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Effect of *Veillonella parvula* on the antimicrobial resistance and gene expression of *Streptococcus mutans* grown in a dual-species biofilm

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Introduction: Our previous studies showed that *Streptococcus mutans* and *Veillonella parvula* dual-species biofilms have a different acid production profile and a higher resistance to chlorhexidine than their single-species counterparts. The aim of the current study was to test whether the susceptibility of *S. mutans* grown in the presence of *V. parvula* is also decreased when it is exposed to various other antimicrobials. Furthermore, the aim was to identify other changes in the physiology of *S. mutans* when *V. parvula* was present using transcriptomics.
Methods: Susceptibility to antimicrobials was assessed in killing experiments. Transcript levels in *S. mutans* were measured with the help of *S. mutans* microarrays.
Results: When *V. parvula* was present, *S. mutans* showed an increase in survival after exposure to various antimicrobials. Furthermore, this co-existence altered the physiology of *S. mutans*. The expression of genes coding for proteins involved in amino acid synthesis, the signal recognition particle-translocation pathway, purine metabolism, intracellular polysaccharide synthesis, and protein synthesis all changed.
Conclusion: Growing in a biofilm together with a non-pathogenic bacterium like *V. parvula* changes the physiology of *S. mutans*, and gives this bacterium an advantage in surviving antimicrobial treatment. Thus, the study of pathogens implicated in polymicrobial diseases, such as caries and periodontitis, should be focused more on multispecies biofilms. In addition, the testing of susceptibility to currently used and new antimicrobials should be performed on a multispecies microbial community rather than with single pathogens.

Key words: biofilm; resistance; species interaction

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Streptococcus mutans is considered one of the primary cariogenic pathogens present in dental plaque. It has been studied extensively as a monoculture grown in suspension. In recent years, the majority of the studies on *S. mutans* have focused on monoculture biofilms, because the natural habitat of *S. mutans*, dental plaque, is a biofilm. Dental plaque is a multispecies community from which hundreds of species have been isolated. Therefore, instead of studying monocultures, it would be more realistic to study the properties of *S. mutans* grown in the presence of other bacteria, especially because other studies have shown that the presence of a second bacterium may influence the gene expression, virulence, and other properties of the other bacterium in the culture (10, 11, 29).

A method that is frequently used to obtain an overview of the changes in

physiology in bacteria after a certain stimulus is transcriptional profiling with the help of microarrays. Microarrays have been used to study the difference in gene expression between *S. mutans* grown in suspension and in biofilm (32), changes in gene expression in mutant strains (16, 24, 40), and for comparative genome hybridization of *S. mutans* strains (39).

Up to now, *S. mutans* microarrays have not been used to study physiological changes in response to the presence of another bacterium. Some reasons for this could be that it is difficult to grow two species with different nutrient requirements together, that the results of the dual-species analyses have to be compared with analyses of the two single species, which triples the number of samples, and that it needs to be verified that the sample of the one species does not bind to the microarray of the other species.

Veillonella parvula lives in the same complex multispecies plaque community as *S. mutans*. It cannot ferment glucose and most other sugars (9, 28), but it metabolizes lactic acid, a waste product of *S. mutans*, and converts it into weaker acids, such as acetic and propionic acid, which have a reduced ability to solubilize enamel (30). For this reason, it is considered a benevolent plaque organism.

Previously, we studied the effect of *V. parvula* on *S. mutans* grown in a dual-species biofilm. The presence of *V. parvula* influenced the acid production profile of the *S. mutans* in the dual-species biofilm. Furthermore, both *V. parvula* and *S. mutans* grown in dual-species biofilm showed increased survival (18, 19) and were able to regrow faster than bacteria grown in single-species biofilms after exposure to the antimicrobial chlorhexidine (19). Therefore, we decided to study the interaction between these two plaque bacteria in more detail.

