Journal of Industrial Microbiology, 13 (1994) 273–278

Published by The Macmillan Press Ltd

Effect of dilution rate on the release of pertussis toxin and lipopolysaccharide of *Bordetella pertussis*

Maria E. Rodriguez, Daniela F. Hozbor, Analia L. Samo, Rodolfo Ertola and Osvaldo M. Yantorno

Centro de Investigacion y Desarrollo en Fermentaciones Industriales (CINDEFI), Facultad de Ciencias Exactas UNLP, Calle 47 y 115, (1990) La Plata, Argentina

(Received 31 August 1993; accepted 13 April 1994)

Key words: Bordetella pertussis; Pertussis toxin; Lipopolysaccharide; Continuous culture; Growth kinetics

SUMMARY

The kinetics of *Bordetella pertussis* growth was studied in a glutamate-limited continuous culture. Growth kinetics corresponded to Monod's model. The saturation constant and maximum specific growth rate were estimated as well as the energetic parameters, theoretical yield of cells and maintenance coefficient. Release of pertussis toxin (PT) and lipopolysaccharide (LPS) were growth-associated. In addition, they showed a linear relationship between them. Growth rate affected neither outer membrane proteins nor the cell-bound LPS pattern.

NOMENCLATURE

X	cell concentration (g L^{-1})
μ	specific growth rate (h^{-1})
μ_m	maximum specific growth rate (h^{-1})
D	dilution rate (h^{-1})
S	concentration of growth rate-limiting nutrient (glutamate) (mmol L^{-1} or g L^{-1})
Ks	substrate saturation constant (μmol L^{-1})
m_s	maintenance coefficient (g g^{-1} h^{-1})
$Y'_{x/s}$	theoretical yield of cells from glutamate (g g^{-1})
$Y_{x/s}$	yield of cells from glutamate (g g^{-1})
$Y_{PT/s}$	yield of soluble PT from glutamate (mg g^{-1})
$Y_{KDO/s}$	yield of cell-free KDO from glutamate (μg g^{-1})
$Y_{PT/x}$	specific yield of soluble PT (mg g^{-1})
$Y_{KDO/x}$	specific yield of cell-free KDO (μg g^{-1})
q_{PT}	specific soluble PT production rate (mg g^{-1} h^{-1})
q_{KDO}	specific cell-free KDO production rate (μg g^{-1} h^{-1})

INTRODUCTION

Whooping cough continues to be a major health problem. The traditional whole cell vaccine for pertussis, although effective in providing immunity, has been associated with many adverse reactions. A toxin present in large amounts in whole cell vaccine is *Bordetella* lipopolysaccharide (LPS endotoxin) which is thought to contribute to the vaccine's reactogenicity [23,29]. For this reason, LPS should be reduced or excluded from vaccine formulations. One of the main advantages of introducing acellular pertussis vaccine composed of one or more antigenic proteins, is the removal of most of the LPS during the purification of soluble antigenic material. Although the number of components to be included in an optimally protective pertussis vaccine remains to be established, the consensus is that the pertussis toxin (PT) will be an essential constituent [2,25,26]. The development of a vaccine based on this toxin will require a high yield of this protein from cultures of *B. pertussis*.

Licari et al. [14] demonstrated that when working in continuous culture with a mod(−) strain of *B. pertussis* growing in an iron-limited modified SS medium, the production of PT was predominantly growth-associated. They concluded that continuous operation at high dilution rate would be the process best suited for PT production. Perera et al. [19] reported that PT could be released in association with outer membrane components such as LPS. However, whether the dilution rate affects the LPS release remains to be studied.

The present work was carried out in continuous culture under energy-limited conditions. We studied kinetics of growth of *B. pertussis* strain Tohama phase I and the release of both PT and LPS in relation to the specific growth rate. Furthermore, the effect of the dilution rate on outer membrane proteins (OMP) and cell-bound LPS expression was also investigated in order to determine if any outer membrane change at different growth rates could be implicated in the release of PT.

