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MEASURING THE GROWTH OF PSEUDOMONAS MEAT SPOILAGE BACTERIA ON SURFACES

KAYE J COATES[1], RICHARD K LUKE[2] AND PHILLIP R WIDDERS[1]
[1] Victorian Institute of Animal Science, Attwood, Victoria, Australia
[2] LaTrobe University, Bundoora, Victoria, Australia

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Background

The behaviour of bacteria on meat and other complex surfaces is not well understood. Typically, generalisations about the growth of bacteria on meat are extrapolations of data generated from liquid laboratory cultures. Bacteria in such cultures may behave differently from those cultured on solid or contoured surfaces. In addition, plate counts from swabbed or stomached samples are generally used to enumerate organisms from the meat surface. These methods rely on removal of bacteria from a surface. If all bacteria are not removed from the surface, counts will not be accurate. If the percent recovery were to remain constant, accurate estimates of surface contamination could be made. However, if percent recovery is affected by colonisation, the accessibility of the microorganisms and/or the number of bacteria present on the meat surface, such counts can lead to inaccurate estimates of surface contamination. These factors serve to decrease the accuracy of measurement of bacterial growth rates on meat surfaces

In the present study, an assay based on the reduction of a tetrazolium salt by bacterial dehydrogenase was used to assess bacterial numbers *in situ*. In this system the intensity of the formazan dye produced is proportional to the number of organisms initially present in the system. Experiments designed to investigate the effect of microtopography on bacterial growth rate are described. Growth on two different forms of dacron, a synthetic surface, is compared to that on meat.

Experimental

Bacteria

A *Pseudomonas* biotype (VIAS 1) isolated from spoiled pork was used (VIAS culture collection). Prior to use, bacteria were cultured on sterile meat for 2d at 25°C

Surfaces

Dacron, in two physical forms ('flat', similar to paper; 'fibrous', similar to cottonwool) was sterilised at 121°C. Sterile meat was prepared by aseptically cutting discs of meat from slices of surface sterilised pigmeat. Replicate dacron pieces and meat discs were placed aseptically in 24-well tissue culture trays for the assays.

MTT Standard Curve

An assay based on bacterial dehydrogenase activity (Peck 1985) was adapted as an alternative to the colony forming unit (CFU) assay in order to estimate bacterial numbers without the need for prior separation of bacteria. A suspension (approximately 10^8 cells/ml) of VIAS1 was prepared in broth. Doubling dilutions (10^8 to <10 organism/ml) were prepared (28 tubes) and from each tube duplicate samples (100µl) were placed in wells of a microtitre tray and incubated at 21°C for 24 hours. After 24h incubation, 10µl of an aqueous solution of 3(4,5-dimethylthiazoyl-2-yl)2,5-diphenyltetrazolium bromide, 5mg/ml (MTT, Sigma) were added to each well of a microtitre tray. Trays were shaken for 25min and incubated at 21°C for a further 15min. Absorbance of the suspension in each well was read at 540nm in a microtitre plate reader (Flow) fitted with Skansoft software. A standard curve of bacterial numbers against OD_{540} in the assay was plotted. There was a strong correlation (R^2=0.98) between absorbance (540nm) in the MTT assay and original numbers of bacteria in assay wells. Points on the curves were averaged and the regression found to be linear, Abs = 0.23 (log counts) + 0.07 (R^2 = 0.98).

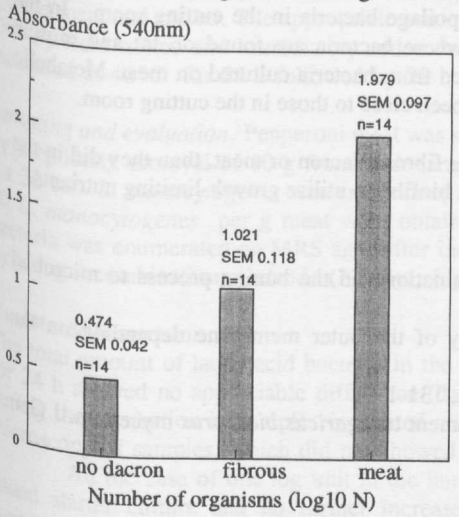

Fig 1. Effect of surface on growth of VIAS1 organisms as assessed by MTT assay.

Dacron Growth Assay

Meat, no dacron, flat dacron and fibrous dacron were added to 24-well tissue culture trays A suspension of VIAS1 (absorbance$_{610}$ 0.13 in broth) was added to test wells (1.5ml/well). Sterile broth (1.5ml/well) was added to control wells. Trays were incubated overnight at 21°C and a MTT assay performed on each well. There was no significant difference in the rate of growth of bacteria in wells containing no dacron or flat dacron. As shown in figure 1 there were significant differences between growth in the absence of dacron, growth in the presence of fibrous dacron and growth on meat ($p<0.005$). These apparent differences in growth rates of VIAS1 in the presence of extra surface were subsequently confirmed by counts of radio-labelled bacteria.

