Title

The effect of environmental variables in the human oral cavity on the balance between epiparasite TM7x and its host bacterium Actinomyces odontolyticus strain XH001.

Author

Fujii, Alison

Publication Date

2016

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The effect of environmental variables in the human oral cavity on the balance between epiparasite TM7x and its host bacterium *Actinomyces odontolyticus* strain XH001.

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Microbiology, Immunology, and Molecular Genetics

by

Alison Fujii

2016

ABSTRACT OF THE THESIS

The effect of environmental variables in the human oral cavity on the balance between epiparasite TM7x and its host bacterium *Actinomyces odontolyticus* strain XH001.

by

Alison Fujii

Master of Science in Microbiology, Immunology, and Molecular Genetics

University of California, Los Angeles, 2016

Professor Wenyuan Shi, Chair

TM7x is a recently cultivated bacterium of the phylum Candidatus Saccharibacteria (formerly TM7). TM7x is an ultrasmall bacterium and an obligate parasite that attaches to the surface of its bacterial host, *Actinomyces odontolyticus* strain XH001 (XH001). This episymbiotic pair are native to human subgingival plaque and display dynamic interaction during *in vitro* cultivation. Furthermore, an inverted relationship in abundance between Saccharibacteria and *Actinomyces* has been documented in healthy and periodontal conditions, implicating their association with periodontal status. In this thesis, I examine the impact of variable physiological factors associated with periodontitis, and other potential changes in the human oral cavity on

the population dynamics of in vitro grown TM7x /XH001 episymbiont. Hydrogen peroxide as well as high concentration of sodium chloride and potassium chloride were found to reduce the amount of TM7x and result in increased relative abundance of TM7x-free XH001 in the *XH001* / TM7x co-culture. In addition, fetal bovine serum increased the amount of TM7x attachment on XH001 cells to the point where XH001 growth was severely inhibited. Furthermore, XH001 monocultures are more resistant to alteration of their preferred microaerobic environment, while heat shock (at 42°C) did not shift the population balance between XH001 and TM7x. These data may lead us toward further understanding the environmental and host factors which modulate the balance between this parasite and its bacterial host.

The thesis of Alison Fujii is approved.

Peter Bradley

Kent Hill

Wenyuan Shi, Committee Chair

University of California, Los Angeles

2016

Table of Contents

Acknowledgments

I would like to acknowledge Batbileg Bor, Xuesong He, Luija Cen, and my graduate advisor Wenyuan Shi, in the assistance in preparation for my thesis. Luija Cen assisted me with laboratory work, while Batbileg Bor, Xuesong He, and Wenyuan Shi offered advice, guidance, and editing assistance.

Figures 1 and 2 include as-yet unpublished material from the manuscript in preparation, Establishment of novel epiparasitic relationship between TM7x and Actinomyces odontolyticus actinosynbacter (XH001), by Batbileg Bor, Alejandro Serrato, Alison Fujii, Lujia Cen, Melissa Agnello, Jeffrey S. McLean, Wenyuan Shi, and Xuesong He

Introduction:

Bacteria surround us; they are in our environments, in our homes, in our food, in our pets, and even in us. While many of us are accustomed to thinking of bacteria as germs that make us sick, the majority of bacteria cause us no harm, and many are actively beneficial: they help us digest food, protect us from pathogenic bacteria, and even maintain a healthy immune system (1-3). The communities of bacteria that live in and on our bodies are often complex ecosystems with hundreds to thousands of different microbial species (4). This complexity may lend the microbiome resilience, but it also creates an obstacle to our understanding of diseases caused by the disruption of these ecosystems (5). Because we cannot attribute a single pathogen to the development of many diseases such as inflammatory bowel disease, dental caries, and periodontitis (6-8), we must look at the entirety of the microbial community's members.

Though culture-independent sequencing and metagenomic techniques have done a great deal to reveal the makeup of the human microbiome, our understanding is far from complete because many members have never been cultivated and studied in the laboratory (9-12). Without the ability to study these species in the laboratory, the physiological properties of uncultured microbes remain unknown.

One clue as to why some species have never been successfully cultivated lies in a member of the ultra-small phylum Candidatus Saccharibacteria, formerly Candidate Phylum TM7. Culture-independent sequencing has revealed that Saccharibacteria are found ubiquitously in natural environments such as soil and seawater, as well as in many human body sites (13-16). Members of Saccharibacteria have small genomes of around 1000 kb and lack biosynthetic pathways that would be expected in free-living bacteria (13, 17). Until recently,

they have not been successfully cultivated in laboratory conditions. The Shi group at UCLA was the first to culture a phylotype of TM7, and dubbed it TM7x (18). He et al. isolated TM7x, an ultra-small, 700-kb genome TM7 phylotype from the human oral cavity, finding its growth required the presence of another oral bacteria, *Actinomyces odontolyticus* strain XH001 (XH001). The reduced genome and lifestyle of TM7x indicates that other TM7 phylotypes as well as many other uncultured bacteria may be obligate symbionts: only be cultivable with support from other species.

While TM7x cannot grow without XH001, the reverse is not true of its partner. Monocultures of TM7x-free XH001 have been successfully cultivated (18). Though the XH001/TM7x co-culture has stable growth that can be maintained indefinitely with both partners persisting in culture, XH001 grows faster and is able to reach a higher cell density when it is in monoculture than when it is associated with TM7x (19). Under nutrient restricted conditions, this trend is exacerbated: TM7x appears to cause extra harm to its host, with XH001 losing viability much faster than in monoculture (18). XH001 monoculture is able to grow to a greater cell density than the XH001/TM7x co-culture, and when attached by TM7x, XH001 up-regulates key stress-response genes (19), which is consistent with the proposed parasitic relationship between the two partners.

Actinomyces odontolyticus strain XH001 has variable morphology, found as short rods, long rods, or in branched, hyphal form (19). When in monoculture, XH001 cells have a smooth surface, whereas when co-cultured with TM7x, XH001 cells appear bumpy, due to the surface attachment of TM7x cocci, forming a “grapes on a vine” structure (18, 19). TM7x has thus been characterized as a parasitic epibiont and its XH001 as its host basibiont. We see from this

previous work by Bor et al. and He et al., that there is a dynamic interaction between these two species and can be modulated by laboratory conditions. It is not just in lab where this interaction might have an affect, but also in their natural habitat: the human microbiome.

Human mucosal inflammatory diseases are often dysbiotic in nature; caused by a shift in the microbial community from one which is well tolerated, to one which is pathogenic (20, 21). Candidatus Sachharibacteria is commonly overrepresented in diseased microbial communities, such as bacterial vaginosis, inflammatory bowel disease, and periodontitis (22-25). Periodontitis is an inflammatory gum disease characterized by the deepening of gingival sulcus, the space between the gums and the tooth, into a periodontal pocket (26). A previous study sampling human oral subgingival plaque, 97% of samples showed presence of TM7 (25). While the majority of people have oral Saccharibacteria, in healthy oral communities, the proportion of Saccharibacteria is generally only ~1% of the total microbial community. Metagenomic analyses comparing healthy communities to periodontial communities reveal an association between periodontitis and a higher proportion of Saccharibacteria in the subgingival plaque, up to ~20% of total microbial load (24, 27). Somewhat contrarily, *Actinomyces* prevalence is associated with healthy gingiva (25). Periodontitis is considered a disease of dysbiosis; it is not associated with a single pathogen, but rather a shift in the subgingival microbial community. The same species are present in both health and in disease, but the relative proportion of each tend to be altered disease as compared to health. As there are hundreds of microbial species in the oral cavity, the details of all these interactions are still unclear, but understanding the dynamics of inter-microbial and host factors are key toward furthering our understanding and the development of therapies to prevent and treat periodontitis and other diseases of dysbiosis.

In periodontitis, a physically deeper pocket develops between the tooth and the gum. This occurs in a cycle of increased microbial load, increased microbial diversity, the dominance of certain species (28), and host immune response, including inflammation and extravasation of immune cells from the tissues and into the gingival sulcus, which can ultimately result in bone loss and the further deepening of the periodontal pocket (26, 29). The progression of periodontitis proceeds as a balance between host, microbial, and environmental factors. TM7x is a member of a candidate phylum, Saccharibacteria, associated with periodontitis, while its host, *Actinomyces odontolyticus,* is associated with periodontal health. We chose to explore the interaction between these two oral symbionts within the frame of physiological oral environmental changes, with a focus on conditions related to periodontitis.

The conditions examined were hydrogen peroxide concentration, high salt and high osmotic concentration, long-term nutrient deprivation, presence of serum, and heat shock.

We examined the effect of hydrogen peroxide on XH001 and TM7x, as hydrogen peroxide naturally occurs in the oral cavity from host and microbial sources, as well as is commonly introduced via oral health products such as toothpaste or mouthwash. Macrophages use hydrogen peroxide to fight pathogens, but periodontitis has been reported to be associated with a decrease in macrophage activation (30). Some bacteria produce hydrogen peroxide. Notably, periodontitis is associated with a reduction in hydrogen peroxide producing bacteria, such as *Streptococcus sanguinis* (28, 31).

XH001 and TM7x were tested in culture medium with added salt. The salt concentration in the gingival crevicular fluid is higher than that of saliva, but lower than blood. This variable

may also be impacted by the depth of the pocket in periodontitis, as well as increased exudate and bleeding, which often accompany periodontal disease (26, 28).

While the gingival sulcus is low in oxygen, it is not completely anaerobic (26). Additionally, as the plaque is a three dimensional and heterogeneous biofilm, there are varying concentrations of O_2 within plaque (32, 33). In deep periodontal pockets, oxygen concentration drops even further than the healthy gingival sulcus (26). XH001 monoculture and XH001/TM7x co-culture were tested in anaerobic, microaerobic, and anaerobic conditions.

Materials and Methods:

Growth conditions. Planktonic cultures of *Actinomyces odontolyticus* strain XH001 monoculture and XH001/TM7x co-culture were grown at 37°C in brain heart infusion (BHI) medium in microaerobic conditions, unless otherwise noted. Planktonic cultures were grown for 24 hours then passaged to partially mimic continuous culture where nutrient is not the limiting factor. Passages were performed by diluting previous culture into fresh BHI medium at a variable total volume, but at a 1:10 volume split ratio, unless otherwise noted.

Incubation conditions. Cultures were grown at 37°C under either aerobic (ambient air, ~21% O_2, 0.04% CO_2, 0.9% Ar, 78% N_2), microaerobic (2.6% O_2, 5% CO_2, balanced with N_2), or anaerobic (0% O_2, 5% CO_2, 5% H_2, balanced with N_2) conditions.

Media preparation. The appropriate amount sodium chloride, potassium chloride, mannitol, or hydrogen peroxide were dissolved in previously autoclaved BHI medium and then sterile

filtered through a 0.22 µm syringe filter (Millipore). Fetal bovine serum at 10%, and non-essential amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine), were added to RPMI media (Life Technologies). Media was incubated for at least 24 hours in the appropriate oxygen concentration environment prior to culture inoculation.

Culture growth and TM7x attachment assessment. Phase contrast imaging was used to visually assess the amount of TM7x attachment on the host cells, XH001. Cells were directly observed and imaged using Nikon Eclipse E400 microscope equipped with a Nikon Plan Fluor ×100/1.30 oil immersion objective.

Also used as an indicator of amount of TM7x attachment was colony morphology, with irregularly shaped colonies used as a proxy for TM7x-attached XH001, and round smooth colonies used a proxy for TM7x-free XH001 (note: TM7x alone has not been found to form colonies without XH001). Colonies were plated at varying serial dilutions from $1/10^0$ – $1/10^7$, incubated for three days in microaerobic conditions at 37°C, and then visually assessed for morphology using a light microscope.

