Effects of Dilution Rate on Biomass and Extracellular Enzyme Production by Three Species of Cutaneous Propionibacteria Grown in Continuous Culture

By J. GREENMAN† AND K. T. HOLLAND*

Department of Microbiology, University of Leeds, Leeds LS2 9JT, UK

(Received 26 November 1984; revised 5 February 1985)

Propionibacterium acnes, *P. avidum* and *P. granulosum* were grown in continuous culture at a range of dilution rates on a semi-synthetic medium. Dilution rates were chosen to allow the bacteria to grow at the same relative growth rates as compared to their respective $\mu_{\max}$ values. The steady-state levels and production rates of biomass and extracellular enzymes were determined. The lipase and hyaluronate lyase of *P. granulosum* and the proteolytic activity of *P. acnes* and *P. avidum* were growth linked enzymes (i.e. they were produced at constant amounts per unit of biomass). In contrast, the lipase, hyaluronate lyase and acid phosphatase of *P. acnes* and the lipase of *P. avidum* were shown to be non-growth linked enzymes.

INTRODUCTION

The interactions of propionibacteria with human skin are poorly defined and controversial. Circumstantial evidence suggests that *Propionibacterium acnes* plays a role in the development of inflammatory acne lesions (Holland *et al.*, 1981). Holland *et al.* (1978) hypothesized that micro-environmental changes in sebaceous follicles are major factors in determining the physiology of the bacterial inhabitants, with consequent effects on the follicle due to the activities of the bacteria. Propionibacteria are not only associated with acne lesions but are also found on normal skin. *P. acnes* constitutes the major fraction of the microflora in sebaceous gland rich areas of adult human skin i.e. face, back and chest (Johnson & Cummins, 1972; McGinley *et al.*, 1978). *P. granulosum* and *P. avidum* have been isolated from these areas in lesser numbers (McGinley *et al.*, 1978). *P. avidum* also colonizes skin of high humidity, i.e. axilla and groin (McGinley *et al.*, 1978). Propionibacteria can be isolated both from the skin surface and from the pilosebaceous ducts (Puhvel *et al.*, 1975; Leeming *et al.*, 1984).

A number of extracellular enzymes are produced by the skin propionibacteria, including lipase (Hassing, 1971; Kellum *et al.*, 1970; Ingham *et al.*, 1981), hyaluronate lyase (Puhvel & Reisner, 1972; Ingham *et al.*, 1979), protease and DNAase (Marples & McGinley, 1974; Ingham *et al.*, 1983) and acid phosphatase (Ingham *et al.*, 1980) which may play a role in the interactions of the bacteria with their host.

The following environmental factors have been shown to affect the growth and physiology of the skin propionibacteria *in vitro*: pH (Greenman *et al.*, 1983), nutritional changes (Holland *et al.*, 1979; Greenman *et al.*, 1981) and O₂ concentration (Cove *et al.*, 1983).

The effects of varying the dilution rate of continuous cultures of organisms on the production of extracellular enzymes is of particular interest. Extracellular enzyme production may be closely linked to the dilution rate and, therefore, to the growth rate of the cells; alternatively, the

† Present address: Department of Science, Bristol Polytechnic, Bristol, UK.

relationship may be a more complicated function. The aim of this study was to determine the effects of dilution rate on the production of extracellular enzymes by cutaneous propionibacteria.

METHODS

Organisms and media. *Propionibacterium acnes* (laboratory strain P37), *P. avidum* [laboratory strain PF77(i)] and *P. granulosum* [laboratory strain PF208(ii)] were isolated, identified and maintained as described by Greenman *et al.* (1981, 1983). The medium was that used by Eaves *et al.* (1979). Glucose solution (10%, w/v) was added aseptically to a final concentration of 0.2% (w/v) for *P. granulosum* which requires glucose in order to produce sufficient biomass (Greenman *et al.*, 1981, 1983).

Continuous culture apparatus and conditions. The organisms were grown in a one litre culture vessel with control modules for temperature, pH, gas flow and stirrer rate (series 500, LH Engineering, Stoke Poges, Bucks, UK). The growth conditions were those used by Greenman *et al.* (1983). The pH was maintained at 5.5 ± 0.1 by the automatic addition of 2 M-NaOH or 2 M-HCl. The dilution rates were set to allow a range of relative growth rates (μ_{rel}) from 0.075 to 0.9 for each of the three species of *Propionibacterium* (Table 1).

Biomass and maximum specific growth rates. These were determined as described by Greenman *et al.* (1983). The dilution rates used for washout were: *P. acnes* 0.25 h^{-1} , *P. avidum* 0.28 h^{-1} and *P. granulosum* 0.2 h^{-1} . Production rate of biomass was expressed as $\text{g l}^{-1} \text{ h}^{-1}$.

