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Research Article

A Novel Binary Biofilm Coinfection Model for the Simultaneous Growth of Gram-Positive and Gram-Negative Bacterial Species

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ABSTRACT

Objectives: The aim of this study was to determine if it is possible to establish and maintain a binary biofilm consisting of Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria, which could be used as a multi-species model for antibiotic action studies. Materials and Methods: A medium controlled, continuous culture biofilm model was developed based upon the previously developed Sorbarod™ model. This new model was designed to enable the growth of *B. subtilis* and *E. coli* at the same time without either out-competing the other. Results: A pseudo-steady-state binary biofilm was established, which could be maintained for a total of 53 hours. This biofilm was tested to confirm the ability of the biofilm model to support the growth of a Gram-positive (*Bacillus subtilis*) and a Gram-negative (*Escherichia coli*) bacterial species under the same conditions of media throughput, aeration and temperature. Conclusions: This paper gives evidence of the ability to develop and control binary biofilm models to maintain the growth of two Gram-dissimilar species of bacteria. We believe this is a novel concept and will aid the future in vitro assessment of antibiotic activity in coinfection models.

Keywords: *Escherichia coli*, *Bacillus subtilis*, biofilm, coinfection.

INTRODUCTION

Most clinically-related microbiological studies investigate the growth and antibiotic sensitivity of bacterial species grown in pure culture. However, many clinical infections are composed of two or more species of bacteria growing in apparent harmony at a critical nidus within the human body. Multi-species infections, sometimes called coinfections, of this nature are notoriously difficult to treat with antibiotics and often require expensive and painful physical removal as well. Examples of such coinfections may include periodontal disease (coinfections with *Treponema denticola*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus* spp.[1] and burn and scald infections (common coinfective bacterial agents include: *Staphylococcus aureus*, Methicillin-resistant *S. aureus*, coagulase-negative staphylococci, *Enterococcus* spp., Vancomycin-resistant enterococci, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter* spp., *Proteus* spp., *Acinetobacter* spp. and *Bacteroides* spp.[2]). Several studies implicate such coinfections as resulting in the horizontal transfer of antibiotic resistance genes between apparently unrelated bacterial species[3,4,5].

The general perception of bacteria existing as autonomous single species populations is largely due to their extensive study in pure culture. In fact true mono-species biofilms are rarely observed in nature and most bacteria occur as part of complex multi-species communities[6]. The numbers of bacteria attached to surfaces have been estimated to be between 1000 and 10,000 times greater than the numbers of planktonic bacteria in any given environment[7]. This may be because the structure of the biofilm provides the bacteria with several advantages compared to those living as planktonic cells, with the development of increased antibiotic resistance being the most crucial among all the other properties conferred[8]. Biofilms also offer enhanced opportunities for interactions, such as horizontal gene transfer and co-metabolism[9]. In addition to all these properties, negative interactions in biofilms, including production of bacteriotoxins[10] and the lowering of environmental pH[11] by one member of the biofilm consortium also has been reported[12]. Biofilms are communities of microorganisms attached to a biotic or an abiotic surface. These are complex microbial associations, embedded in a self-produced extracellular matrix consisting of polysaccharide, protein and DNA[13]. Most of the bacteria found in natural, clinical and industrial settings have been observed to form biofilms which can either consist of a single microorganism or different types of microbes[14]. Studies indicate that biofilms are a stable point in a biological cycle that includes initiation, maturation, maintenance and dissemination. Bacteria seem to initiate biofilm development in response to specific environmental cues, such as nutrient availability, resistance to antimicrobial agents and predation and other factors[15]. The growth of biofilms is governed by the availability of