Total culture (both XH001 monoculture as well as XH001/TM7x co-culture) growth and health was assessed by optical density (OD) using 600nm wavelength spectrophotometric reader, and colony forming units (CFU). Plates for colonies were 5% sheep blood 1.5% agar in Brain Heart Infusion (BHI) medium.

PCR. TM7x detection was performed via PCR amplification. We used TM7x-specific 16S primers (400F: 5'- TATGAGTGAAGAATATGAC -3' and 1110R: 5'-CAGTCCAAGTAGAAAAATAC -3'). Each 50-µL PCR assay contained 100 ng of purified genomic DNA, 40 pmol of each primer, 200 µM each dNTP, 4.0 mM MgCl2, 5 µL of 10× PCR buffer, and 2.5 units of Taq DNA polymerase (Invitrogen). Cycling conditions were 96 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min, with a final extension period of 5 min at 72 °C. The resulting PCR products were evaluated by 1% agarose gel electrophoresis.

Results:

TM7x-associated and TM7x-free XH001 exhibit different colony morphologies. When in co-culture with TM7x, not all XH001 cells are TM7x-attached (Figure 1), but quantifying attachment, in terms of how many XH001 cells are free of TM7x and how many are TM7x-attached, has previously been elusive. However, recent work I assisted with, led by Dr. Batbileg Bor of the Shi research group, has developed a method to quantify the portion of XH001 cells attached by TM7x. This method relies on the visual assessment of agar-plated colonies grown from XH001/TM7x co-culture. While XH001 monoculture almost exclusively forms smooth, round colonies when plated on BHI blood agar plates, the XH001/TM7x co-culture shows a mixed population of colony morphologies: some round with smooth edges as seen in the XH001 monoculture, and others with jagged edges and irregular shapes (Figure 2A). We termed these "regular" and "irregular" colonies, respectively. I performed colony PCR on 20 individual colonies of each morphology type to detect the presence of TM7x. Of the irregular colonies, 95% were positive for TM7x, while only 15% of the regular colonies were positive for TM7x

(Figure 2B). This finding has led us to use colony morphology as a reasonable proxy for proportion of TM7x attachment within a mixed population.

Hydrogen peroxide decreases TM7x in XH001/TM7x co-culture. To assess the effect of hydrogen peroxide on XH001 and TM7x, mono- and co-cultures were grown in a range of hydrogen peroxide concentrations from 0 to 5 mM, in BHI, under microaerobic conditions at 37C. Increased hydrogen peroxide concentration led to overall inhibition of bacterial growth as indicated by the reduction in CFU, with a concentration of 2 mM or higher resulting in no colony growth. While total CFU/mL had an inverse correlation with H_2O_2 concentration, the percentage of irregular colonies also decreased with increased H_2O_2 concentration (Figure 3A), indicating that there were fewer TM7x-associated XH001 cells at higher H_2O_2 concentrations. This dose-dependent effect was observed after 24-hour incubation.

To investigate the effect of hydrogen peroxide on TM7x attachment over a longer exposure period, XH001 monoculture as well as XH001/TM7x co-culture were grown in 0.5 mM H_2O_2 for six days, passaged at a 1:10 ratio and plated for CFU and colony morphology each day. While the CFU/mL stayed relatively stable over the experiment, the percentage of irregular colonies in the co-culture was reduced from 73% irregular at day 0, to 4% at day 6 (Figure 3B). Interestingly, the lowest CFU counts for the co-culture occurred during the days in which the irregular colony percentage was decreasing. At those same time points, the XH001 monoculture did not have reduced CFU, indicating that the TM7x-free XH001 cells may have been less sensitive to the hydrogen peroxide condition than the TM7x-attached cells.

High salt concentration reduces TM7x attachment. To test whether high salt concentrations affect the balance between XH001 and TM7x, XH001 monoculture and XH001/TM7x co-culture were grown in BHI alone, BHI supplemented with 250 mM NaCl, or 250 mM KCl, or 500 mM mannitol as no-salt condition with the same osmolarity. Phase contrast images of XH001/TM7x co-cultures indicate that high salt medium reduces the amount of TM7x attachment, while mannitol does not (Figure 4A). Cultures in their respective salt conditions were grown for 5 days, and passaged each day. Samples were plated each day for CFU and colony morphology assessment. In the XH001/TM7x co-culture, the percentage of irregular colonies in the 250 mM NaCl and 250 mM KCl media dropped from a starting point of 31% at time zero, to 12% and 3% after one day, respectively (Figure 4B). By the end of the five-day experiment, there were no detectable irregular colonies in either high-salt condition. The BHI-only and BHI + mannitol, by contrast, maintained a high proportion of irregular colonies throughout the experiment.

To test whether the TM7x had been completely eliminated from the co-culture, I performed PCR to detect TM7x on the passage 5 cultures. No TM7x was detected in the high-salt co-cultures, but it was detected in the BHI and mannitol co-culture samples (Figure 4C).

The CFU results show some fluctuation in both the monoculture and the co-culture, but there is no strong trend to indicate that high salt had an extreme effect on the growth of XH001. The CFU for the co-culture in the mannitol treatment was the lowest. This result is also accompanied by the observation that under phase contrast, there sometimes appears to be greater TM7x attachment and greater distended morphology of the XH001 cells than in the BHI only sample (data not shown) although the irregular colony proportion is not significantly higher.

A shorter term study was conducted over a 24 hour time frame in order to elucidate details in the changes of TM7x attachment that occur during the lag, log, and stationary growth cycle. Mono- and co-culture were inoculated into BHI only, BHI + 250 mM NaCl, BHI + 250 NaCl, or 500 mM mannitol. With samples plated for CFU and colony morphology at time 0, 6, 12, 18, and 24 hours post inoculation (Figure 4D). The trend seen over this 24-hour time course is consistent with that of the five-day experiment (Figure 4B). All cultures experienced increased CFU over the experiment, as expected. Co-cultures in the mannitol and BHI-only conditions had increased irregular colony percentage over time, while the NaCl and KCl conditions had decreased irregular colony percentage. Notably, the strongest trend in terms of colony irregularity was the mannitol co-culture: colony irregularity percentage increased steadily over the time course, which is consistent with my observations of TM7x over-attachment on XH001 by phase contrast imaging (Figure 4A).

Although the high salt conditions in this five-day experiment indicate that TM7x is entirely eliminated (Figure 4B), this may not necessarily be the case. In a similar experiment, I grew the XH001 mono- and XH001/TM7x co-cultures in the various salt conditions for seven passages, and then passaged them into BHI only. After 6 more passages in BHI, I performed PCR to detect TM7x (Figure 4E). In all three biological replicates of co-culture in BHI + 250 mM KCl, TM7x was absent. However, in two of three biological replicates of co-culture in BHI + 250 mM NaCl, TM7x was amplified. It may be the case that a small number of TM7x cells were able to persist throughout the initial seven passages in high concentration NaCl, then able to expand once normal salt concentration medium was available. Overall, the phase contrast, colony

morphology, and PCR data indicate that high salt reduces TM7x preferentially while having minimal effect on its host.

TM7x and XH001 both persist under long-term nutrient depletion. In nutrient replete conditions, with passaging of culture at a 1:10 ratio into fresh media each 24 hours, the balance between TM7x and XH001 remains relatively stable. Over a ten-day experiment, the amount of TM7x attachment fluctuated between ~40-75% irregular colony proportion (Figure 5A) In 2015, He et al. (18) demonstrated that in nutrient-depleted conditions (96 hours post-inoculation with no new media added), XH001 cells co-cultured with TM7x lose viability to a greater degree than those in monoculture.

In order to test what happens to the balance between XH001 and TM7x under longer-term nutrient depletion conditions, I inoculated XH001 monoculture and XH001/TM7x co-culture in BHI and incubated them in microaerobic conditions at 37°C for 36 days without adding fresh media. Samples were plated for colony morphology at days 1, 3, 7 and 10. Over the ten days with no nutrient addition, the percent of irregular colonies dropped to an average of 4%, indicating there were fewer viable TM7x-attached XH001 cells in the nutrient-depleted cultures than in the nutrient-replete cultures (Figure 5B).

Small volumes were taken from the long-term cultures at day 7, day 14, day 21, and day 36 post-inoculation, and secondarily inoculated in fresh BHI medium. These secondary cultures were passaged each day for a series of days until TM7x was detected (Figure 5C). After a 24-hour period post-secondary-inoculation, XH001 growth was visible in both mono- and co-cultures (Figure 5D). TM7x, however, was not immediately detectable by phase contrast or by

colony morphology. At time points day 7, day 14, and day 21, each secondary co-culture eventually gave rise to visible TM7x, though the longer the nutrient depleted culture had incubated, the more passages it took for TM7x to re-emerge, with day 21 secondary culture taking eight passages for re-emergence (Table 1). At day 36, the secondary culture was not passaged until re-emergence, but PCR was performed after three 1:10 split passages. TM7x was detected in faint bands in both co-culture biological duplicates, but not in the XH001 monoculture (Figure 5E). While this hints that TM7x persists to at least 36 days under starvation conditions, the presence of TM7x in the 3X passaged secondary culture does not necessarily indicate the presence of viable TM7x cells, as the PCR product may have been an amplification of trace TM7x DNA from the original culture. We conclude that under starvation conditions, fewer viable XH001 cells are TM7x-attached, but both species co-exist viably for at least 21 days.

Serum induces TM7x over-attachment. In the inflammatory state of periodontitis, there is an increased amount of serum exudate released into the gingival crevicular fluid (18, 20). To test whether serum has any effect on the population dynamic between XH001 and TM7x, we started with a serum-free, chemically defined medium, RPMI. This medium alone could not support growth of either the mono- or co-culture (Figure 6A), but when 10% FBS was added, XH001 monoculture was able to grow to its maximum OD of ~1 within 24 hours (Figure 6B). The co-culture was only able to grow to an OD of ~1 for the first 24 hours after being subcultured from a BHI-grown culture. At each passage in RPMI + FBS thereafter, the OD of the co-culture diminished dramatically (Figure 6B). At passage 3, co-culture OD was so low, the culture was

not diluted by passaging, but allowed to recover. After 48 hours of no passaging, the OD of the co-culture had returned to its normal maximum level, of ~0.6-1.0. Upon passaging again, the co-culture OD again was greatly reduced.

Alongside this slow growth, phase contrast images show XH001 cells with much higher than normal amounts of TM7x attachment (Figure 6C). Additionally, the XH001 forms highly distended and hyphal morphology, which is indicative of stress (19). Because the XH001 monoculture did not display these stress markers, it may be the over-attached TM7x causing stress and lowering the viability or growth rate of XH001.

Microaerobic environments are preferred by XH001 in mono- or co-culture, but XH001 in monoculture tolerates anaerobic and aerobic conditions better than XH001 in TM7x co-culture. To assess how oxygen concentration affects XH001 and TM7x, XH001 monocultures and XH001/TM7x co-cultures were grown in ambient aerobic conditions, in anaerobic conditions, and in microaerobic conditions (2.6% O_2). Passages were performed such that all cultures at the beginning of each 24-hour growth cycle had equal OD. As monitored by OD, the aerobic and anaerobic mono- and co-cultures grow poorly when compared to the microaerobic cultures, with the aerobic condition showing the least growth (Figure 7A).

Mono- and co-cultures were initially grown in microaerobic conditions, then inoculated 1:10 into BHI pre-incubated in microaerobic, anaerobic, or aerobic conditions. Cultures were followed for 48 hours with no nutrients added post inoculation, and plated for CFU counting and colony morphology observation. Irregular colony percentage rose and then dropped for co-cultures in all oxygen conditions (Figure 7B). Comparing XH001 monoculture to XH001/TM7x

co-culture by CFU, the co-culture was more sensitive to non-preferred oxygen concentration, with lowest growth in the aerobic environment (~21% O_2) (Figure 7C). Preliminarily, microaerobic and anaerobic growth conditions did not show a trend in terms of increase or decrease in irregular colony percentage over a three-day experiment (Figure 7D). However, a longer time course experiment may yield different results.