Extracellular enzymes. Lipase (EC 3.1.1.3), hyaluronate lyase (EC 4.2.2.1) and acid phosphatase (EC 3.1.3.2) activities were assayed by the methods used by Holland *et al.* (1979). Proteolytic activity was assayed by the method of Millet (1970). Ingham *et al.* (1983) have reported that several proteinases are produced by propionibacteria. Therefore, the term proteolytic activity is used in this paper because no attempt was made to assay independently the different proteinases. All units of activity (U) are $\mu\text{mol end-product h}^{-1}$, apart from the proteolytic activity which is $A_{440} \times 200/T$ where T is the time of incubation of the assay mixture. Specific enzyme activities are expressed as U (mg biomass) $^{-1}$, and production rate of extracellular enzyme activity as U (mg biomass) $^{-1} \text{ h}^{-1}$. The extracellular enzymes have different stabilities at different pH values. The corrected production rates at pH 5.5 were calculated from the measured activities using a correction formula $[(\lambda + D) \text{ measured activity}]/D$, where D is the dilution rate and λ is the denaturation constant for the enzyme at a particular pH (Eaves *et al.*, 1979; Greenman *et al.*, 1983).

Steady state. Biomass and extracellular enzyme activities were determined on samples taken from the chemostat during steady state growth conditions at the different relative growth rates that were selected. Only after a minimum of six culture volume changes had occurred between particular conditions was a steady state condition considered to have been achieved.

Statistical analysis. Linear regression analysis was used to determine the slope of the curve for the wash-out data for calculation of μ_{max} . For biomass and extracellular enzyme production data the standard error of the mean was computed, and with the number of samples the $\pm 95\%$ confidence limits calculated.

RESULTS

Cell biomass and extracellular enzyme production were measured for each species of *Propionibacterium* grown at a steady state using a range of dilution rates. The dilution rates chosen (Table 1) enabled the three species to be compared whilst growing at the same fraction of their respective μ_{max} values obtained under the same conditions of temperature and pH.

Table 1. Dilution rates used to obtain the relative specific growth rates for *P. acnes*, *P. avidum* and *P. granulosum*

Relative growth rate	Steady-state dilution rate, D (h^{-1})		
	<i>P. acnes</i>	<i>P. avidum</i>	<i>P. granulosum</i>
0.075	0.013	0.014	0.010
0.150	0.026	0.027	0.021
0.300	0.051	0.055	0.041
0.600	0.102	0.109	0.082
0.900	0.154	0.164	0.123

Fig. 1

Fig. 2

Fig. 1. Effect of relative growth rate on biomass production rate of *P. acnes* (○), *P. avidum* (●) and *P. granulosum* (□). The bars represent 95% confidence limits about the mean.

Fig. 2. Effect of relative growth rate on production of lipase (corrected for denaturation) by *P. acnes* (○), *P. avidum* (●) and *P. granulosum* (□). The bars represent 95% confidence limits about the mean.

Biomass. Biomass production increased with increasing growth rates over the range 0.075 to $0.6 \mu_{\max}$ for all three species of *Propionibacterium* (Fig. 1). At the highest dilution rate ($0.9 \mu_{\max}$) *P. acnes* biomass production was higher than at $0.6 \mu_{\max}$ whereas biomass production by *P. avidum* remained the same. In contrast, the biomass production rate for *P. granulosum* decreased from the value obtained at $0.6 \mu_{\max}$.

Lipase. The specific activities of lipase for the three species at the different dilution rates are shown in Fig. 2. For *P. acnes*, the production of lipase per unit biomass decreased with increasing relative growth rate, whilst the other two species maintained an approximately constant production of this enzyme. *P. avidum* had a lower production of lipase per unit biomass at relative growth rates approaching a value of 1. The production rates for lipase for *P. acnes* and *P. avidum*, for measured activity and corrected for denaturation, are shown in Fig. 3. The results demonstrate the difference in production rate of active enzyme and total enzyme, and the variation of this difference with the increasing relative growth rate of the bacteria. *P. avidum* and *P. granulosum* (results not shown) have increased production rates with increasing relative growth rate up to $0.6 \mu_{\max}$ whilst *P. acnes* shows only minor variation.

Hyaluronate lyase. The specific activities of hyaluronate lyase for *P. acnes* and *P. granulosum*, the two species that produce this enzyme, are shown in Fig. 4. *P. granulosum* production per unit biomass did not alter with increasing relative growth rate, whilst *P. acnes* had an optimum production at $0.15 \mu_{\max}$ which decreased with increasing relative growth rate. The production rates of hyaluronate lyase (results not shown) increased with increasing relative growth rate up

Fig. 3

Fig. 4

Fig. 3. Effect of relative growth rate on lipase production rate by *P. acnes* (○) and *P. avidum* (●). —, Values corrected for denaturation; ----, values uncorrected. The bars represent 95% confidence limits about the mean.