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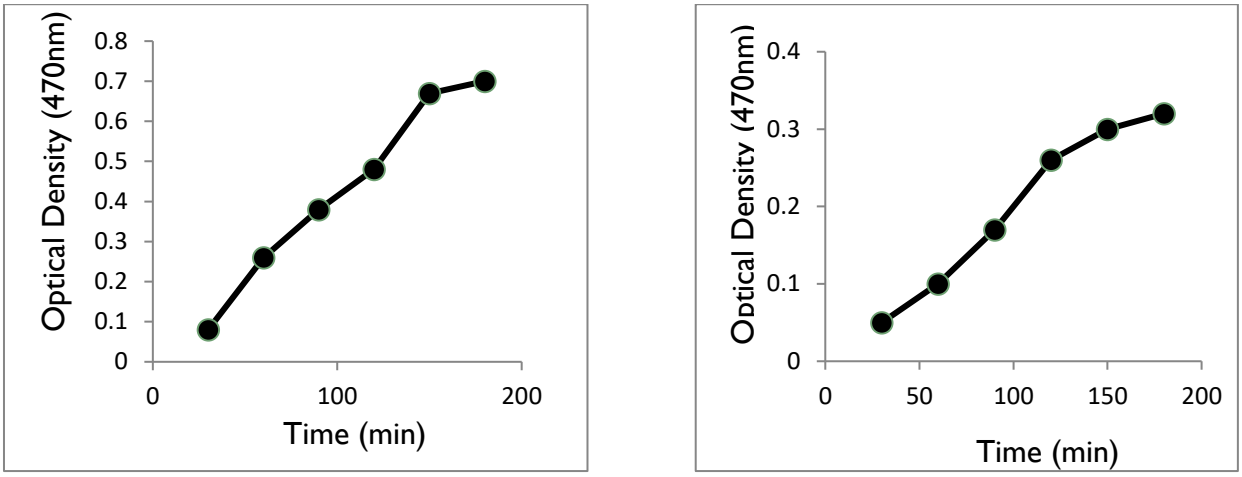

a b

Figure 1: The growth curve of Optical Density (470 nm) against time for (a) *Escherichia coli* and (b) *Bacillus subtilis* grown in Brain Heart Infusion Broth at 37ºC.

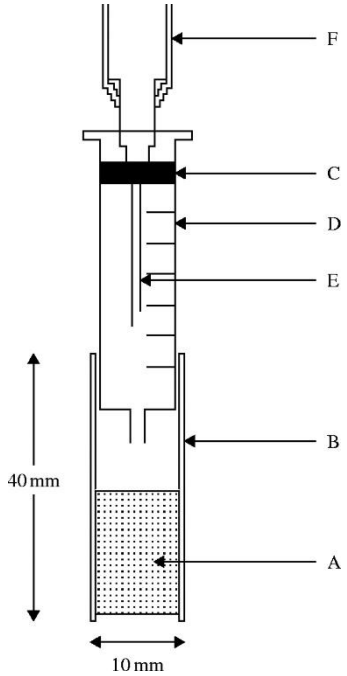

Figure 2: Schematic representation of a Sorbarod™ model. The Sorbarod™ filter (A) contained within a length of PVC tubing (B) has bacteria loaded onto it from a syringe. The plunger is withdrawn from a 2 mL syringe, leaving only the rubber seal within the syringe lumen (C). The syringe (D) is introduced into the PVC tubing containing the Sorbarod™ and a sterile needle (E) inserted through the rubber seal. The filter unit is then clamped upright and media inlet tubing (F) attached to the needle through which fresh medium is delivered. (From Gander *et al.*, 2005 [42]).

nutrients and if these are deprived, the microbes forming the biofilm can detach and return to a planktonic mode of growth[16,17].

Recent studies[18,19] have attempted to identify the species composition of multi-species biofilm infections *in situ* using techniques such as Pulsed-Field Gel Electrophoresis (PFGE). Whilst such techniques are useful in identifying the major causal agents of such infections, they do not allow the experimental control of these bacterial collaborations *in vitro*, nor their subsequent analysis for antibiotic sensitivity. It is the necessity to develop controllable multi-species coinfection biofilm models that underpins this study. Previous work from this laboratory[20] has shown that it is possible to develop simple and controllable biofilm coinfection models that allow the determination of antibiotic sensitivity of two Gram-negative bacterial species (*E. coli* and *Pseudomonas aeruginosa*) in a modified chemically defined medium (MCDM). In this study we exhibit the development of a novel binary biofilm model that supports the growth of a Gram-negative (*E. coli*) and a Gram-positive bacterial species (*Bacillus subtilis*) in the same complex medium (Brain Heart Infusion Medium), which is commonly used in antibiotic sensitivity tests.

