DNase. None of the transconjugants carried plasmid DNA bands that corresponded to pAM378 indicating that this plasmid was not stably maintained in any of the streptococcal recipients. Although replication of pAM373 has not been observed in non-enterococcal species, pAM378 may deliver a plasmid-borne transposon which may subsequently be maintained, presumably in the recipient chromosome [16, 28]. Therefore, one hundred randomly-selected transconjugants from each mating were picked to tetracycline (Tc) agar to determine maintenance of the Tc^R determinant on Tn*918* carried on pAM378. Although we did not detect Tc resistance in any of the pAMS470-carrying transconjugants (Table 2), this may be attributable to the low frequency of this event or the short duration of our matings.

BIOLOGICAL AND MEDICAL IMPLICATIONS

Our results support a model that in multi-species communities where bacteria grow in close proximity as occurs in biofilms, streptococcal peptides such as s.g.cAM373 may

Table 2. Pheromone-induced co-resident plasmid transfer

Recipient strain	Transconjugation frequencies of pAMS470 from JH2-2/pAM378/pAMS470 donors*		
	Donors incubated with s.g.cAM373†	Donors incubated with s.g.cAM373-R	Donors incubated with TH medium
S. gordonii CH1-S	$4.0\times10^{-4}\pm1.1\times10^{-4}$	$<1.1\times10^{-8}\pm5.7\times10^{-9}$	$<4.2\times10^{-8}\pm4.4\times10^{-8}$
S. gordonii CH9278	$4.1\times10^{-4}\pm1.3\times10^{-4}$	$<1.6\times10^{-8}\pm1.1\times10^{-8}$	$<1.3\times10^{-8}\pm7.4\times10^{-9}$
S. sanguinis SK36-S	$7.9\times10^{-4}\pm1.1\times10^{-3}$	$<1.2\times10^{-8}\pm1.4\times10^{-8}$	$<3.2\times10^{-8}\pm4.0\times10^{-8}$
S. anginosus ATCC 33397 s	$2.4\times10^{-4}\pm2.2\times10^{-4}$	$<1.1\times10^{-8}\pm7.1\times10^{-9}$	$<1.4\times10^{-7}\pm2.1\times10^{-7}$
S. mutans ATCC 25175 s	$2.1\times10^{-6}\pm2.0\times10^{-6}$	$<6.3\times10^{-9}\pm7.3\times10^{-9}$	$<2.3\times10^{-8}\pm2.5\times10^{-8}$
S. suis ATCC 43765 s	$2.6\times10^{-6}\pm4.9\times10^{-6}$	$<6.3\times10^{-9}\pm7.3\times10^{-9}$	$<1.3\times10^{-8}\pm1.4\times10^{-8}$

*Average [number of c.f.u. of transconjugants (Sm^R Em^R or Sp^R Em^R) divided by the number of c.f.u. of donors (Rf^R Fa^R)]±S.D.; *N*>3 independent biological replicates. Selected Em^R transconjugants from each mating were incubated for 48 h at 37 °C on 25 µg Rf ml^{-1}+25 µg Fa ml^{-1} agar to confirm their sensitivity; none grew on Rf+Fa, thereby ruling out Sm^R or Sp^R *E. faecalis* donor breakthrough. No selected transconjugants grew after 48 h incubation at 37 °C on 10 µg Tc ml^{-1} agar indicating that the Tc^R determinant from pAM378 had not been stably maintained.

†Mean log values for all strains are significantly different when compared to the cAM373-R ($P\leq0.02$) and medium alone ($P\leq0.007$) values for each streptococcal strain. Negative control matings of s.g.cAM373-induced JH2-2/pAMS470 donors with each recipient strain resulted in no transconjugants (frequencies<3.3×10^{-9}) for all strains.

induce *E. faecalis* cells carrying pheromone-responsive plasmids to conjugatively transfer DNA to neighbouring non-pheromone producing recipient species. Genome database searches have identified a number of oral streptococcal species that encode s.g.cAM373 in the signal sequences of CamG-like lipoproteins (Fig. S2) indicating the prevalence of this signaling molecule. In the present studies, the potential recipients were limited to members of the genus *Streptococcus* so that the co-resident plasmid, used as a molecular tool to monitor mating-response-related conjugation, could be readily detected. *In vivo,* conjugatively acquired DNA could include stable plasmids, or alternatively, if co-transferred plasmids cannot replicate independently in the new recipient, the DNA could be incorporated into the chromosome or additional co-resident plasmids via homologous recombination or independent mechanisms encoded within many mobile genetic elements [20]; the absence of stable plasmids in any bacterial isolate does not mean that it did not acquire DNA conjugatively. *In vivo,* s.g.cAM373-induced *E. faecalis*/pAM373 cells could transfer DNA to both Gram-positive and Gram-negative recipients. Based upon nucleotide sequence similarities of promoters and structural genes and comparison of codon usage with the host chromosome, it is highly probable that transfer of antibiotic resistance determinants from Gram-positive to Gram-negative readily occurs [29]. Although not demonstrated in the present studies, in addition to antibiotic resistance determinants, conjugatively acquired DNA may carry genes that contribute to the fitness or virulence of recipient cells. Even if such conjugative events occur at relatively low frequency *in vivo*, they may confer genetic plasticity that remains quiescent, but could become clinically relevant when oral dysbiosis occurs due to systemic disease or antibiotic treatment [30]. Since enterococci are known to harbour a multitude of resistance and virulence determinants, factors such as s.g.cAM373 that could facilitate genetic dissemination among members of the oral microbiota may be highly significant genetic signals. Novel antibiotic resistance determinants have been found among commensals in human gut metagenomic studies [21]; *E. faecalis* has been postulated to play a pivotal role in this genetic exchange [20]. Similar biological potential exists in the oral microbiome. In addition to facilitating DNA transfer, streptococcal pheromones may contribute to *E. faecalis* carriage and pathogenicity. Pheromone-induced AS proteins that are functionally similar to AS-373 affect biofilm structure [31] which may influence bacterial colonisation, and have been implicated as virulence factors in endocarditis [32]. AS proteins have also been implicated in immune system evasion by increasing enterococcal survival in polymorphonuclear leukocytes [33] and human macrophages [34]. Through actions as benign as chewing, oral bacteria may be dispersed into the bloodstream, and in rare but significant cases, both streptococci and enterococci can cause serious systemic complications such as infective endocarditis [35]. All the recipient streptococcal species that acquired DNA from s.g.cAM373-induced *E. faecalis* cells in the present studies have been associated with endocarditis and/or abscess formation. The induction of AS-373 by s.g.cAM373 and the accompanying potential for acquisition of additional resistance and virulence determinants by commensal bacteria from *E. faecalis* could make both donors and recipients more difficult to eradicate in oropharyngeal, cardiac, or other systemic infections.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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