Fig. 4. Effect of relative growth rate on production of hyaluronate lyase (corrected for denaturation) by *P. acnes* (○) and *P. granulorum* (□). The bars represent 95% confidence limits about the mean.

to 0.3 for *P. granulorum* and 0.6 for *P. acnes*. The production rate of undenatured enzyme was approximately 90% of the production rate of total enzyme.

Proteolytic activity. The specific activities of the proteinases for *P. acnes* and *P. avidum*, the two species that produce these enzymes, are shown in Fig. 5. The activity produced per unit biomass rose from 0.075 to 0.3 μ_{rel} , and then changed little whilst production of this activity by *P. avidum* showed a minor peak at 0.3 μ_{rel} . The production rates of proteolytic activity (results not shown) for both species increased steadily with increasing relative growth rate.

Acid phosphatase. Extracellular acid phosphatase is produced only by *P. acnes*; its production per unit biomass decreased steadily (by about 5-fold) with increasing relative growth rate from 0.15 to 0.9 (Fig. 6). The production rate of acid phosphatase increased with increasing relative growth rate up to 0.6 (results not shown). The rate of production of undenatured enzyme was approximately half that of the total enzyme.

DISCUSSION

All experiments were performed at dilution rates which allowed the bacteria to grow at the same relative growth rates compared to their respective μ_{max} values (Tempest, 1976). This method (rather than using the same absolute dilution rates) allowed for comparisons of *P. acnes* and *P. avidum*. *P. granulorum* has a requirement for glucose in a tryptone base medium (Greenman *et al.*, 1981). In the absence of glucose, cultures are, therefore, limited by amino acids supplied by the tryptone which act as nitrogen and carbon/energy sources (Holland *et al.*, 1979; Greenman *et al.*, 1981). Addition of 2% (w/v) glucose to *P. granulorum* cultures merely spares amino acids that would otherwise be used as carbon/energy sources. Consequently, strict comparisons between *P. granulorum* and the other two species cannot be made.

Fig. 5

Fig. 6

Fig. 5. Effect of relative growth rate on production of proteolytic activity by *P. acnes* (○) and *P. avidum* (●). The bars represent 95% confidence limits about the mean.

Fig. 6. Effect of relative growth rate on production of acid phosphatase (corrected for denaturation) by *P. acnes* (○). The bars represent 95% confidence limits about the mean.

In general, where conditions in the experiments such as pH, oxygen tension and dilution rate are like those used by Greenman *et al.* (1983) and Cove *et al.* (1983), the results for biomass and extracellular enzyme production are in good agreement.

The production rates of bacterial extracellular enzymes were estimated taking into account the denaturation constant (λ) of these enzymes under the conditions of growth of the bacteria (Eaves *et al.*, 1979). The constants were determined experimentally using a mixture of the enzymes produced by the particular species. This method accounts for non-specific and proteinase effected denaturation of extracellular enzymes and, therefore, gives a more accurate value of production rate than that measured directly.

The lipase and hyaluronate lyase of *P. granulosum* and the proteinases of *P. acnes* and *P. avidum* are growth linked since they are produced in nearly constant amounts per unit biomass at varying relative growth rates. This suggests that different carbon/energy sources are used by these bacteria in their natural environment, *P. granulosum* using products from triglycerides of sebum and hyaluronic acid of the host intercellular matrix, and *P. acnes* and *P. avidum* using protein. It is interesting to note that there are no reports of production of extracellular proteinases by *P. granulosum*, and that this species requires some form of carbohydrate for growth (Greenman *et al.*, 1981; Holland *et al.*, 1979). The importance and function of the non-growth linked enzymes of *P. acnes* and *P. avidum* to the growth and survival of these bacteria on human skin has yet to be determined. Ingham *et al.* (1981) suggested that fatty acids may be a substitute for biotin for these bacteria. In this case, the lipase acting on sebum triglycerides may be the means by which biotin limitation is averted.

The authors wish to acknowledge the financial support of the Medical Research Council throughout this study.