The first aim of the current study was to determine whether *V. parvula* and *S. mutans* grown together in dual-species biofilms are more resistant to antimicrobials with a mode of action related to that of chlorhexidine and antimicrobials with a mode of action completely different from that of chlorhexidine. For this purpose the survival of bacteria in the dual-species biofilm was compared with the survival of bacteria in single-species biofilms after exposure to five antimicrobials with different modes of action: (3, 8, 31): cetylpyrimidinium chloride (CPC), zinc chloride, erythromycin, hydrogen peroxide, and amine chloride, the chloride salt of Olaflur. The second aim was to use transcriptional profiling with the help of microarrays to determine what other changes in the physiology of *S. mutans* occur in response to the presence of *V. parvula*.

Materials and methods

Bacterial strains and growth conditions

Forty-eight-hour single-species *S. mutans* UA159 biofilms, single-species *V. parvula* DSM 2008 (DSMZ) biofilms, and dual-species biofilms with both *S. mutans* and *V. parvula* were grown as described previously (19) on the bottom of 96-well polystyrene microtiter plates (19) (antimicrobial treatment) or on the bottom of polystyrene culture plates (23) (RNA isolation). Medium was refreshed after 24 h.

Antimicrobial treatment

The following antimicrobials were used: amine chloride [bis(hydroxyethyl)-aminopropyl-*N*-hydroxyethyl-octadecylamine dihydrochloride; GABA International (Therwil, Switzerland); 0.014%], zinc chloride (0.1 mmol/l), CPC (VWR International [Fontenay Sous Bois, France]; 0.2 mmol/l), erythromycin (Sigma-Aldrich, St Louis, MO; 0.015 g/l) and hydrogen peroxide (Merck [Darmstadt, Germany]; 2.5%). Killing experiments were performed in triplicate as described previously ('neutralized killing') (19), with the following exceptions: biofilms were exposed to antimicrobial for 5 min instead of 10 min and after exposure to amine chloride, zinc chloride, and erythromycin cysteine peptone water (19) was used as a neutralizer. Percentage survival was calculated by dividing the viable counts of a treated sample by the average of the viable counts of the control samples. To test the hypothesis that the survival of *S. mutans* or *V. parvula* was not influenced when they co-existed with each other, we compared the survival percentage of a species grown in single-species biofilms to the same species' survival percentage in dual-species biofilms. For this purpose a permutation test was used (35) ($\alpha \leq 0.05$). We used this non-parametric test because normality could not be assumed.

RNA isolation

Forty-eight-hour biofilms were harvested as follows: the medium was poured off the polystyrene plates, 15 ml fresh warmed (37°C) medium was added and the biofilm cells were removed from the surface by swabbing. The resulting suspension was centrifuged once (15 min, 3939 ***g***, 37°C) and the liquid was removed. The pellets were resuspended in 1 ml RNAPro solution (FastRNA Pro Blue Kit, MP Biomedicals [Illkirch, France]), snap frozen in liquid nitrogen and stored at –80°C until further use. Total RNA was extracted by bead beating followed by the steps described in the protocol of the FastRNA kit, and a subsequent step using Trizol (Invitrogen [Breda, The Netherlands]) followed by a DNase treatment with RNase-Free DNase. Isolated total RNA was further purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). Quantification of total RNA was performed in a NanoDrop ND-100 UV/VIS spectrometer (Nanodrop Technologies, Wilmington, DE). RNA quality was validated using the RNA 6000 NanoAssay on an Agilent 2100 BioAnalyzer (Agilent Technologies, Amstelween, The Netherlands) and by recording the ribosomal RNA ratios (28S : 18S) and the RNA Integrity Number (RIN). For each biofilm type, five biological replicates were used to isolate total RNA and to hybridize with microarrays.

