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Observation with microcalorimetry: Behaviour of *P. aeruginosa* in mixed cultures with *S. aureus* and *E. coli*

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ABSTRACT

In this work, isothermal microcalorimetry (IMC) was used to study the behaviour of mixed cultures of *Pseudomonas aeruginosa* with *Staphylococcus aureus* and *Escherichia coli*. The species were firstly studied as pure culture at 10^6 CFU/mL densities in nutrient broth (NB). The species were then introduced into NB to give an equivalent inoculum of 10^6 CFU/mL of each. Additionally, the inoculum density of one species was maintained at 10^6 CFU/mL and the other varied between 10^3–10^5 CFU/mL. The IMC power-time curves were characteristic for the species in the medium. The total heat output (Q_t), amplitude of first peak (P_1), time of registration of first peak (t_1), final metabolic/maximum peak (P_{fmax}) and time of registration of the final metabolic/maximum peak (t_{fmax}) were calculated from the power-time data. A mixed culture of *P. aeruginosa* and *S. aureus* at equal densities exhibited metabolism synonymous to *P. aeruginosa* alone. When the density of *P. aeruginosa* was decreased, *S. aureus* gradually recovered showing power-time profiles that demonstrated this. Mixed cultures of *P. aeruginosa* and *E. coli* at equal densities showed power-time profiles representative of both species.

1. Introduction

Traditionally, *in vitro* microbial characterization, particularly susceptibility testings, are routinely conducted in pure culture. While these tests are useful, giving important insight on how a specific organism or species survives or adapts to a myriad of variables, the scenarios created may not accurately be representative of real-life situations. For example, the many infections in humans and animals, which occur as associations of two or more bacterial species, are not truly represented by these tests [1,2]. In isolation, a pure culture may behave much differently than when it is combined with other species. Within a community, it is expected that the different species will interact with each other with a resultant effect, which may differ from the effect of the individual component species [3,4]. This could have consequences on the response of the organism to a treatment, for instance, the efficacy of a selected therapeutic.

One main challenge is the lack of simple, fast, high throughput and inexpensive assays for the routine conduct of mixed species characterization. The constraints with traditional microbiological techniques (based on selective or differential spread plate methods) make it difficult for the routine performance of such assays to inform real-time interspecies relationships. The advent of molecular tools such as denaturing gradient gel electrophoresis (DGGE; [5]), randomly amplified polymorphic DNA (RAPD; [6]), real-time polymerase chain reaction (PCR), terminal restriction fragment length polymorphism analysis (T-RFLP; [7]), and length heterogeneity (LH)-PCR [8] which do not require culture for analysis have enabled the characterization of the functional diversity of mixed microbial samples. However, they have not been particularly useful for species-specific enumeration of mixed cultures, which is important for understanding of microbial interactions and the mechanisms underlying such relational dynamics. Nonetheless, quantitative PCR (qPCR; [9]), quantitative T-RFLP (qT-RFLP; [10,11]) and fluorescent in situ hybridization (FISH; [12]) have been successfully employed for quantitative analysis of mixed cultures, providing useful information about interactions between the different species of bacteria. These advanced techniques can however be expensive, require some expertise, reagents and the pretreatment of the culture before analysis. This warrants exploring other methods.

Isothermal microcalorimetry (IMC) is a technique which measures the heat flow of chemical, physical and biological processes. It has been reported to circumvent some of the limitations of traditional microbiologicial assay techniques and been widely applied in microbial characterization [13]. For instance, it has been used for monitoring and quantification of soil microbial activity and contamination [14,15]

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detection of infection and contamination of clinical products and samples [16]; determination of effects of antimicrobial compounds [17,18] including the mode of action of antimicrobial compounds-bacteriostatic or bacteriocidal [19], viral infections and activities of antiviral compounds [20,21] spoilage of food [22] amongst others. However, with all the microbiological applications, only a few studies have been conducted on mixed cultures with the aim of differential detection or to investigate the relationship between two or more bacteria [23].

In this study, we explored the potential of IMC to investigate the growth and behaviour of *Pseudomonas aeruginosa* in mixed culture with *Staphylococcus aureus* (which present as mixed infection in lung and chronic wound infections; [24] and with *Escherichia coli* (which occur in urinary tract infections).

2. Materials and methods

Staphylococcus aureus NCIMB 9518 and *Pseudomonas aeruginosa* NCIMB 8628 were obtained from ConvaTec Ltd. *Escherichia coli* ATCC 25922 was purchased from American Type Culture Collection, USA. The strains were grown in Nutrient broth (NB, Oxoid) in a shaking incubator (Innova 4080, New Brunswick Scientific, UK) at 37 °C. The cells were harvested, washed in phosphate buffered saline (PBS) and resuspended in 15% v/v glycerol in ¼ th strength Ringer's solution to a density of 10^8 CFU/mL when they reached the stationary phase of growth. The resuspended cells were frozen in 1.8 mL aliquots in vials over liquid nitrogen and stored in liquid nitrogen until required [25].