Heat shock does not affect XH001 viability or TM7x attachment. In order to assess what effect heat shock may have on XH001 and TM7x, mono- and co-cultures were incubated in a 42C water bath for 0, 1, 5, 10, 30, and 60 minutes. No appreciable difference in CFU or in proportion of irregular colonies were observed for all the time points tested (Figure 8).

Discussion:

The hydrogen peroxide data is compelling in that there was a marked reduction of TM7x at even low concentration of H_2O_2, 0.5 mM, which is within the relevant range for peroxide-producing plaque bacteria (34). Hydrogen peroxide produced by host cells or hydrogen peroxide-producing oral commensal bacteria could play a crucial role in inhibiting TM7x growth and keep its population in check. Under periodontal disease condition, the shift of the commensal microbiome, such as the decrease in the abundance of H_2O_2-generating Strepctococci (28), as well as the impaired function of macrophages (30) could result in imbalance of population between TMx and its host, leading to the over-growth of TM7x, which could potentially further impact the progression of disease. As TM7x is the first cultured member of Candidatus Saccharibacteria, more phylotypes of this clade and their putative hosts

should be characterized and evaluated to determine whether the lifestyle and phenotype of TM7x is representative of other members of this clade.

In periodontitis, inflammation and increased serum exudate are common (26). The XH001/TM7x co-culture in RPMI with 10% FBS showed over-attachment and high amounts of free-floating TM7x by phase contrast imaging, concurrent with decreased growth rate of XH001, as measured by OD (Figure 6). *In vivo*, excess serum in the gingival crevicular fluid might increase the attachment of TM7 to the point of suppressing the growth of host *Actinomyces*, partially explaining why periodontitis is associated with greater abundance of TM7 and a lower abundance of *Actinomyces* (28). We are focusing on a very specific model here; while 97% of people may have TM7 in their subgingival plaque, that doesn't mean they all have this TM7x/XH001 pairing. More examples of widespread TM7 parasitic behavior and its role in periodontitis have yet to be discovered, but future work along this avenue could be elucidating.

While the 250 mM NaCl and KCl conditions led to a decrease in TM7x attachment (Figure 4B), it is unlikely that physiological conditions would allow for salt concentrations as high as 250 mM, as blood serum sodium concentration is only ~140 mM (35), and potassium concentration is only ~5 mM (36). A titration of salt concentrations in a physiologically relevant might be useful to assess whether salt concentration could modulate the *Actino*myces/TM7 balance *in vivo*. In a preliminary experiment culturing TM7x/XH001 co-culture in BHI + 196 mM KCl, there was no full clearance of TM7x as assessed by phase contrast imaging (data not shown). However, whether TM7x attachment was reduced is unknown, as colony morphology was not analyzed in this experiment.

Mannitol was used in order to increase osmolarity without the ionic and specific physiological effects of NaCl and KCl. Mannitol was chosen because it is non-fermentable by *Actinomyces odontolyticus*. My data indicates that mannitol may increase TM7x attachment on XH001 (Figure 4A). Whether this trend is consistent and the reason for it still needs to be explored. Raising the osmolarity of the culture medium with amino acids or organic buffers may be another method to determine if increased osmolarity leads to increased TM7x attachment.

There is an interesting phenomenon regarding the re-appearance of TM7x in co-cultures after it seems to have disappeared. TM7x appeared to be eliminated from the co-culture following seven days in 250 mM NaCl, only to be detected via PCR after six more days in BHI-only (Figure 4E). In the long-term starvation experiment, TM7x re-emerged after days when no TM7x was visible (Figure 5D, E, Table 1). How TM7x is able to persist through these conditions is under further investigation at this time. Based on unpublished EM data from the Shi research group showing what looks to be a TM7x cell inside of an XH001 cell, TM7x may survive by becoming intracellular when environmental conditions are harsh.

Though we have identified some potentially influencing factors, the physical and molecular mechanisms of how TM7x attachment is increased or decreased are not known. Transmission EM images from He et al. indicate that TM7x-attached cells have a severely compromised membrane (18). Thus, it is possible that TM7x attachment to XH001 sensitized the XH001 to hostile environments due to loss of membrane integrity. The decreased proportion of irregular colonies in hydrogen peroxide and high salt may be due to TM7x-attached XH001 cells dying, allowing TM7x-free cells to take over, or these conditions may be directly cytotoxic to TM7x. More experimentation is in progress to determine the physical and

biochemical mechanisms involved in the shifting balance between the parasite and host populations.

While Saccharibacteria abundance in microbial communities is associated with inflammatory disease (22-25), it is unknown at this time whether TM7x or other Saccharibacteria members are directly pathogenic. Additionally, it is implausible that all human-associated species within the candidate phylum are equally disease-associated. However, TM7x and XH001 represent a novel inter-bacterial relationship, and greater understanding of this type of bacterial symbiosis is integral in understanding human microbiota and dysbiosis. Whether Saccharibacteria abundance is incidental rather than causal in disease, shifting the microbial balance back toward a community that is more health-associated by reducing Saccharibacteria may be a worthwhile aim for therapeutic interventions. Studying this partnership, or rather, rivalry, could lead to the discovery, characterization, and cultivation of other such relationships in the bacterial domain.

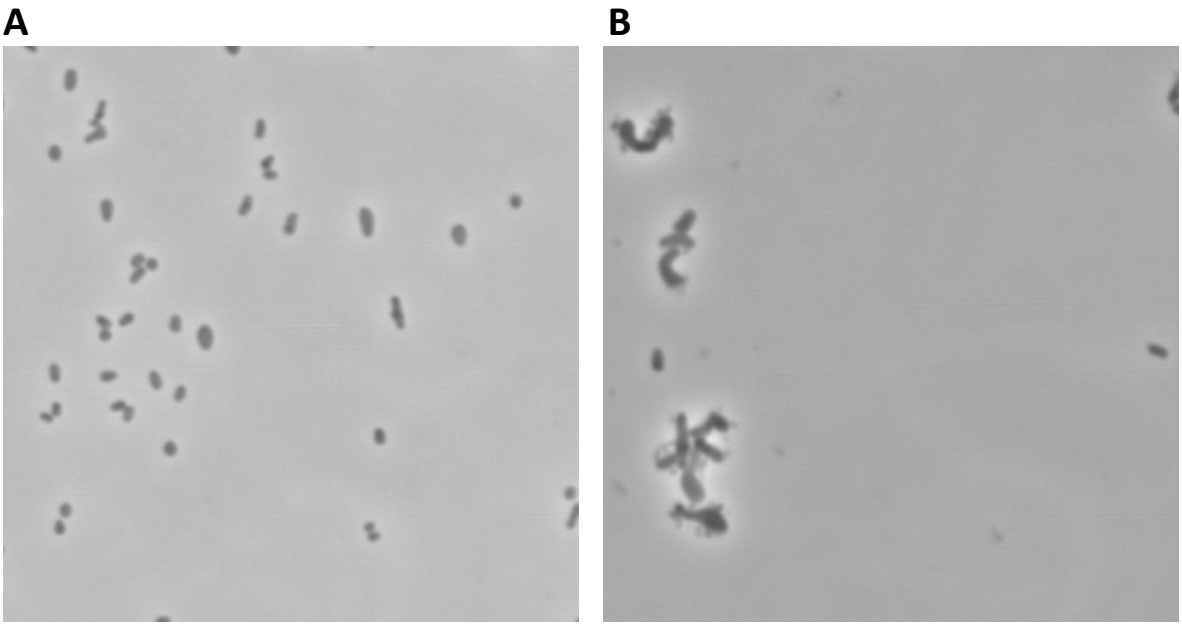


Figure 1. Morphology of *Actinomyces odontolyticus* strain XH001 (XH001) and TM7x under phase contrast imaging. A) Monoculture of XH001. All cells appear as short rods. B) Co-culture of XH001 and TM7x. TM7x-attached XH001 cells are long, branched, and decorated with small cocci. TM7x-free XH001 cells appear as short rods with no surface-attached cocci. Note: both images are equally scaled.

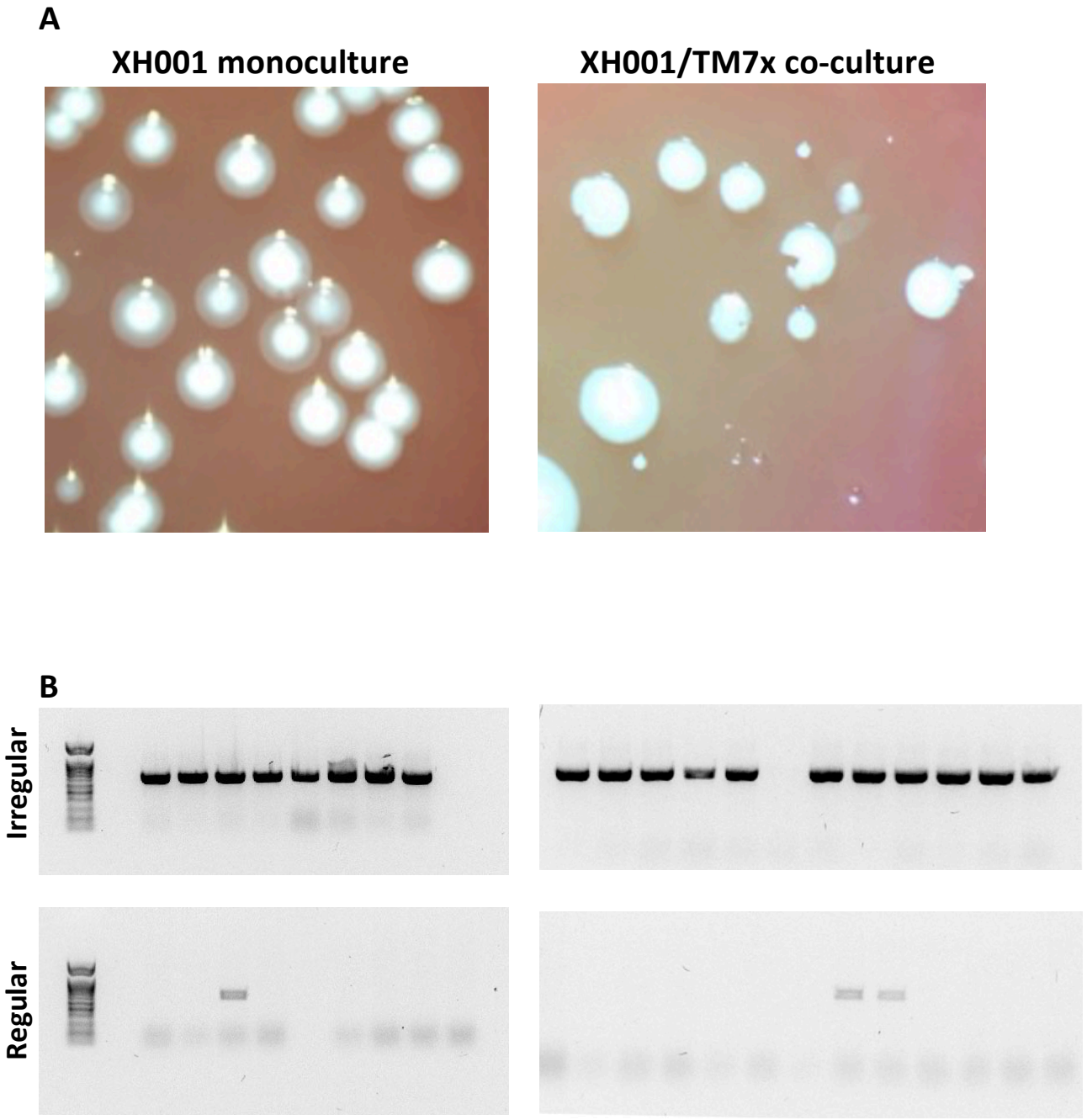


Figure 2. Colony morphology is indicative of TM7x attachment. A) *Left,* colony morphology of XH001 monoculture is round and smooth. *Right*, colony morphology of TM7x/XH001 co-culture contains round, smooth colonies and irregular colonies. B) Colony PCR of regular and irregular colonies from plated co-culture. *Top*, TM7x was detected in 19/20 irregular colonies. *Bottom*, TM7x was detected in 3/20 regular colonies.