Microarrays, target preparation, and hybridization

Microarrays specific for *S. mutans* were obtained from the Pathogen Functional Resource Centre (TIGR). The libraries represent a total of 1948 oligonucleotides and 500 controls printed in fourfold. The oligonucleotide library was printed with a Lucidea Spotter (Amersham Pharmacia Biosciences, Uppsala, Sweden) on commercial Epoxy slides (Schott Nexterion, Jena, Germany) and processed according to the manufacturer's instructions. Total RNA samples were hybridized, according to a common reference design without dye swap, with a pool of test samples as common reference (containing equal amounts of dual-species RNA, *S. mutans* single-species RNA and *V. parvula* single-species RNA). In a common reference design the fluorescence intensity of the experimental sample is compared to that of the common reference on the same slide, which makes an accurate comparison between the slides possible. From the total RNA samples with RIN-value >7, 15 μg (single-species samples) or 30 μg (dual-species samples and common reference sample) was labeled using a random primed procedure with SuperScript II Reverse Transcriptase (Invitrogen), dCTP Cy5 (experimental samples), and dCTP Cy3 (common reference) according to the manufacturer's instructions. A doubled

amount (30 μg) of RNA sample was used for the dual-species samples because we assumed that only half of the RNA from these samples would bind to the slide, because *V. parvula* RNA hardly hybridized to the *S. mutans* microarray slide (see below) and because we assumed that approximately half of each dual-species sample consisted of *S. mutans* mRNA. This last assumption was based on microscopic cell counts and was confirmed by the fact that there were no major differences in overall intensity levels between the slides hybridized with dual-species samples and *S. mutans* single-species samples. We normalized for the modest difference in overall intensity between dual-species slides and *S. mutans* single-species slides using lowess-normalization (see below). Labeled complementary DNA (cDNA) was assessed for the amount of incorporated label using a NanoDrop ND-100 UV/VIS spectrometer. The microarrays were hybridized overnight with 200 μl hybridization mixture, consisting of 50 μl Cy3- and Cy5-labeled cDNA, 100 μl formamide and 50 μl 4 × Micro-Array Hybridization Buffer (Amersham Pharmacia Biosciences) at 37°C, washed in an Automated Slide Processor (Amersham Pharmacia Biosciences), and subsequently scanned in an Agilent DNA MicroArray Scanner (Agilent Technologies).

Data analysis and statistics

Microarray spot intensities were quantified using FEATURE EXTRACTION software (version 8.5). Data were processed further using R [version 2.2.1 (27)] and BIOCONDUCTOR (http://www.bioconductor.org/) MAANOVA package (version 0.98.8). All slides were subjected to quality control checks. Quality checks included visual inspection of the scans, examining the consistency among the replicated samples by principal components analysis, testing against criteria for signal to noise ratios, for consistent performance of the labeling dyes, consistent pen performance, and visual inspection of pre- and postnormalized data with box plots and Ratio-Intensity (RI) plots. The quality checks revealed that the RNA from *V. parvula* samples only resulted in background expression values caused by non-specific binding. Further analysis was therefore focused on the difference between samples from *S. mutans* biofilms and *S. mutans* + *V. parvula* biofilms. After log2 transformation, the data were normalized using a spatial lowess smoothing procedure and statistically analyzed using a two-stage mixed analysis of variance (ANOVA) model (20, 43). First, array, dye and array-by-dye effects were modeled globally. Subsequently, the residuals from this first model were fed into the gene-specific model to fit biofilm and spot effects on a gene-by-gene basis using a mixed model ANOVA. These residuals are reported as expression values. To test the hypothesis that *S. mutans* genes were not influenced when *S. mutans* co-existed with *V. parvula*, a permutation based F1 test was applied, which allows the assumption that the transcript data are normally distributed to be relaxed (6). To account for multiple testing, *P*-values from the permutation procedure were adjusted to represent a false discovery rate (FDR) of 5% (2). An overview of pathways (and protein complexes) that contained one or more of the differentially expressed genes was constructed with the help of database mining and gene ontology. The pathway complexes were related to differences in gene expression through gene set enrichment analysis on the F1 statistics (25), using the gene-set-test facility in the bioconductor package LIMMA. Only the pathways that showed a significant change are mentioned in the Results section. Microarray data have been deposited in the ARRAYEXPRESS database (http://www.ebi.ac.uk/aerep?) with experiment accession number E-MEXP-1034.