A vial was removed from the storage liquid nitrogen container, thawed at 40 °C for 3 min and vortexed for 1 min prior to use. 30 µL of the thawed culture of respective species was inoculated into 2970 µL of pre-warmed NB in a sterile 3 mL calorimetric glass ampoule giving a density of 10^6 CFU/mL for pure culture experiments. For mixed culture experiments, one species was inoculated at a constant density of 10^6 CFU/mL and mixed with different densities of the other (10^3–10^6 CFU/mL) in a 3 mL calorimetric ampoule. The ampoules were sealed with crimped caps and vortexed for 10 s. They were placed in the thermal equilibration position of a Thermometric Thermal Activity Monitor 2277 (TAM 2277, TA Instruments Ltd., UK) set at 37 °C (± 0.1 °C). The loaded ampoules were allowed to equilibrate at this intermediate position for 30 min before being lowered into the measurement position. Data were captured with Digitam 4.1 every 10 s with an amplifier range of 1000 µW until the power-time data returned to baseline. Experiments were performed in triplicate. Data were analysed with Origin Pro 8.6 (Microcal Software Inc.). The reference ampoule was loaded with 3 mL of sterile media. The instrument was calibrated at regular intervals.

Relative growths of the species were determined after microcalorimetric measurements by plating 50 µL of serially diluted cultures on Cetrimide agar (Oxoid, Basingstoke, UK), Mannitol salt agar (Oxoid, Basingstoke, UK) and MacConkey agar (Oxoid, Basingstoke, UK). pH measurements (pHenomenal®, UK) were also done post calorimetric experiments. All procedures were carried out aseptically.

3. Results and discussion

IMC records power (µW or μJs^{-1}) as a function of time (t). The growth of microorganisms in the microcalorimeter typically results in an exponentially increasing signal (representative of the heat produced by the growing microorganism) until the concentration of the energy source becomes limiting, and there is a build up of toxic metabolites, in which case the power signal approaches baseline. The data in Fig. 1, which compare the power-time growth curves of *P. aeruginosa*, *S. aureus* and their mixed culture at equal densities, illustrate this. The power-time curve of *P. aeruginosa* shows increasing signal with two metabolic peaks; the second with greater energy and without significant lag after the first. *S. aureus* shows initial peak as *P. aeruginosa* but shows another

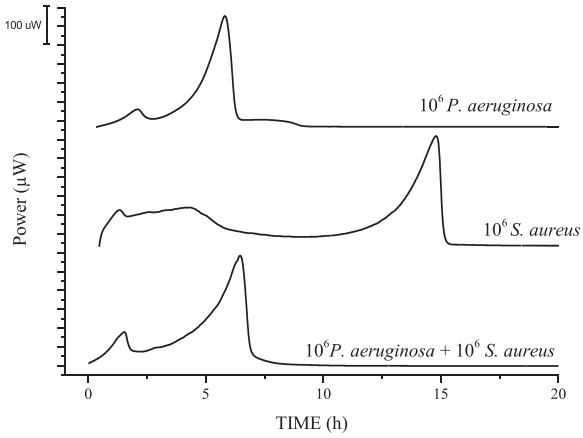

Fig. 1. Comparison of the power-time growth curves of pure cultures of *P. aeruginosa, S. aureus* and a mixed culture in NB at same inoculum density (10^6 CFU/mL). Growth curve of mixed culture share similarities with *P. aeruginosa.*

metabolic phase, followed by a long period of latency then a final metabolic peak of intensity greater than the first two metabolic phases. The main difference between the profile of *P. aeruginosa* and *S. aureus* is the duration and metabolic activity preceding the final exponential metabolic phase.

To express the data in a more quantitative way, the total heat output (Q_t) which is the total area under growth curve (AUC), amplitude of first peak (P_1), time of registration of first peak (t_1), final metabolic/maximum peak (P_{fmax}) and time of registration of the final metabolic/maximum peak (t_{fmax}) were calculated from the power-time data and this shows significant differences ($p < 0.05$) in Q_t, P_1, t_1 and t_{fmax} between the two species (Table 1). The growth profile of mixed cultures of the two species shows an initial metabolic peak and a second of greater intensity. There is insignificant lag between the two peaks but a slight metabolic activity can be seen in the curve prior to the final exponential phase. The power-time data for the mixed culture shows significant differences ($p < 0.05$) in P_1 and t_{fmax} between the mixed culture and pure *S. aureus* but no significant differences were seen between the former and pure *P. aeruginosa*. Overall, when the duration and metabolic phases are considered, the power-time profile of the mixed culture shows more similarities to *P. aeruginosa* than *S. aureus.* These factors are one reason that perhaps the growth of *P. aeruginosa* dominated in the mixed culture although it can also be argued that an interaction occurred between the two species.

The mixed cultures of *P. aeruginosa* and *S. aureus* at different densities of the two are compared in Fig. 2. The profile of the mixed cultures appears to revert from the dominating characteristic profile of *P. aeruginosa* as the density of *P. aeruginosa* is decreased to curves that show prominence of *S. aureus*. The derivatives from the power-time data support this observation. Although not very reflective in the Q_t values, the P_1 and t_1 values show significant differences ($p < 0.05$) between pure *S. aureus* and its mixed cultures with *P. aeruginosa* at higher densities of *P. aeruginosa. P. aeruginosa* also shows significant differences ($p < 0.05$) in P_1, t_1 and t_{fmax} values when its density is smaller relative to *S. aureus* in the mixed cultures. The data may imply some interaction between the two species in mixed cultures. The data also suggest that *P. aeruginosa* may have dominated at low densities of *S. aureus* while *S. aureus* dominated at lower densities of *P. aeruginosa*. However, overall, the profiles of the mixed cultures share more similarities to that of *P. aeruginosa*. The pH measurement and viable cell count determination after microcalorimetric experiments are given in Table 2. The metabolism of *S. aureus* showed more acidic waste than that of *P. aeruginosa*. The plate count data showed that *S. aureus* was inhibited in growth especially when its inoculum density was lower than that of *P. aeruginosa* in the mixed cultures.

The growth curves of *P. aeruginosa* and *E. coli* are shown and