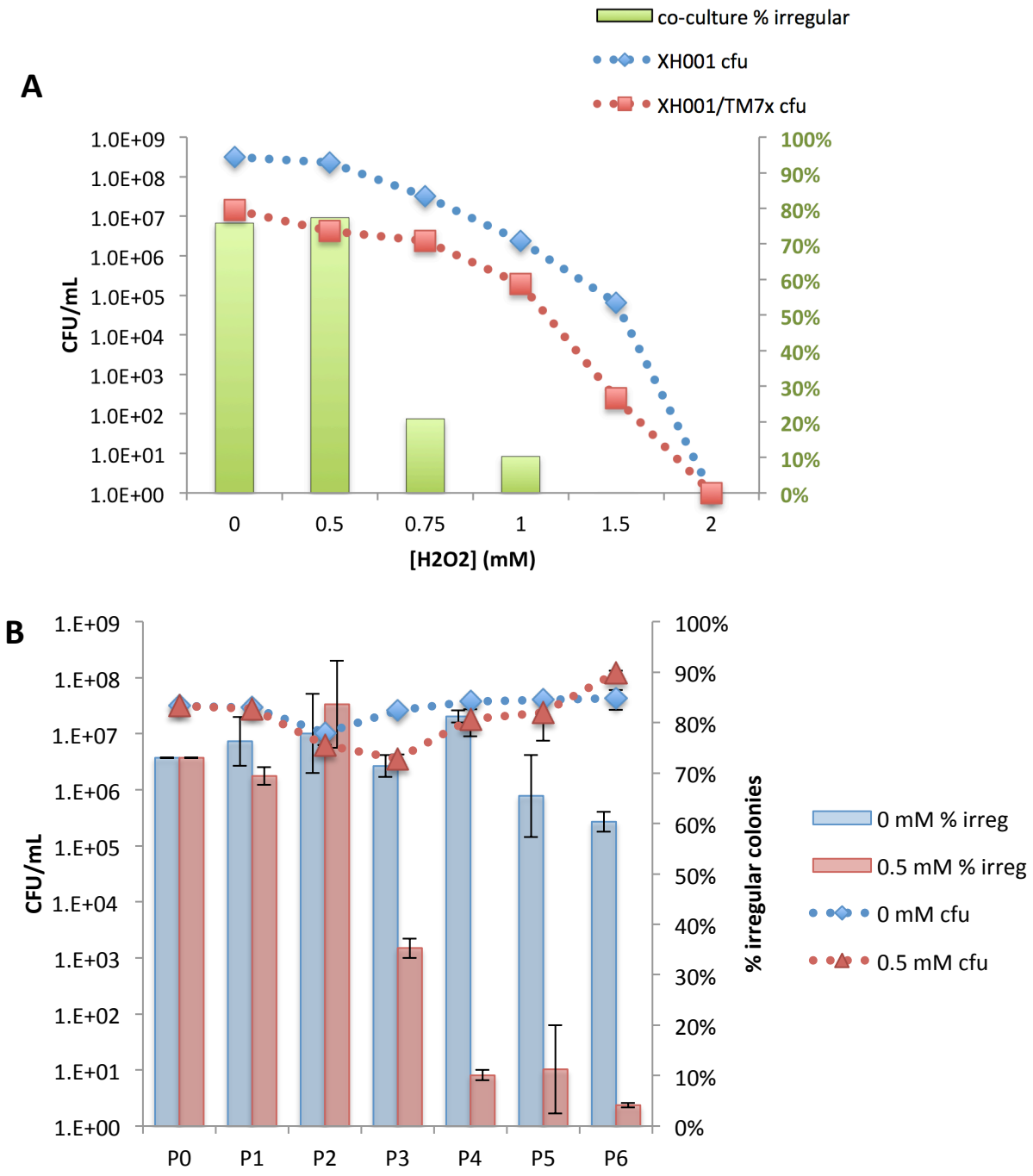
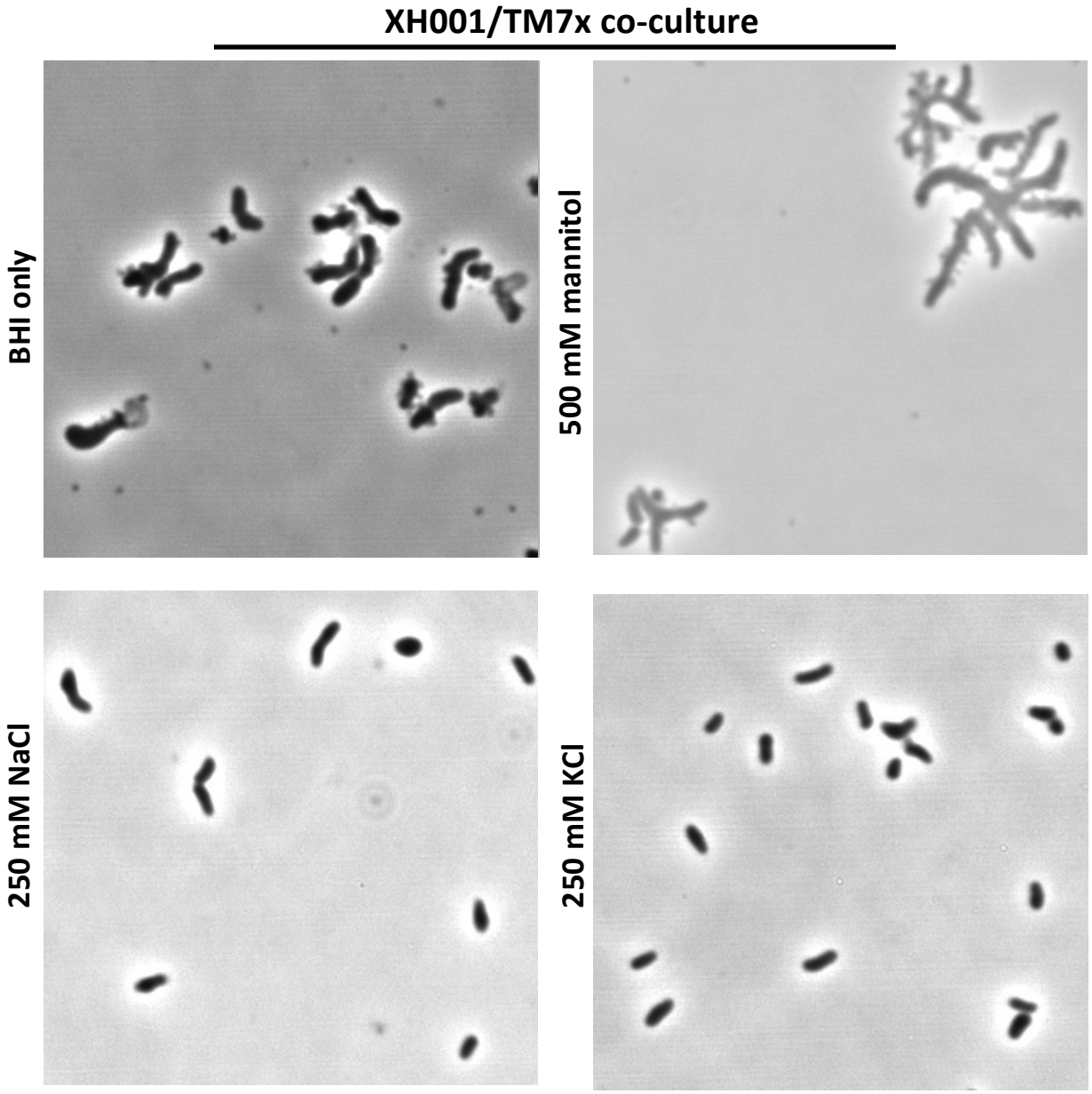
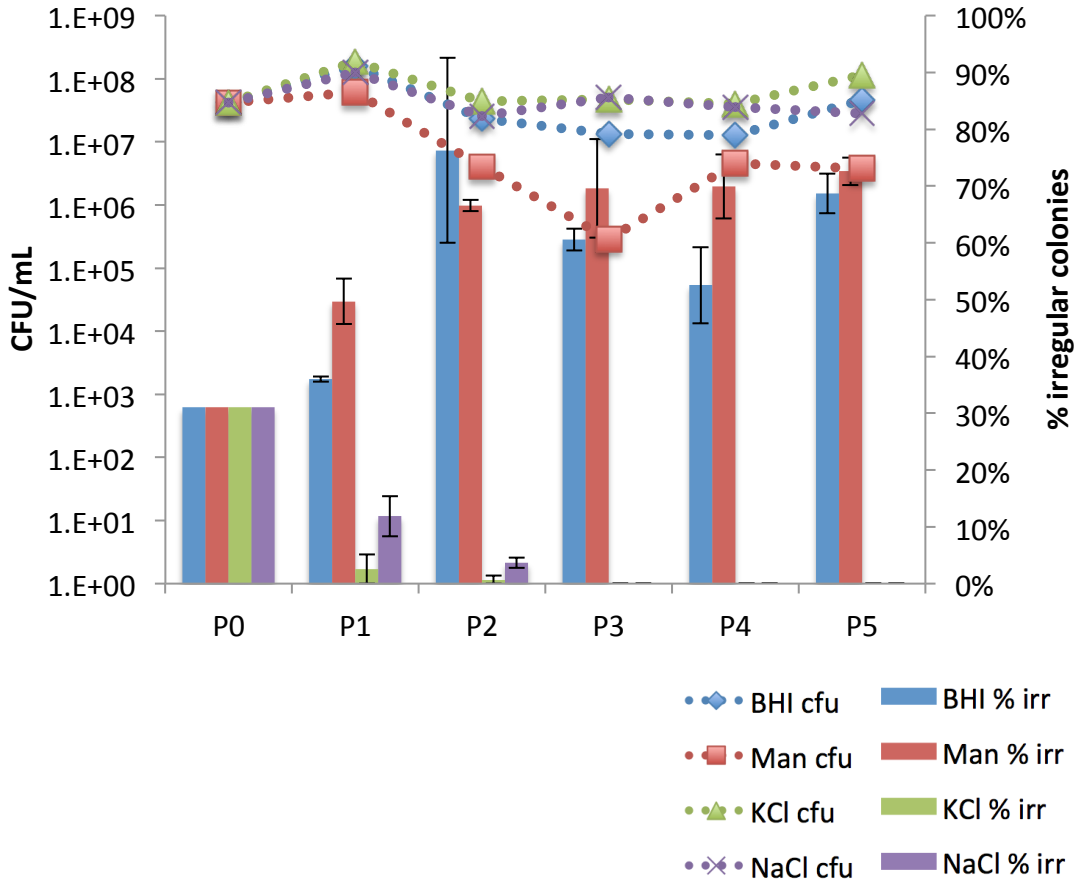


Figure 3. Hydrogen peroxide reduces TM7x attachment on XH001. A) Dose response of mono- and co-culture to H2O2, after 24 hours incubation. B) CFU and colony morphology of co-culture grown and passages for six days in either 0 mM (blue) or 0.5 mM (red) H2O2.