Results

S. mutans UA159 and *V. parvula* were grown separately in single-species biofilm and together in dual-species biofilms for 48 h. All biofilms contained 3×10^8 to 10^9 colony-forming units/cm^3. In a previous study with *S. mutans* C180-2, we exposed these three types of biofilms to chlorhexidine and found that the bacteria in the dual-species biofilms survived better than the bacteria in the single-species biofilm (19). Similar results were found for *S. mutans* UA159 exposed to chlorhexidine (data not shown). In the present study, the bacteria in the single-species biofilms and dual-species biofilms were exposed to five other antimicrobials with different modes of action.

V. parvula survived erythromycin treatment better when *S. mutans* was present (Fig. 1). The results for survival of *V. parvula* after exposure to hydrogen peroxide are not reliable because the counts were below the detection limit, and are therefore not shown. The average percentage of *S. mutans* that survived

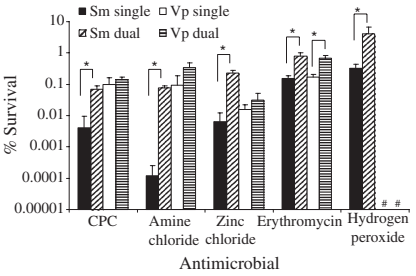

Fig. 1. Percentage survival of single- and dual-species biofilm bacteria after exposure to 0.2 mmol/l CPC, 0.014% amine chloride, 0.1 mmol/l zinc chloride, 0.015 g/l erythromycin and 2.5% hydrogen peroxide. Sm single, *Streptococcus mutans* in single-species biofilm; Sm dual, *S. mutans* in dual-species biofilm; Vp single, *Veillonella parvula* in single-species biofilm; Vp dual, *V. parvula* in dual-species biofilm. 100% survival values (average ± SD) are $2.5 \pm 0.93 \times 10^8$ (Sm single), $2.8 \pm 0.55 \times 10^8$ (Sm dual), $3.9 \pm 2.2 \times 10^8$ (Vp single) and $4.1 \pm 1.3 \times 10^8$ colony-forming units/well (Vp dual). *Significant difference between single-species and dual-species biofilm viable counts of the same bacterium ($P = 0.05$). #Value below the detection limit (Vp single, 0.08%; Vp dual, 0.11%). Error bars represent the standard deviation.

antimicrobial treatment was higher in dual-species biofilm than in single-species biofilm ($P = 0.05$) for all five antimicrobials used. Thus, *S. mutans* growing in these dual-species biofilms was better able to survive exposure to the six antimicrobials than *S. mutans* growing in single-species biofilm.

These findings suggested that there are differences in physiology between *S. mutans* grown in single-species biofilm and *S. mutans* grown in dual-species biofilm together with *V. parvula*. To gain more insight into these differences we compared, with the help of *S. mutans* microarrays, the transcript levels in *S. mutans* grown alone directly with those of *S. mutans* grown with *V. parvula*. This direct comparison was possible because hybridization of the *V. parvula* samples to the *S. mutans* microarrays only resulted in background expression values caused by non-specific binding (Fig. 2). The distribution of the spot fluorescence intensities of *S. mutans* microarrays hybridized with a *V. parvula* sample was similar to that of spots on the microarrays that did not contain oligonucleotides (empty spots).

Analysis showed that in *S. mutans* the transcript levels of 15 genes were significantly ($P < 0.05$) higher in the presence of *V. parvula* than in the absence of *V. parvula* and 19 were lower (Tables 1 and 2). Of the 15 upregulated genes, seven