Assessment of bacterial growth through incorporation of tritiated thymidine (^{3}HTdR)

Sterile (24 well) tissue culture plates were set up with 12 wells containing no dacron and 12 wells containing fibrous dacron. For each group, 6 wells were designated "labelled" and 6 were designated "unlabelled". To the "unlabelled" wells, 1ml suspension of VIAS1 (65%T) in RPMI was added. "Labelled" wells were treated identically except that ^{3}HTdR was added to a final concentration of 4µCi/ml in the suspension. Plates were incubated at 21°C for 48h and on ice for a further 30min

prior to filtration. A sample (100µl) was removed from each well for estimation of bacterial numbers as determined by plate counts. Well contents, collected by filtration, were monitored by scintillation for incorporation of label into the trapped bacteria (label was added to "cold" wells immediately prior to collection). There was no significant quenching of scintillation counts by any component of the system. There was no significant radioactivity in any wells where bacteria grew in the absence of label. Where wells contained labelled bacteria there were significantly ($p<0.001$) more scintillation counts where cells had grown in the presence of fibrous dacron (table 1).

Table 1. Effect of surface on growth of VIAS1 organisms as assessed by uptake of ^{3}HTdR in replicate experiments. Activity is expressed as percent of maximum incorporation of label.

Surface type	With label	No label
No dacron	41.7%	0
Fibrous dacron	100%	<0.1%

While the ^{3}HTdR results confirms the earlier result that growth is stimulated in the presence of fibrous dacron, MTT tests on the contents of these wells revealed no such difference (no dacron 0.7984, SEM 0.022: fibrous dacron 0.8752, SEM 0.0979). This discrepancy may be a function of the difference in assay formats in that there is no allowance in the MTT assay for dead cells or for enzyme which may have lost its activity. Only live capable of producing dehydrogenase are measured in the MTT assay. The inability of the MTT assay to measure, at a single time point, all enzyme produced from all cells for the duration of the culture could explain the discrepancy in estimated cell numbers between the MTT assay and ^{3}HTdR counts. The MTT assay may be limited to measurement of bacterial cells in exponential phase of growth.

Discussion

The MTT assay has the advantage of measuring numbers of bacteria *in situ*. In most studies where contamination of meat with bacteria has been examined, counts have been based on removal of bacteria from the surface. Disadvantages of the methods include failure to remove all bacteria and inconsistent removal. Neither meat nor dacron affected the development of the formazan dye, and colour development was found to be linearly related to the initial numbers of bacteria. With a similar assay, a detection sensitivity of 12 to 12800 bacteria/100µl was achieved (Peck, 1985).

Dacron was used as the artificial surface in the growth assays, in part because it is available in two different physical forms, one flat and the other fibrous. Confirmation of increased growth by bacterial uptake of tritiated thymidine would not be possible on a meat surface as endogenous thymidine would have masked uptake of exogenous, labelled thymidine.

The present study has shown that the growth rate of *Pseudomonas* VIAS1 was increased in the presence of additional surface, whether this was dacron or meat. It was assumed that this improved growth rate was a reflection of improved growth conditions in the micro-environment of a biofilm, perhaps by increased nutrient availability and/or synergism between neighbouring bacteria.

Generally, bacteria cultured in laboratory broth medium have been used as the seeding organisms in studies of growth of bacteria on meat. Rainey (1991) has shown that the medium in which bacteria are cultured has a significant effect on the ability of the organisms to attach to surfaces. In studies on the growth of organisms in liquid environments (Horsky et al, 1993), it was found that pre-culturing in liquid medium did not significantly alter the findings. However, in order to predict accurately the behaviour of meat spoilage organisms, it is important to use seeding bacteria grown under conditions closest to those encountered by spoilage bacteria in the cutting room. In the cutting room, the major sources of spoilage organisms are the carcasses and the boards, where bacteria are found on fat and muscle residues (Coates et al, 1995). In the studies described here, seeding suspensions were prepared from bacteria cultured on meat. Metabolic characteristics of these organisms and the conformation of the surface, should therefore have been close to those in the cutting room.

In summary, bacteria were shown to grow faster in association with complex surfaces, such as fibrous dacron or meat, than they did in the absence of such surfaces. This increase in growth may reflect enhanced ability of bacteria in a biofilm to utilise growth-limiting nutrients.

References

Coates KJ, Beattie JC, Morgan IR, Widders PR. 1995. The contribution of carcass contamination and the boning process to microbial spoilage of aerobically stored pork. Food Microbiology 12, 49-54.

Horsky E, Pokorny J, Labajova M. 1993. Changes of surface charge and hydrophobicity of the outer membrane depending on the cultivation method. Biologia, Bratislava. 48, 343-347.

Peck R. 1985. A one-plate assay for macrophage bactericidal activity. J Immunol Methods 82, 131-140.

Rainey PB. 1991. Phenotypic variation of *Pseudomonas putida* and *P. tolaasii* affects attachment to *Agaricus biosporus* mycelium. J Gen Microbiol. 137, 2769-2779.