A

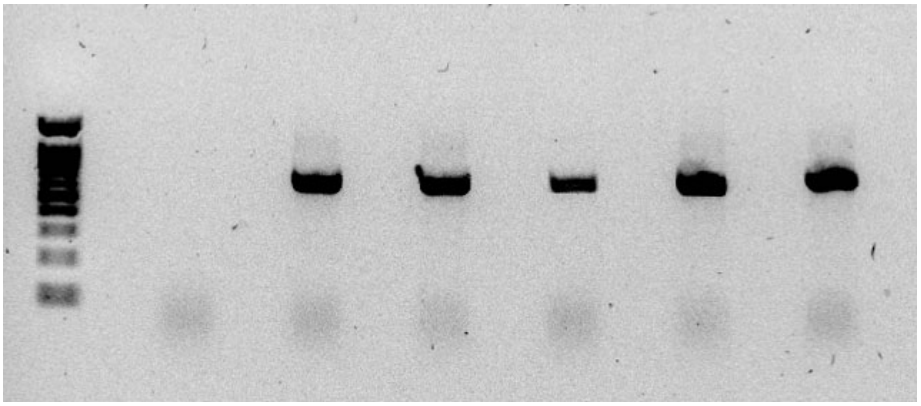

XH001/TM7x co-culture
BHI only
500 mM mannitol
250 mM NaCl
250 mM KCl

B

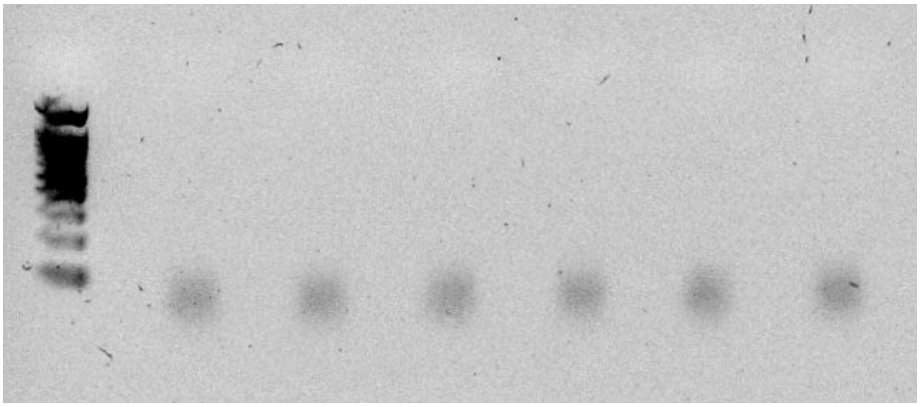

1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
CFU/mL
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% irregular colonies
P0
P1
P2
P3
P4
P5
BHI cfu
Man cfu
KCl cfu
NaCl cfu
BHI % irr
Man % irr
KCl % irr
NaCl % irr

C

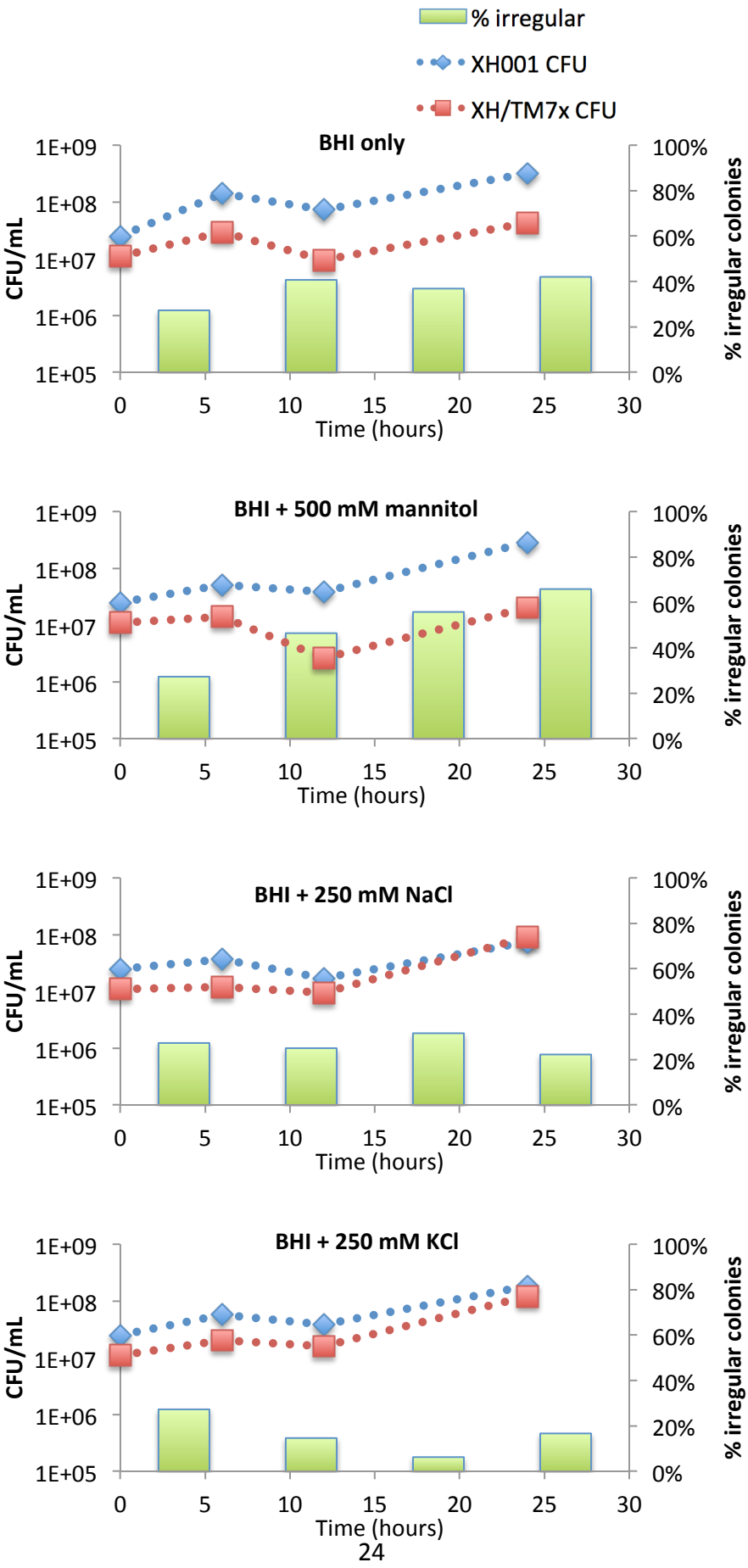
BHI XH001	BHI XH/TM-1	BHI XH/TM-2	Mannitol XH001	Mannitol XH/TM-1	Mannitol XH/TM-2



KCl XH001	KCl XH/TM-1	KCl XH/TM-2	NaCl XH001	NaCl XH/TM-1	NaCl XH/TM-2



D


% irregular
XH001 CFU
XH/TM7x CFU
BHI only
BHI + 500 mM mannitol
BHI + 250 mM NaCl
BHI + 250 mM KCl
CFU/mL
1E+09
1E+08
1E+07
1E+06
1E+05
% irregular colonies
100%
80%
60%
40%
20%
0%
0
5
10
15
20
25
30
Time (hours)

E

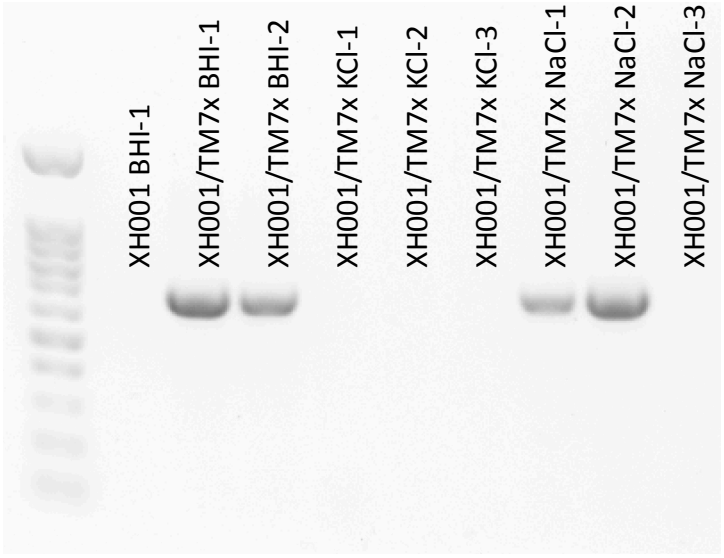
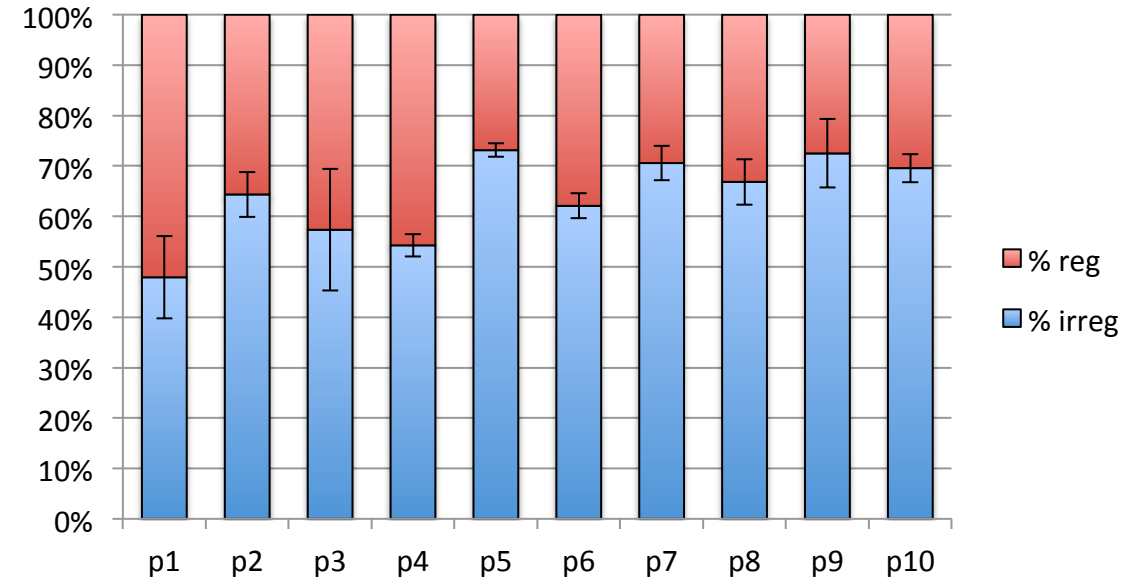
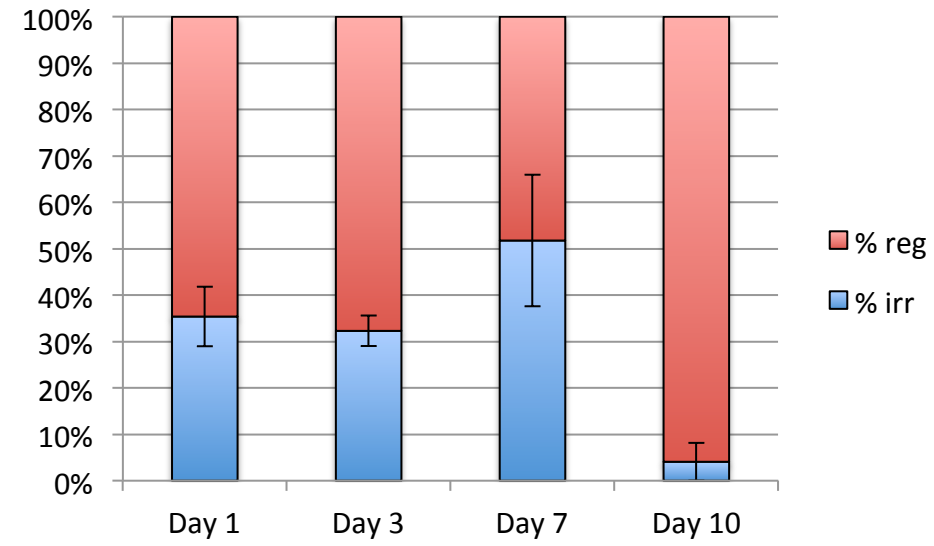


Figure 4. High NaCl and KCl concentrations reduce TM7x attachment.
A) Representative images from phase contrast imaging of XH001/TM7x co-culture after seven passages in BHI only, BHI + 500 mM mannitol, BHI + 250 mM NaCl, or BHI + 250 mM KCl. B) HX001/TM7x co-cultures grown in BHI, salt or mannitol conditions for five days and analyzed for colony forming units (CFU/mL) and colony morphology. Dotted lines, CFU/mL. Columns, percentage irregular colonies out of total colonies. C) Agarose gel of PCR products for detection of TM7x. Amplified DNA was isolated from culture after five days of growth and passaging in BHI only, high concentration NaCl, KCl or mannitol. XH/TM, XH001/TM7x co-culture. Numbers (-1, -2) represent biological duplicates. D) 24-hour time courses of XH001 monoculture (blue) and XH001/TM7x co-culture (red) in various salt and osmotic conditions. Dotted lines, CFU/mL. Columns, percent irregular colonies from plated co-culture. E) Agarose gel of PCR products for detection of TM7x. Amplified DNA was isolated from culture after seven days of growth and passaging in conditions as labeled followed by six days of growth and passaging in BHI only. Numbers (-1, -2, -3) represent biological triplicates.