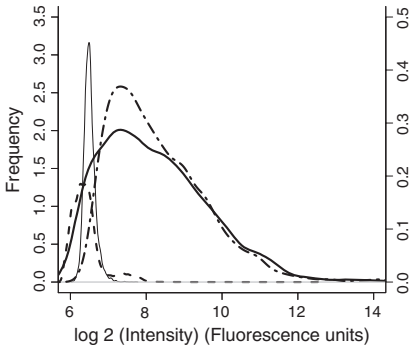

Fig. 2. Frequency plots of the intensities of the spots from an arbitrary microarray hybridized with a *Streptococcus mutans* sample (black thick line, right-hand *y*-axis), from an arbitrary microarray hybridized with *S. mutans* and *Veillonella parvula* dual-species sample (dashed-dotted line, right-hand *y*-axis), from empty control spots on the microarrays (dashed line, left-hand *y*-axis), and from an arbitrary microarray hybridized with *V. parvula* sample (black thin lines, left-hand *y*-axis). Empty spots are spots that deliberately do not contain oligonucleotides. The *x*-axis indicates the $\log_2$ of the Cy5 fluorescence signal and the *y*-axes indicate the relative concentration of spots with the corresponding fluorescence intensity.

encoded ribosomal proteins and one encoded trigger factor (*ropA*, *SMU.91*), a ribosome-associated chaperone (17, 21). The transcript levels of an additional four genes encoding ribosomal proteins were higher in *S. mutans* grown in dual-species biofilm than in *S. mutans* grown in single-species biofilm at a lower significance level ($P < 0.10$). Gene-set enrichment analysis showed that one specific group of 23 genes encoding for ribosomal proteins was upregulated ($P = 2 \times 10^{-5}$, data not shown). Furthermore, almost all (47 out of 51) ribosomal protein gene transcript levels were higher in *S. mutans* grown in dual-species biofilm than in *S. mutans* grown in single-species biofilm (data not shown).

Another upregulated gene coded for PurN (*SMU.35*), which is involved in purine synthesis. Gene-set enrichment analysis showed that the expression of the genes of the entire purine synthesis pathway (*SMU.29*, *SMU.30*, *SMU.32*, *SMU.34*, *SMU.35*, *SMU.37*, *SMU.48*, *SMU.50*, *SMU.51*, *SMU.59*, and *SMU.268*) in which L-glutamine and 5-phosphoribosyl 1-pyrophosphate are joined and converted to adenosine 5′ monophosphate, increased ($P = 0.035$). *SMU.33*, a gene that encodes for a hypothetical protein and lies between two of the genes coding for enzymes of the purine synthesis pathway, was also significantly upregulated.

Two of the 19 downregulated genes are involved in amino acid metabolism: ThrC (*SMU.70*), which catalyzes the conversion of *O*-phospho-L-homoserine to L-threonine and GdhA (*SMU.913*), which converts L-glutamate to 2-oxoglutarate and ammonia. In addition, the transcript level of the gene encoding GlnA (*SMU.364*), the enzyme that converts L-glutamine to L-glutamate and ammonia, was down 10.3-fold ($P = 0.31$, data not shown).

In *S. mutans* that was grown in dual-species biofilm, expression of the peptide chain release factor 3 gene (*pfrC*, *SMU.608*) was significantly decreased. PfrC is involved in translation, but is a dispensable factor (38).

Glycogen in *S. mutans* is called intracellular polysaccharide (IPS). In addition to *glgC* (*SMU.1538*), which showed a significant decrease in expression, other genes involved in IPS metabolism (36) were also downregulated: *SMU.1535* ($P = 0.126$), *SMU.1536* ($P = 0.092$), *SMU.1537* ($P = 0.062$), and *SMU.1539* ($P = 0.104$). Gene-set enrichment analysis showed that the expression of *SMU.1535* to *SMU.1539* together decreased significantly ($P = 0.0091$). *SMU.1565*, another significantly downregulated gene, codes for a putative 4-α-glucanotransferase (synonym of amylomaltase). In *S. mutans* it may be involved in the production of IPS from maltose (33, 44).