MATERIALS AND METHODS

Microorganisms and Chemicals

Stock cultures of *Bacillus subtilis* (NCIMB 12900) and *Escherichia coli* (NCIMB 10000) were obtained from the National Collections of Industrial & Marine Bacteria (NCIMB), Aberdeen, UK. Cultures were sub-cultured in Brain Heart Infusion (BHI) prepared as either a broth or solid agar by the addition of 1.5% (w/v) technical agar. Culture identification was determined using Gram-stain reaction and the API 20 E and API 50 CH tests (Biom-erieux, France).

Preparation of Media

Brain Heart Infusion Medium (BHI) was prepared by adding 37g of the medium to 1L of distilled water, which was then mixed well and dissolved by heating with frequent agitation. In order to dissolve the media completely, it was boiled for a minute and then sterilised by autoclaving at 121˚C, 15psi for 15 minutes. The prepared medium was amber in colour and was stored at 2 to 8˚C until required. Technical agar (1.5% w/v) was added to BHI prior to autoclaving if a solid medium was required. Chromogenic *E. coli*/Coliform Medium (CM956; Oxoid LTD., Basingstoke, UK) is a differential agar, which

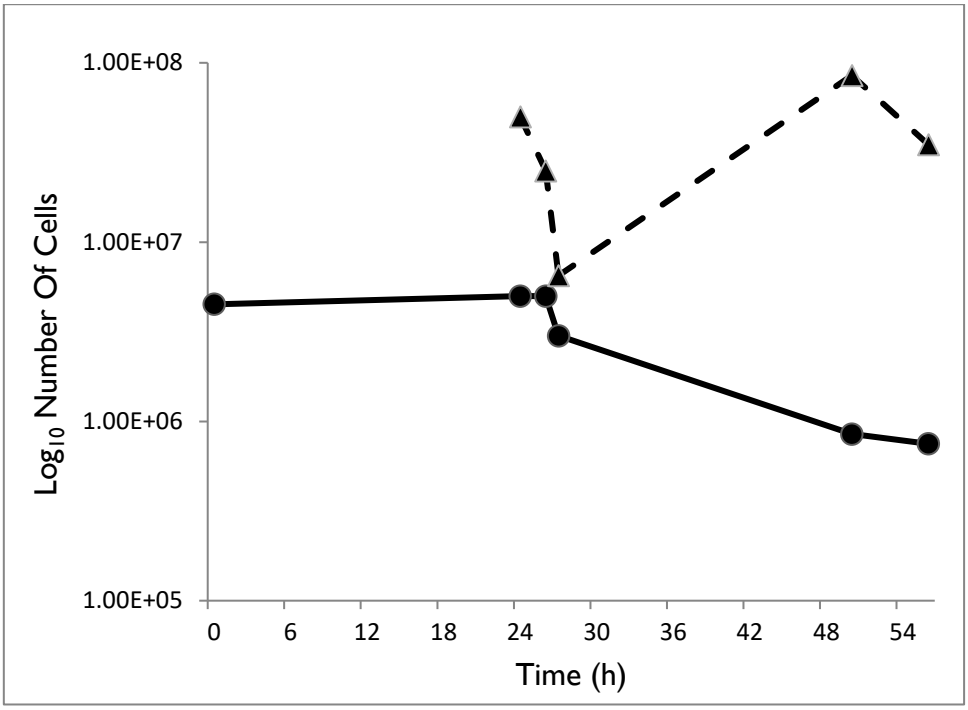

Figure 3: Logarithm (base10) of bacterial numbers assessed by plate count technique on Chromogenic *E. coli*/Coliform Medium (CM956; Oxoid LTD., Basingstoke, UK) against time (hours) exhibiting the attainment of "pseudo-steady state" in a binary biofilm model growing *Escherichia coli* (Solid triangles, broken line) and *Bacillus subtilis* (Solid circles, solid line) in Brain Heart Infusion Broth (BHI) at 37ºC. *Escherichia coli* was added to the Sorbarod™ model at 24 hours after addition of the *Bacillus subtilis* in order to allow the *B. subtilis* to become established prior to the addition of the faster growing *E. coli*.