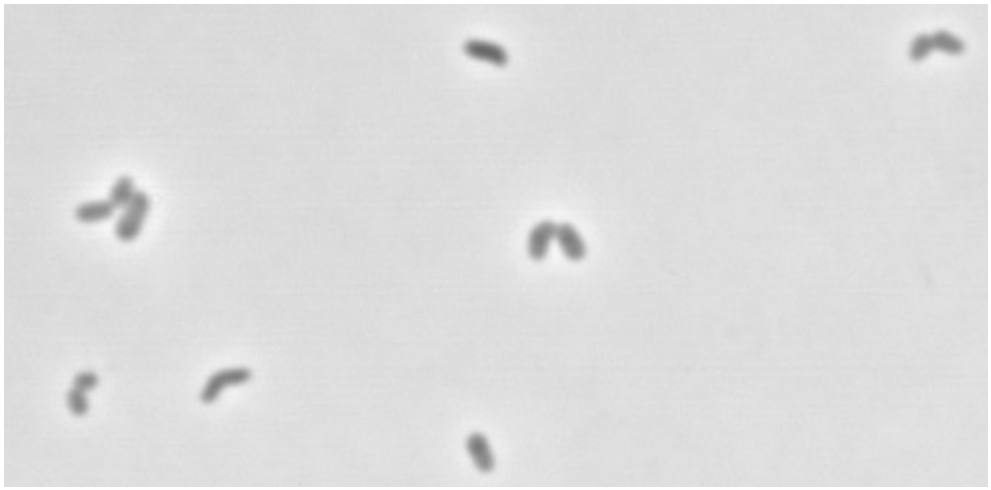
A


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
p1
p2
p3
p4
p5
p6
p7
p8
p9
p10
% reg
% irreg

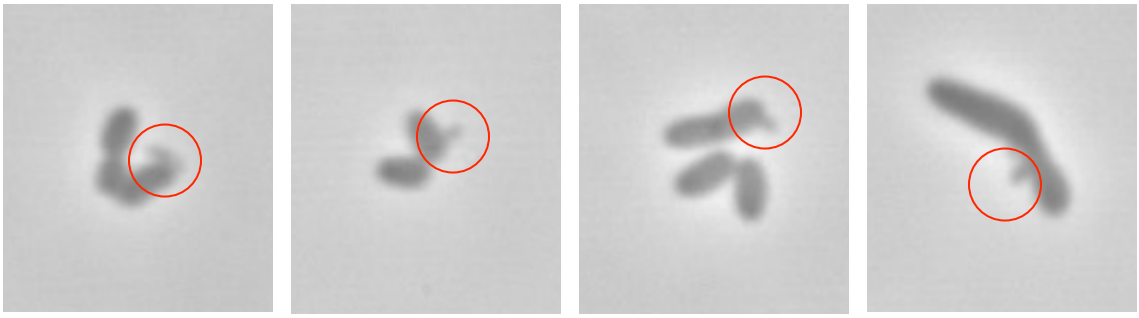
B


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Day 1
Day 3
Day 7
Day 10
% reg
% irr

C



D



E

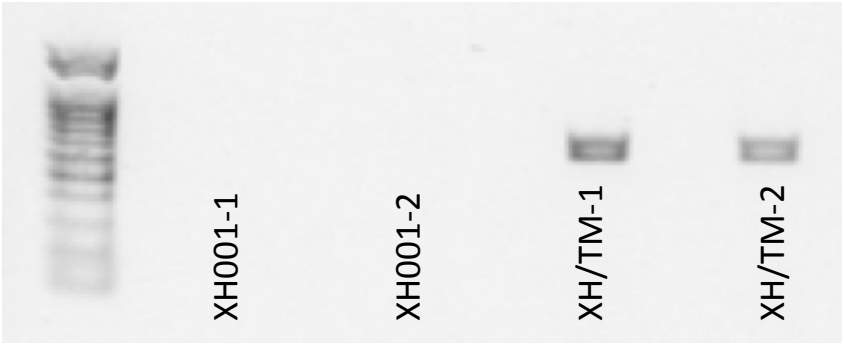
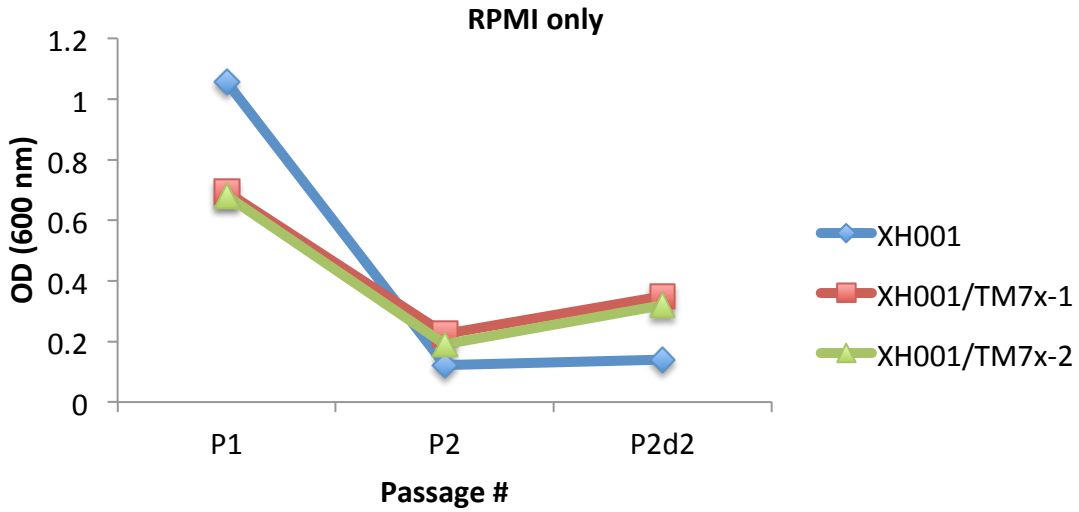


Figure 5. XH001 and TM7x re-emerge after long-term nutrient depletion. A) Proportion of irregular colonies in XH001/TM7x co-culture under nutrient-replete conditions. Co-cultures were passaged every 24 hours for 10 days, using one part previous culture to nine parts fresh BHI medium. B) Proportion of irregular colonies in XH001/TM7x co-culture under nutrient-depleted conditions. Co-cultures were inoculated into fresh BHI medium, with no fresh medium added for 10 days. C) Representative phase contrast image of XH001/TM7x co-culture, after long-term nutrient-depletion conditions, then re-inoculation into fresh medium, but before TM7x re-emergence. D) Representative phase contrast images of initial TM7x re-emergence after secondary inoculation, post-long-term nutrient depletion. Red circles indicate presence of TM7x (small cocci) attached to surface of XH001 (larger rods). E) Agarose gel of PCR products for detection of TM7x after 36 days of nutrient-depleted incubation. Volumes from 36-day-old mono- and co-cultures were inoculated into fresh medium and allowed to grow for 3 days (passaging 1:10 each day). DNA was isolated from these cultures and amplified using TM7x-specific PCR primers. XH/TM indicates XH001/TM7x co-cultures. Numbers (-1, -2) represent biological duplicates.

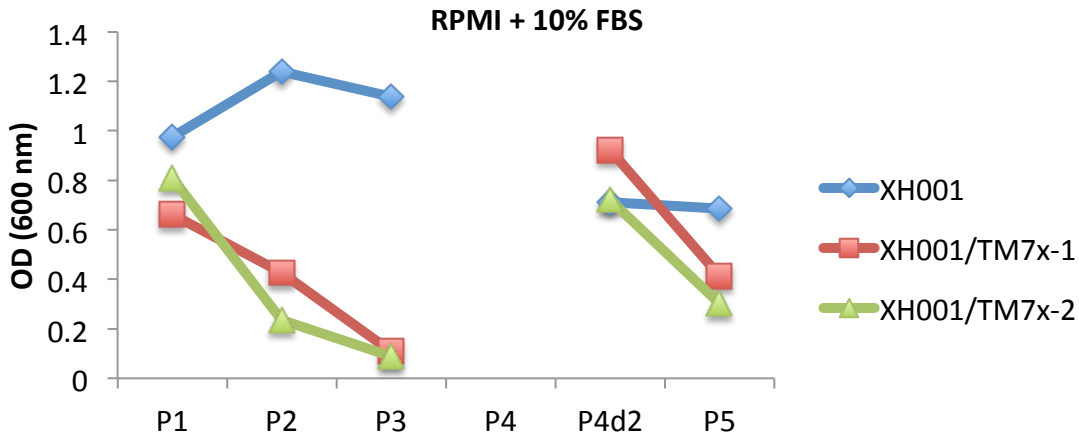
Day post-inoculation, continuous culture	Passages for TM7x re-emergence
7	3
14	4
21	8
36	N/A

Table 1. Longer-term starvation leads to slower TM7x re-emergence when nutrients are added. *Left,* number of days XH001/TM7x co-culture was incubated for without adding any fresh media. *Right,* the numbers of days, passaged at 1:10 split volume each day, it takes for TM7x to be observed via phase contrast, after inoculation into fresh medium from starved co-cultures.