Discussion

In a previous study we have shown that the susceptibility to chlorhexidine of *S. mutans* and *V. parvula* grown in biofilm decreases in each other's presence. The current study shows that this is also true when these biofilms are exposed to other antimicrobials. *V. parvula* was able to survive erythromycin treatment better in dual-species biofilm than in single-species biofilm. Survival after exposure to the hydrogen peroxide concentration that was used to kill *S. mutans* was too low to be detected, as would be expected for a bacterium with a very low oxygen tolerance. *S. mutans* was better able to survive

Table 1. Overview of gene transcripts upregulated dual-species biofilms[1]

Locus[2]	Definition[3]	Gene name[3]	Fold change	*P*-value
Translation: ribosomal proteins: synthesis and modification				
SMU.2017c	50S ribosomal protein L14	*rplN*	2.2	0.041
SMU.2018c	30S ribosomal protein S17	*rpsQ*	2.0	0.033
SMU.2019c	50s ribosomal protein L29	*rpmC*	1.9	0.045
SMU.2023c	30S ribosomal protein S19	*rpsS*	2.8	0.034
SMU.2024c	50S ribosomal protein L4	*rplD*	2.9	0.041
SMU.2025c	50S ribosomal protein L3	*rplC*	2.9	0.041
SMU.2026c	30S ribosomal protein S10	*rpsJ*	2.8	0.030
Cellular processes: protein and peptide secretion				
SMU.91	Peptidyl-prolyl isomerase, trigger factor	*ropA*	1.3	0.046
Purines, pyrimidines, nucleosides, and nucleotides: purine ribonucleotide biosynthesis				
SMU.35	Putative phosphoribosylglycinamide formyltransferase	*purN*	1.6	0.046
Unassigned				
SMU.641	Oxidoreductase	*qor*	2.3	0.034
SMU.679	Oxidoreductase, aldo/keto reductase family	*ycgG*	1.4	0.046
Unknown				
SMU.543	Conserved hypothetical protein	–	1.4	0.027
Hypothetical				
SMU.33	Hypothetical protein	–	1.7	0.048
SMU.642	Hypothetical protein	–	2.3	0.034

[1]Gene transcripts in *S. mutans* grown together with *V. parvula* were compared to gene transcripts of *S. mutans* grown in single-species biofilms (fold-change ≥1.3, $P < 0.05$).
[2]GenBank locus tag.
[3]http://www.oralgen.lanl.gov/.

Table 2. Overview of gene transcripts downregulated dual-species biofilms[1]

Locus[2]	Definition[3]	Gene name[3]	Fold change	*P*-value
Amino acid biosynthesis: aspartate family				
SMU.70	Threonine synthase	*thrC*	6.1	0.017
Amino acid biosynthesis: glutamate family				
SMU.913	NADP-specific glutamate dehydrogenase	*gdhA*	4.0	0.042
Protein synthesis: tRNA aminoacylation				
SMU.158	Cysteinyl-tRNA synthetase	*cysS*	4.2	0.030
SMU.773c	Lysyl-tRNA synthetase	*lysS*	4.3	0.032
SMU.1311c	Asparaginyl-tRNA synthetase	*asnS*	3.7	0.035
Protein synthesis: protein modification				
SMU.466	Cysteine aminopeptidase C	*pepC*	6.7	0.029
Protein synthesis: translation factors				
SMU.608	Peptide chain release factor 3	*prfC*	2.0	0.034
Protein fate: protein and peptide secretion and trafficking				
SMU.744	Cell division protein; signal recognition particle	*ftsY*	2.1	0.030
Transport and binding proteins: ABC superfamily: ATP-binding protein				
SMU.803c	ABC transporter, ATP-binding protein	*ykhF*	2.1	0.041
SMU.1120c	Sugar ABC transporter, ATP-binding protein	*psaA*	6.6	0.047
Energy metabolism: sugars				
SMU.1538c	Glucose-1-phosphate adenylyltransferase	*glgC*	1.6	0.046
SMU.1565c	4-α-glucanotransferase	*malM*	7.1	0.013
Energy metabolism: fermentation				
SMU.1021	Citrate lyase alpha chain	*citF*	3.4	0.032
Cellular processes: cell division				
SMU.1003	Glucose-inhibited division protein	*gidA*	6.6	0.020
Cellular processes: adaptations to atypical conditions				
SMU.1060c	Signal recognition particle	*ffh*	6.3	0.039
Cellular processes: toxin production and resistance, transport and binding proteins; other				
SMU.71	MATE efflux family protein	–	3.5	0.046
Unassigned				
SMU.1693c	Uncharacterized hemolysin	–	4.1	0.046
Unknown				
SMU.575c	Conserved hypothetical protein	–	5.3	0.045
SMU.862	Conserved hypothetical protein	–	2.4	0.034