REFERENCES

- COVE, J. H., HOLLAND, K. T. & CUNLIFFE, W. J. (1983). Effects of oxygen concentration on biomass production, maximum specific growth rate and extracellular enzyme production by three species of cutaneous propionibacteria grown in continuous culture. *Journal of General Microbiology* **129**, 3327–3334.
- EAVES, G., GREENMAN, J. & HOLLAND, K. T. (1979). Decay rates of exocellular enzymes produced by bacteria – correction factor of production rates in chemostat cultures. *FEMS Microbiology Letters* **6**, 333–336.
- GREENMAN, J., HOLLAND, K. T. & CUNLIFFE, W. J. (1981). Effects of glucose concentration on biomass, maximum specific growth rate and extracellular enzyme production by three species of cutaneous propionibacteria grown in continuous culture. *Journal of General Microbiology* **127**, 371–376.
- GREENMAN, J., HOLLAND, K. T. & CUNLIFFE, W. J. (1983). Effects of pH on biomass, maximum specific growth rate and extracellular enzyme production by three species of cutaneous propionibacteria grown in continuous culture. *Journal of General Microbiology* **129**, 1301–1307.
- HASSING, G. S. (1971). Partial purification and some properties of lipase from *Corynebacterium acnes*. *Biochimica et biophysica acta* **242**, 381–394.
- HOLLAND, K. T., CUNLIFFE, W. J. & ROBERTS, C. D. (1978). The role of bacteria in acne – a new approach. *Clinical and Experimental Dermatology* **3**, 253–257.
- HOLLAND, K. T., GREENMAN, J. & CUNLIFFE, W. J. (1979). Growth of cutaneous propionibacteria on synthetic medium; growth yields and exoenzyme production. *Journal of Applied Bacteriology* **47**, 383–394.
- HOLLAND, K. T., INGHAM, E. & CUNLIFFE, W. J. (1981). The microbiology of acne. *Journal of Applied Bacteriology* **51**, 195–215.
- INGHAM, E., HOLLAND, K. T., GOWLAND, G. & CUNLIFFE, W. J. (1979). Purification and partial characterization of hyaluronate lyase (EC 4.2.2.1) from *Propionibacterium acnes*. *Journal of General Microbiology* **115**, 411–418.
- INGHAM, E., HOLLAND, K. T., GOWLAND, G. & CUNLIFFE, W. J. (1980). Purification and partial characterization of an acid phosphatase (EC 3.1.3.2) produced by *Propionibacterium acnes*. *Journal of General Microbiology* **118**, 59–65.
- INGHAM, E., HOLLAND, K. T., GOWLAND, G. & CUNLIFFE, W. J. (1981). Partial purification and characterization of lipase (EC 3.1.1.3) from *Propionibacterium acnes*. *Journal of General Microbiology* **124**, 393–401.
- INGHAM, E., HOLLAND, K. T., GOWLAND, G. & CUNLIFFE, W. J. (1983). Studies of the extracellular proteolytic activity produced by *Propionibacterium acnes*. *Journal of Applied Bacteriology* **54**, 263–271.
- JOHNSON, J. L. & CUMMINS, C. S. (1972). Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria and strains of *Arachnia propionica*. *Journal of Bacteriology* **109**, 1047–1066.
- KELLUM, R. E., STRANGFELD, K. & RAY, L. F. (1970). Acne vulgaris. Studies in pathogenesis: triglyceride hydrolysis by *Corynebacterium acnes* in vitro. *Archives of Dermatology* **101**, 41–47.
- LEEMING, J. P., HOLLAND, K. T. & CUNLIFFE, W. J. (1984). The microbial ecology of pilosebaceous units isolated from human skin. *Journal of General Microbiology* **130**, 803–807.
- MARPLES, R. R. & MCGINLEY, K. J. (1974). *Corynebacterium acnes* and other anaerobic diphtheroids from human skin. *Journal of Medical Microbiology* **7**, 349–357.
- MCGINLEY, K. J., WEBSTER, G. F. & LEYDEN, J. J. (1978). Regional variations of cutaneous propionibacteria. *Applied and Environmental Microbiology* **35**, 62–66.
- MILLET, J. (1970). Characterization of proteinases excreted by *Bacillus subtilis* Marburg strain during sporulation. *Journal of Applied Bacteriology* **33**, 207–219.
- PUHVEL, S. M. & REISNER, R. M. (1972). The production of hyaluronidase (hyaluronate lyase) by *Corynebacterium acnes*. *Journal of Investigative Dermatology* **58**, 66–70.
- PUHVEL, S. M., REISNER, R. M. & AMIRAN, D. A. (1975). Quantitation of bacteria in isolated pilosebaceous follicles in normal skin. *Journal of Investigative Dermatology* **65**, 525–531.
- TEMPEST, D. W. (1976). The concept of 'relative' growth rate: its theoretical basis and practical application. In *Continuous Culture 6: Applications and New Fields*, pp. 349–352. Edited by A. C. R. Dean, D. C. Ellwood, C. G. T. Evans & J. Melling. Chichester: Ellis Horwood.