A


RPMI only
1.2
1
0.8
0.6
0.4
0.2
0
OD (600 nm)
P1
P2
P2d2
Passage #
XH001
XH001/TM7x-1
XH001/TM7x-2

B


RPMI + 10% FBS
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD (600 nm)
P1
P2
P3
P4
P4d2
P5
XH001
XH001/TM7x-1
XH001/TM7x-2

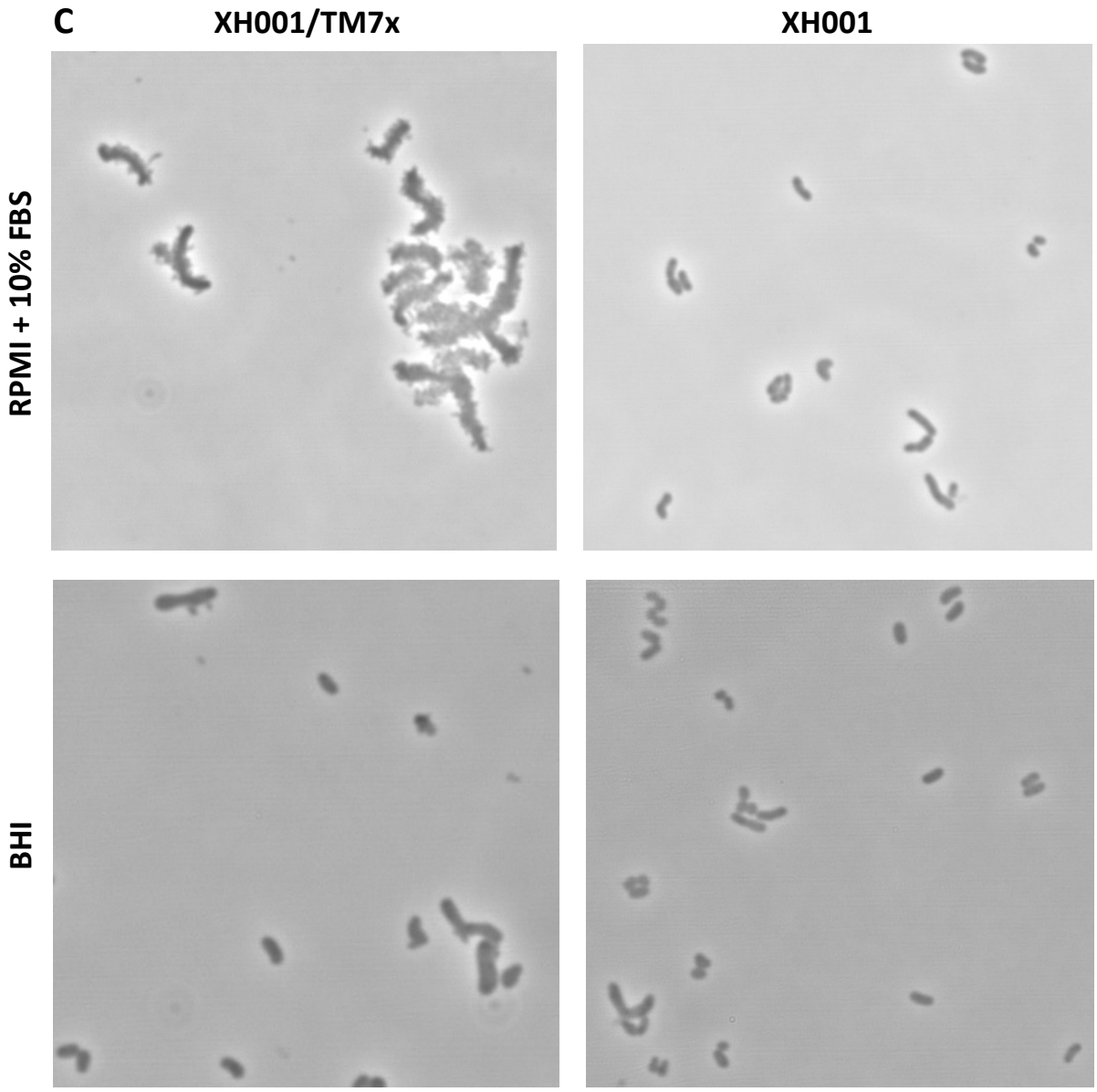
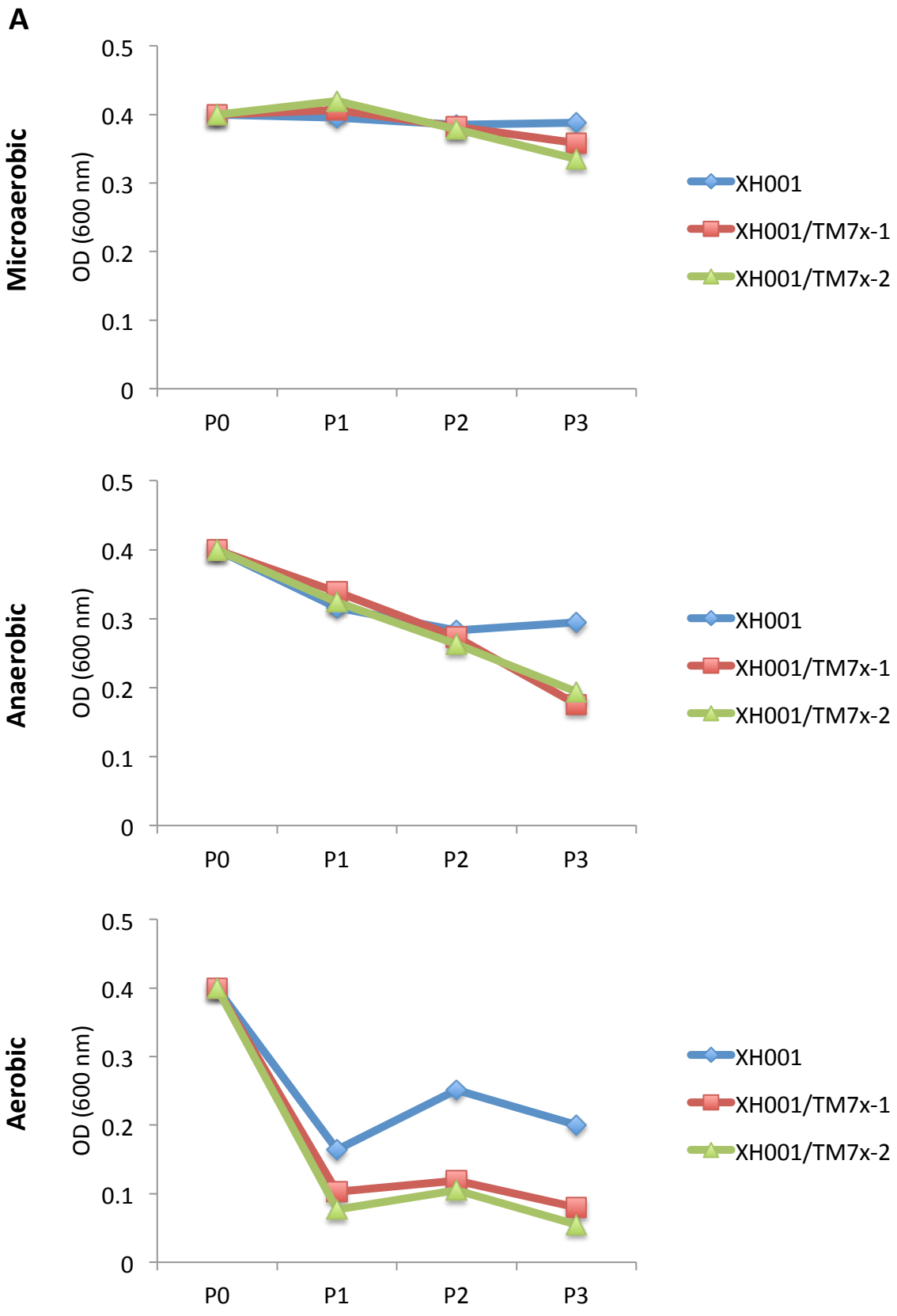
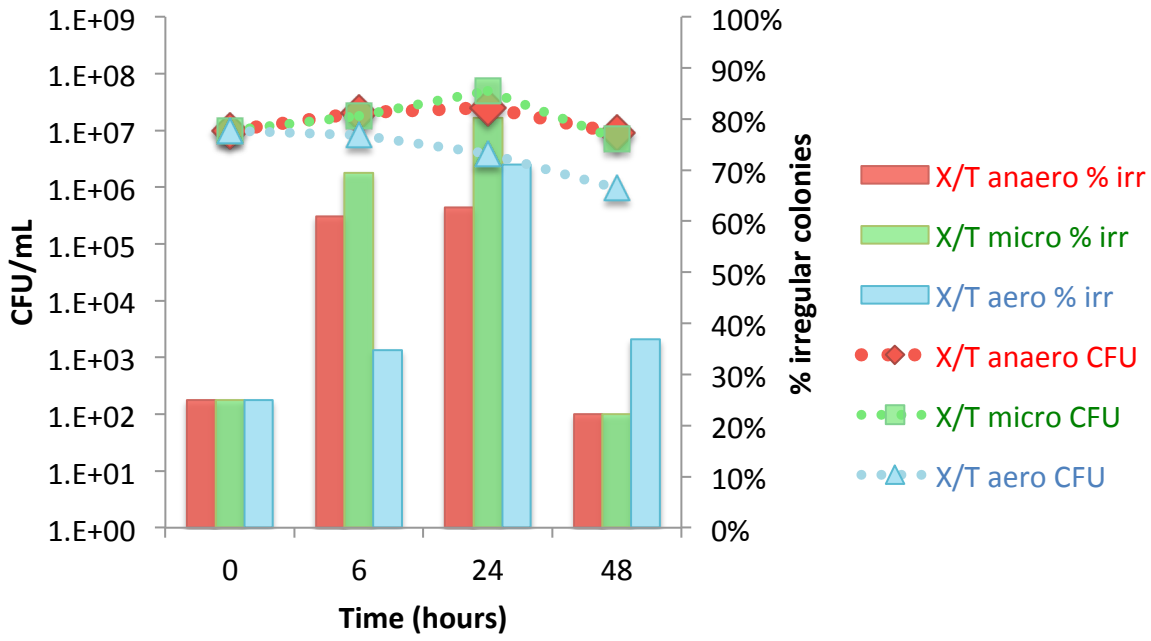


Figure 6. RPMI + fetal bovine serum increases TM7x attachment and decreases XH001 growth. A) XH001 monoculture and XH001/TM7x co-culture initially grown in BHI and then passaged into RPMI only. *Y axis*, optical density of cultures read at 600nm wavelength. *X axis*, P indicates passage number, with P2d2 indicating passage 2 day 2 (no dilution of culture into fresh media). Numbers (-1, -2) represent biological duplicates. B) XH001 monoculture and XH001/TM7x co-culture initially grown in BHI and then passaged into RPMI + 10% fetal bovine serum + non-essential amino acids. OD was not read on passage 4 day 1, but was read again on passage 4 day 2 (P4d2). C) Representative phase contrast images from XH001 monoculture and XH001/TM7x co-culture in RPMI + FBS or BHI. All four images are equally scaled.

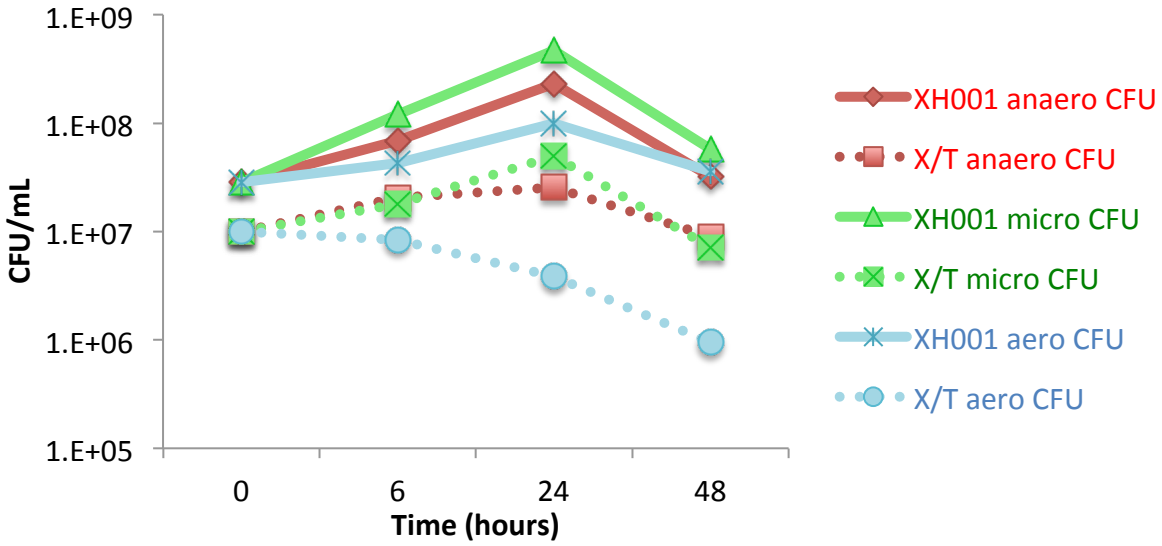
A


Microaerobic
OD (600 nm)
0.5
0.4
0.3
0.2
0.1
0
P0
P1
P2
P3
XH001
XH001/TM7x-1
XH001/TM7x-2
Anaerobic
OD (600 nm)
0.5
0.4
0.3
0.2
0.1
0
P0
P1
P2
P3
XH001
XH001/TM7x-1
XH001/TM7x-2
Aerobic
OD (600 nm)
0.5
0.4
0.3
0.2
0.1
0
P0
P1
P2
P3
XH001
XH001/TM7x-1
XH001/TM7x-2