[1]Gene transcripts in *S. mutans* grown together with *V. parvula* were compared to gene transcripts of *S. mutans* grown in single-species biofilms (fold-change ≥1.3, $P < 0.05$).
[2]Genbank locus tag.
[3]http://www.oralgen.lanl.gov/.

antimicrobial exposure when grown in dual-species biofilm than in single-species biofilm. This was not only true after exposure to antimicrobials with a mode of action related to that of chlorhexidine (CPC and amine chloride), something that may have been expected, but was even true after exposure to antimicrobials with a completely different mode of action (hydrogen peroxide, erythromycin, and zinc chloride). An increased resistance to antimicrobials has also been found in other mixed-species biofilms compared to single-species biofilms (4, 12, 34).

Our results suggest that *V. parvula* changes the physiology of *S. mutans*. Recent studies on the behavior of multi-species communities have shown that the presence of one or more bacteria can change the virulence and gene expression in other pathogens. Animal experiments with pathogenic *Pseudomonas aeruginosa* and avirulent oropharyngeal flora showed that avirulent strains enhance lung damage by *P. aeruginosa* (10). Strains of *Veillonella* and *Lactobacillus* were shown to decrease the expression of *aggR*, which codes for a global transcriptional regulator of enteroaggregative *Escherichia coli* virulence factors, whereas *Enterococcus faecalis* and *Clostridium innocua* were shown to increase the expression of this gene (29). A study on the interaction between *Streptococcus gordonii* and *Veillonella atypica* showed that *S. gordonii* increases its expression of the α-amylase-encoding gene *amyB* in the presence of *V. atypica* (11).

In the current study, comparative analysis of transcript levels in *S. mutans* growing in dual-species biofilm and in single-species biofilm showed that the physiology of this bacterium is indeed changed by the presence of *V. parvula*. Quite some differences were found, even though these cells were in stationary phase which may make it more difficult to detect changes. According to our results the most important changes that occurred when *V. parvula* was present were those listed below.

1 The signal recognition particle-translocation pathway genes *ftsY* and *ffh* (14) were downregulated. FtsY and Ffh are needed for normal growth at low pH in *S. mutans* (14, 15). However, in our previous experiments we have shown that the overall pH of the bulk medium is constant for the three types of biofilms (19), because of the presence of a buffer. Apparently, *ftsY* and *ffh* can also be controlled by regulators that are independent of pH fluctuations.

2 Changes occurred in the amino acid metabolism. We concluded this from the lower expression of enzymes involved in aspartate and glutamate/glutamine metabolism, and from the lower expression of *pepC*, which shows 85% identity with the thiol aminopeptidase *pepC* from *Streptococcus thermophilus* (5). Aminopeptidases are believed to contribute to the final degradation of short peptides. An explanation for the change in amino acid metabolism could be that the amino acid concentration of the outside medium was higher because *V. parvula* breaks down proteins extracellularly to amino acids. *V. parvula* has previously been shown to degrade various proteins extracellularly (42). *S. mutans* could take up these amino acids, perhaps even specifically glutamine

and aspartate, and therefore would need to synthesize fewer amino acids by itself. This may also explain why *pepC* is expressed less: there would be less need for breakdown of small peptides in the cell, because they would already have been broken down to amino acids extracellularly. Glutamine and glutamate are also important for other cellular processes, such as amine metabolism, cell wall synthesis, pyrimidine and purine synthesis, and oxidation and reduction. However, there was no indication that a change in these processes took place when *V. parvula* was present, because the transcript levels of the genes coding for the other enzymes involved in these pathways did not show a significant change or a change in ratio (data not shown). The only exception to this was the change in purine metabolism.