provides presumptive identification of *E. coli* and coliforms in food and environmental samples. The agar contains two enzyme substrates to improve differentiation between *E. coli* and other coliforms. The first substrate allows specific detection of *E. coli* through the formation of purple colonies. This substrate is cleared by the enzyme glucuronidase, which is produced by approximately 97% of *E. coli* strains. The other substrate is cleaved by the enzyme galactosidase, which is produced by the majority of coliforms, resulting in rose/pink colonies[21]. In this study this medium was used to differentiate between two types of bacteria; *B. subtilis*, forming straw colonies and *E. coli*, forming purple colonies. Aliquots (55.8g) of this medium were suspended in 1L of distilled water. This medium was sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

Enumeration of Bacteria

The growth assay of *E. coli* and *B. subtilis* were performed at 37ºC by measuring their optical density at 470 nm against a medium blank (BHI) at measured time intervals. After observing each reading, the sample was replaced into the culture flask so as to prevent any significant changes in the volume and hence, surface area to volume ratio. This was performed for three hours at intervals of 30 minutes. In order to avoid shadowing effects[22], if the optical density at 470 nm was greater than 0.5, a dilution of the culture was made (1:10). The optical density of the dilution was taken and multiplied by 10 to give the true value. The diluted sample was discarded after observing the optical density.

A typical bacterial growth curve (Figure 1a) with a brief lag phase was followed by a distinct exponential phase was observed. Clear entry into stationary phase was not apparent for either species. For *E. coli*, the lag phase lasted for approximately 30 minutes and was followed by a comparatively longer log phase of approximately 2 to 2.5 hours. Thus, mid exponential phase was at 2.25 hours after inoculation. The doubling time of *E. coli* was approximately 35 minutes.

For *Bacillus subtilis* (Figure 1b), the cells were in a lag phase for approximately 40 minutes after which they entered the exponential phase. The exponential phase of the growth curve was long and lasted for about 2.5 to 3 hours with the mid exponential phase at 2.75 hours after inoculation. The generation time for *Bacillus subtilis* was calculated to be approximately 75 minutes. The final optical density (470 nm) achieved by *B. subtilis* was 0.32 as compared with a final optical density of 0.7 for *E. coli*, indicating a much poorer ability of *B. subtilis* to reproduce within the BHI medium.

Since, the generation/doubling time of *E. coli* (approximately 35 minutes) was faster when compared to that of *Bacillus subtilis* (approximately 75 minutes), it was decided that the Sorbarod™ device should be loaded with *B. subtilis* prior to *E. coli* in order to allow the *B. subtilis* to become established prior to the addition of the potentially out-competing *E. coli*[20].

Establishing the Binary Biofilm (Sorbarod™ Model)

In order to establish a binary biofilm, the variation on the Sorbarod™ model was used (Figure 2[20,23]). A Sorbarod™

filter (20 mm x 10 mm), which encases a compacted concertina of cellulose fibres, was inserted into one of the ends of PVC tubing (10 mm internal diameter). To the free end of this tube a 3 ml volume syringe was inserted whose plunger was removed, leaving the rubber seal within the syringe. This entire assembly was then autoclaved to ensure absolute sterility.

Sterile normal saline (0.9% w/v) was prepared and was used to pre-wet the Sorbarod™ filter. 5 ml of saline (0.9% w/v) was added on the surface of Sorbarod™ using aseptic technique. This was followed by the addition of 3 ml of an overnight culture of *B. subtilis* in BHI from a syringe in a drop-wise manner. A sterile, disposable needle (0.8 x 40 mm) was then inserted through the rubber seal held in the 3 ml syringe in order to allow the delivery of the medium to the Sorbarod™ surface. The media inlet tube was attached via the needle and delivered the desired amount of sterile BHI into the assembly[20]. The entire assembly was incubated at 37ºC and continuously perfused with fresh, aerated sterile BHI. The *B. subtilis* biofilm was allowed to mature for 24 hours after which 3 ml of an overnight culture of *E. coli* grown in BHI was introduced in the system in a drop wise fashion using aseptic technique. The Sorbarod™ biofilm was then run for a total of 53 hours so as to achieve a pseudo-steady-state for both the bacterial species and to establish a stable binary biofilm within the apparatus. During this period a series of eluate (spent medium overflow) samples were taken and tested for viable count on solid Chromogenic *E. coli* / coliform medium in order to determine viable counts of both *E. coli* and *B. subtilis*. Aliquots of eluate were taken at three-hour intervals.