B


1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
CFU/mL
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% irregular colonies
0
6
24
48
Time (hours)
X/T anaero % irr
X/T micro % irr
X/T aero % irr
X/T anaero CFU
X/T micro CFU
X/T aero CFU

C


1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
CFU/mL
0
6
24
48
Time (hours)
XH001 anaero CFU
X/T anaero CFU
XH001 micro CFU
X/T micro CFU
XH001 aero CFU
X/T aero CFU

D

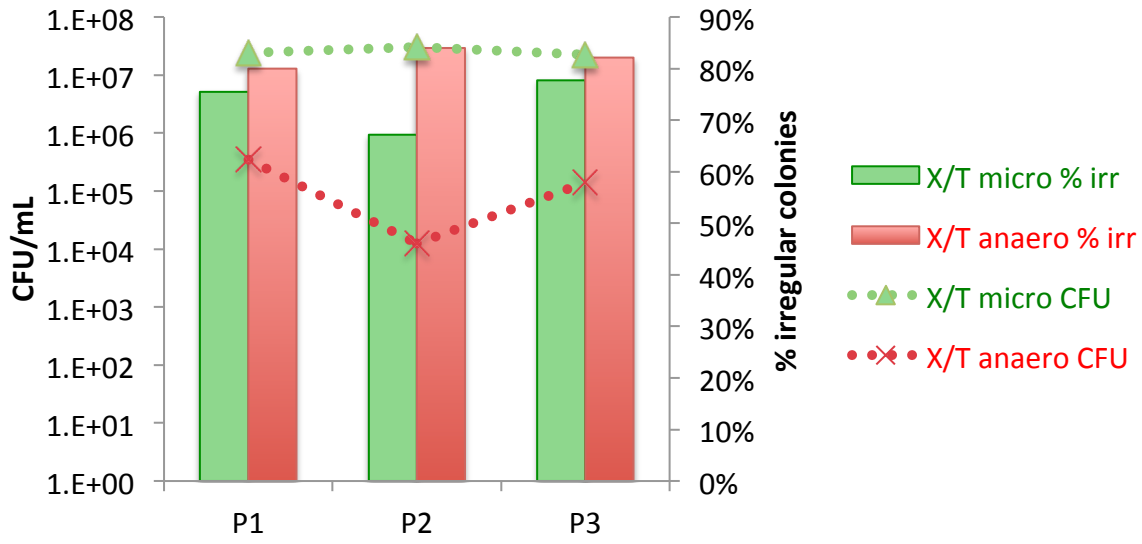


Figure 7. XH001 prefers microaerobic oxygen concentrations, and fares better in mono, rather than co-culture. Mono- and co-cultures were first grown in microaerobic conditions before inoculated into media that had been prepared in the desired oxygen environment. A) Optical density measurement of XH001 monoculture and XH001/TM7x co-culture in microaerobic (2.6% O2), anaerobic (0% O2), and aerobic (~21% O2) conditions, over 3 passages, split by OD. B) and C) Mono- and co-cultures were incubated in microaerobic, anaerobic, and aerobic conditions for 48 hours. Samples at time points indicated were plated for CFU and colony morphology. Dotted lines represent co-culture CFU, solid lines represent monoculture CFU, and columns represent percent irregular colonies. D) CFU and colony morphology of co-culture in microaerobic and anaerobic conditions, passaged for 3 days, split 1:10 each day.

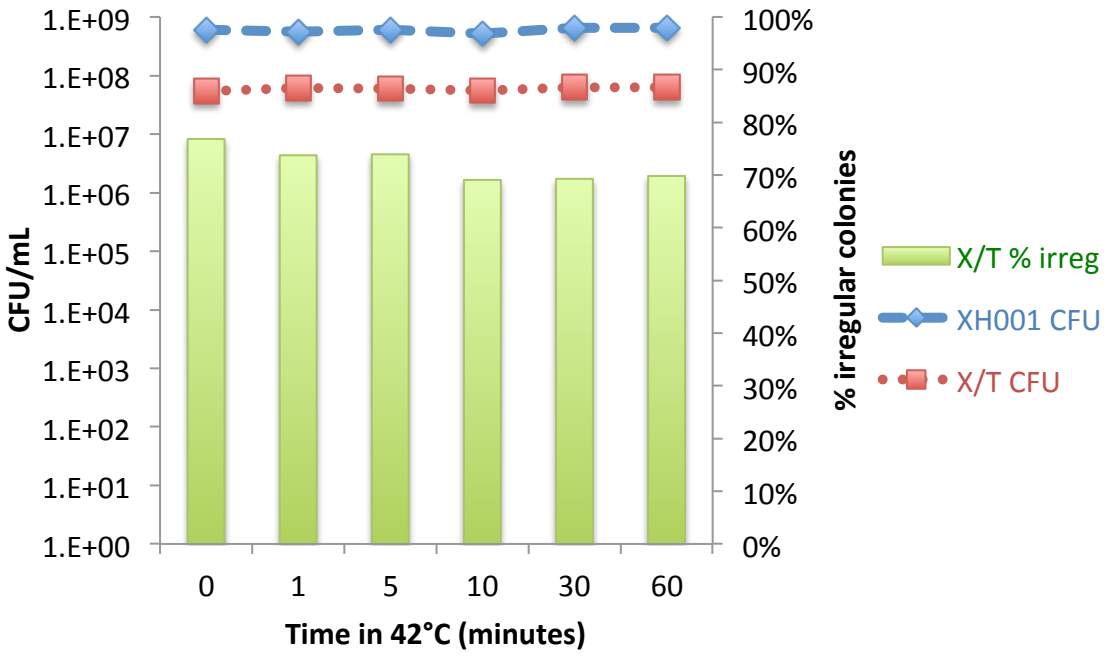


Figure 8. Heat shock at 42C does not affect CFU or colony morphology proportion within a 60-minute time span. XH001 monoculture and XH001/TM7x co-culture were incubated in a 42C water bath from between 0 and 60 minutes. Dotted lines represent CFU/mL and column represent percent irregular colonies.

References

1. Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. *The Lancet*, *361*(9356), 512-519.

2. Vollaard, E. J., & Clasener, H. A. (1994). Colonization resistance. *Antimicrobial agents and chemotherapy*, *38*(3), 409.

3. Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews Immunology, 9(5), 313-323.

4. Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207-214.

5. Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature, 489(7415), 220-230.

6. Tamboli, C. P., Neut, C., Desreumaux, P., & Colombel, J. F. (2004). Dysbiosis in inflammatory bowel disease. Gut, 53(1), 1-4.

7. Simón-Soro, A., & Mira, A. (2015). Solving the etiology of dental caries. Trends in microbiology, 23(2), 76-82.

8. Liu, B., Faller, L. L., Klitgord, N., Mazumdar, V., Ghodsi, M., Sommer, D. D., ... & Stine, O. C. (2012). Deep sequencing of the oral microbiome reveals signatures of periodontal disease. PloS one, 7(6), e37919.

9. Lasken RS, McLean JS (2014) Recent advances in genomic DNA sequencing of microbial species from single cells. Nat Rev Genet 15(9):577–584.

10. Marcy Y, et al. (2007) Dissecting biological “dark matter” with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. Proc Natl Acad Sci USA 104(29):11889–11894.

11. Peterson J, et al.; NIH HMP Working Group (2009) The NIH Human Microbiome Project. Genome Res 19(12):2317–2323.

12. Chen T, et al. (2010) The Human Oral Microbiome Database: A web accessible resource for investigating oral microbe taxonomic and genomic information. Database 2010:baq013.

13. Albertsen M, et al. (2013) Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat Biotechnol 31(6):533–538.

14. Rheims H, Rainey FA, Stackebrandt E (1996) A molecular approach to search for diversity among bacteria in the environment. J Ind Microbiol 17(3-4):159–169.

15. Abrams M, et al. (2012) Genomic characteristics of an environmental microbial community harboring a novel human uncultivated TM7 bacterium associated with oral diseases. Open Access Scientific Reports 1:276.

16. Hugenholtz P, Tyson GW, Webb RI, Wagner AM, Blackall LL (2001) Investigation of candidate division TM7, a recently recognized major lineage of the domain Bacteria with no known pure-culture representatives. Appl Environ Microbiol 67(1):411–419.

17. McCutcheon, J.P. & Moran, N.A. (2012) Extreme genome reduction in symbiotic bacteria. Nat. Rev. Microbiol. 10, 13–26.

18. He X, McLean JS, Edlund A et al (2015) Cultivation of a human- associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. Proc Natl Acad Sci U S A 112:244–249.

19. Bor, B et al. (2016) Phenotypic and physiological characterization of the epibiotic interaction between TM7x and its basibiont Actinomyces. Microb Ecol 71:243–255.

20. Hajishengallis, G. and Lamont, R.J. (2012) Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Molecular oral microbiology 27.6: 409-419.

21. Cho, Ilseung, and Martin J. Blaser. (2012) The human microbiome: at the interface of health and disease. Nature Reviews Genetics 13.4: 260-270.

22. Fredricks DN, Fiedler TL, Marrazzo JM (2005) Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med 353(18):1899–1911.

23. Kuehbacher, T. et al. (2008) Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. J. Med. Microbiol. 57, 1569–1576.

24. Liu B, et al. (2012) Deep sequencing of the oral microbiome reveals signatures of periodontal disease. PLoS ONE 7(6):e37919.

25. Brinig, M.M. et al. (2003) Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. Appl. Environ. Microbiol. 69, 1687–1694.

26. Costalonga, M. and Herzberg, M (2014) The oral microbiome and the immunobiology of periodontal disease and caries. Immunology letters 162.2: 22-38.

27. Rylev, M., et al. Microbiological and immunological characteristics of young Moroccan patients with aggressive periodontitis with and without detectable Aggregatibacter actinomycetemcomitans JP2 infection. Molecular oral microbiology 26.1 (2011): 35-51.

28. Abusleme, L. et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. The ISME journal 7.5 (2013): 1016-1025.

29. Kinane, Denis F. Causation and pathogenesis of periodontal disease. Periodontology 2000 25.1 (2001): 8-20.

30. Chapple, C.C. et al. (1998) Failure of macrophage activation in destructive periodontal disease. The Journal of pathology 186.3: 281-286.

31. Kreth, Jens, et al. (2005) Competition and coexistence between Streptococcus mutans and Streptococcus sanguinis in the dental biofilm. Journal of bacteriology 187.21: 7193-7203.

32. Marsh, P. D. (2004) Dental plaque as a microbial biofilm. Caries research 38.3: 204-211.

33. Xu, K.D. et al. (1998) Spatial physiological heterogeneity in Pseudomonas aeruginosa biofilm is determined by oxygen availability. Applied and environmental microbiology 64.10: 4035-4039.

34. Ryan, C. S., & Kleinberg, I. (1995). Bacteria in human mouths involved in the production and utilization of hydrogen peroxide. Archives of oral biology, 40(8), 753-763.

35. Biggins, S. W. et al. (2006). Evidence-based incorporation of serum sodium concentration into MELD. Gastroenterology, 130(6), 1652-1660.

36. Yang, T., & Roden, D. M. (1996). Extracellular potassium modulation of drug block of IKr implications for torsade de pointes and reverse use-dependence. Circulation, 93(3), 407-411.