3 Purine metabolism was upregulated, which would coincide nicely with the presumed higher concentration of glutamine in the cell, because this amino acid is necessary for the first step of purine synthesis.

4 IPS metabolism was downregulated. *S. mutans* is known to produce IPS from sugars such as glucose and sucrose, which can serve as metabolic substrates for acid production during periods of carbohydrate limitation (13, 37). The downregulation of this metabolism means that either less IPS was made in the dual-species biofilm in this stage of growth or less IPS was broken down. The latter probability seems the most plausible because in stationary phase there is probably a sugar shortage and so no synthesis of IPS. *S. mutans* growing in dual-species biofilm used less of the IPS, possibly because the cells needed less energy for synthesis of amino acids, and for transport of lactate out of the cell. *V. parvula* consumes the lactic acid that is produced by *S. mutans*, which results in a lower lactic acid concentration in the medium after 48 h for dual-species biofilms than for single-species biofilms (19).

5 The most prominent change that took place in the dual-species biofilm was that expression of a large number of genes coding for ribosomal proteins, and ropA, a ribosome associated chaperone (17, 21), were upregulated. This indicates the presence of more ribosomes. Similarly, Len *et al*. (22) found that all detected ribosomal proteins were upregulated when planktonic *S. mutans* cells were grown under low pH stress. Studies on related gram-positive bacteria have shown similar changes in gene expression patterns in response to antimicrobial exposure. In *Streptococcus pneumoniae*, *Bacillus subtilis*, and *Enterococcus faecalis* the relative transcript levels of the majority of the genes that code for ribosomal proteins increase in response to the presence of various antibiotics (1, 7, 26). The decrease in transcript levels of the genes coding for amino acyl tRNA synthetases that was found in the current study also occurred in *E. faecalis* exposed to erythromycin and in *S. pneumoniae* and *B. subtilis* growing in the presence of various antibiotics (1, 7, 26). Also in accordance with our results is the increase in relative transcript amounts of the genes involved in purine metabolism in *S. pneumoniae* exposed to four antibiotics (26). Apparently, *S. mutans* responds to the presence of *V. parvula* in a manner similar to the response of other gram-positive bacteria to antibiotics.

We speculate that with a higher number of ribosomes present, cells exposed to antimicrobials may be better able to synthesize new proteins, a mechanism that is necessary for repair of the damage caused by the antimicrobials. A finding that could support this theory is the involvement of ropA in the resistance of *S. mutans* to hydrogen peroxide (41). However, whether the statistically significant 1.3-fold upregulation of *ropA* also makes a physiologically significant difference is not clear. The proposed theory to explain the increased survival rate of *S. mutans* after antimicrobial exposure in the presence of *V. parvula* needs to be tested in future studies.

In conclusion, growing in a biofilm together with a non-pathogenic bacterium like *V. parvula* changes the physiology of *S. mutans*. The expression of genes coding for proteins involved in amino acid metabolism, IPS metabolism, the signal recognition particle-translocation pathway, purine metabolism, and protein synthesis changed. Furthermore, the presence of *V. parvula* gives the cariogenic *S. mutans* an advantage in surviving antimicrobial treatments. These findings show that in dental plaque the presence of *V. parvula* cannot solely be considered as an advantage. Besides its beneficial property of lactate consumption and conversion to less cariogenic acids, *V. parvula* could have an adverse effect. It can increase the survival of *S. mutans* after exposure to antimicrobials that are intended to reduce *S. mutans* numbers in dental plaque.

This study shows that the presence of another bacterium can change the phenotype of a pathogen and can increase its resistance to antimicrobials. Thus, the study of pathogens implicated in polymicrobial diseases, such as caries and periodontitis, should be focused more on multispecies biofilms, and the testing of the susceptibility to the currently used and new antimicrobials should be performed on a multispecies microbial community rather than with single pathogens.

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