RESULTS

A logarithmic plot (Figure 3) between the viable counts and time established the presence of a peudo-steady-state binary biofilm which was the main aim of the study. A steady-state biofilm is defined as one that has neither net growth nor decay over time[24]. The binary biofilm established as such achieved a pseudo-steady-state after 53 hours, hence underpinning the results obtained in the previous experiments using two Gram-negative bacteria (*E. coli* and *P. aeruginosa*[20]). The formation of the biofilm could be further confirmed by observing a sudden fall in the population size of *Bacillus subtilis* on the addition of *E. coli* to the biofilm model, which is due to the greater ability of *E. coli* to grow in the BHI medium than *B. subtilis* as exhibited by their differing growth rates in the medium in pure culture.

DISCUSSION

Leeuwenhoek's[25] early observations of bacterial growth were largely those of sessile or biofilm bacterial populations growing on other biotic surfaces (green weeds growing in water). It is an irony that in spite of such an early observation of biofilm growth, most subsequent research drifted away from these microbial associations towards their less common planktonic counterparts[26]. Research into biofilm formation began only in the early 1970s and was based on the assumption that biofilms are simple homogenous systems comprising of bacteria having similar properties as their planktonic counterparts[27]. However, in the more recent past and due to the negative impact of biofilms on human activities, research has aimed at providing clues for combating the detrimental effects of complex biofilms and their associated coinfections in clinical situations. Biofilms are one of the most ubiquitous entities found naturally occurring in the environment and possess the capacity to invade biotic and abiotic surfaces alike. In most natural settings, bacteria are found predominantly in biofilms[28,29,30]. Outside the host organism, a biofilm exists in the environment, such as on rock surfaces in liquid media and on pipelines used to transport fluids. Biofilms also infect the surfaces of medical implants, such as pacemakers, prosthetics and catheters. Inside the host, biofilms allow the pathogens to evade the immune response and are hence, associated with long-term illness[31].

Among a myriad of options available to grow the biofilm such as flow cell[32,33], microtiter dish assay system[34,35,36,37,38] and a number of bioreactors, the Sorbarod™ Model was chosen since it was very convenient to establish and was also cost effective. Furthermore, previous work by Novick[39] showed that a continuous bacterial growth can be best achieved in a continuous culture device, in this case the Sorbarod™ model[20,23]. According to Campbell[40] "*It is logically necessary that as long as the constant conditions prevail, growth will be balanced over any time interval"* and according to Herbert *et al.*,[41] microbial growth in a continuous culture takes place under steady state conditions, i.e. growth occurs at a constant rate and in a constant environment.

The objective of this study was to develop a binary biofilm of two Gram-dissimilar bacterial species (*E. coli* and *B. subtilis*) and to observe its stability by checking for the attainment of a pseudo-steady-state within the experimental model. This was achieved in our experiment due to a balance in bacterial numbers being maintained between the number of cells forming within the biofilm and the numbers of cells being collected as the eluate (biofilm spent medium) over a period of time. The growth curve of the binary biofilm clearly indicated a fall in the population size of *B. subtilis* upon the addition of *E. coli* to the binary culture. However, if there was no interaction between the two species, both of them would show independent growth and no such change in the population size would be observed. These results confirm that the aim of this experiment has been achieved in that it is possible to establish and maintain a binary biofilm consisting of Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*) bacteria.

CONCLUSION

Most research in clinical microbiology has been undertaken using pure cultures. However, in this study the two bacteria chosen markedly differ from each other such that one is a Gram-positive (*Bacillus subtilis)* and the other is a Gram-negative bacterium (*Escherichia coli*). These

two bacteria also differ in their habitat, as one exists as a commensal in the human gut (*E. coli*) while the other is extensively present in the environment usually associated with soil (*B. subtilis*). The main idea of selecting these two contrasting bacteria was to check for the development of a binary biofilm between them and further corroborating the same by achieving a steady-state in the system which was established using a Sorbarod™ model[20,23]. A continuous culture provides the opportunity to study the growth characteristics and antimicrobial sensitivity of the microorganisms over a period of time, which is not possible in case of a batch culture because of the constant depletion of nutrients.

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