Correspondence to: Maria E. Rodriguez, CINDEFI, Facultad de Ciencias Exactas UNLP, Calle 47 y 115, (1900) La Plata, Argentina.

MATERIALS AND METHODS

Microorganism

B. pertussis strain Tohama (8132 of the Pasteur Institute Collection) was maintained in 15% glycerol, 1% casamino-acids at −70 °C.

Growth conditions

Cells were grown for 48 h on Bordet Gengou agar (Difco Laboratories, Detroit, MI, USA) supplemented with 1% (w/w) peptone and 15% (v/v) defibrinated sheep blood (BGs). Cells harvested from these plates were transferred into 500-ml flasks containing 125 ml of Stainer Scholte (SS) [28] medium and incubated on an orbital shaker (160 r.p.m.) for 24 h at 35.5 °C before inoculating the chemostat. After 6 h of batch growth, the medium (SS) feed for the chemostat operation was started.

Continuous culture was conducted in a LH 210 series 2 modular fermentation system (LH Engineering Co., Bell's Hill, Stoke Poges, Bucks, UK), with a working volume of 700 ml. The dissolved oxygen was controlled at 40% by the agitation rate. The pH was maintained at 7.4 by addition of HCl and the temperature was maintained at 35.5 °C. The medium in the reservoir was replaced daily. Growth rates were fixed by adjusting the dilution rate (D) in the range of 0.03 to 0.06 h^{-1}, and then checked at least twice each day. Cultures were allowed to achieve the steady state over at least ten generations before samples were taken. Cell growth was monitored by optical density at 650 nm and dry cell weight. Samples were removed for analysis of PT, LPS, OMP and cell-bound LPS.

LPS extraction

Cells harvested from cultures maintained at different dilution rates were centrifuged at 8000 × *g* for 15 min at 4 °C and washed twice in distilled water. The bacterial concentrations were adjusted to the same optical density and LPS was extracted by the hot phenol–water method of Westphal and Jann [31]. The volume of phenol used to suspend the organisms was 55% (v/v). The extracts were dialyzed overnight at 4 °C against 4 L of water and then centrifuged at 100000 × *g* for 2 h. The sediment (LPS) was resuspended in distilled water and stored at −20 °C until used.

SDS-PAGE of cell-bound lipopolysaccharides

The Laemmli [11] discontinuous buffer system was employed with an acrylamide concentration of 15% in the separating gel. LPS preparations of whole cells were solubilized in sample buffer and heated at 100 °C for 10 min. Twenty-five μg of proteinase K in 10 μl of buffer was added per 50 μl of LPS suspension. The mixtures were incubated in a water bath at 60 °C for 1 h with occasional vortexing. Proteinase-K-treated samples were applied to gels.

Determination of extracellular LPS

LPS [13] from *B. pertussis* contains lipid A and an oligosaccharide core containing 2-keto-3-deoxyoctonate (KDO) [17], but not the long-chain polysaccharide characteristic of the enterobacterial LPS [8]. LPS in culture supernatant fluids was determined by means of KDO using the method of Karkanis et al. [10]. KDO (Sigma, St Louis, MO, USA) was employed as reference. Samples taken at each dilution rate were centrifuged at 8000 × *g* for 15 min at 4 °C. Under these conditions, cell-free supernatant fractions were obtained as shown by microscopic examination.

Preparation of outer membrane

Envelope fractions (outer membrane plus peptidoglycan) were prepared using the method of Schnaitman et al. [27], with minor modifications as reported by Hozbor et al. [9].

Protein assay

Protein content was estimated by the Lowry method, using bovine serum albumin as the standard [15].

SDS-PAGE of outer membrane proteins

Separating and stacking gels were 12.5% (w/v) and 4% (w/v), respectively. Envelope proteins were solubilized by heating at 100 °C for 5 min in 0.0625 M Tris/HCl buffer pH 6.8 containing SDS (2% w/v), glycerol (10% w/v), bromophenol blue (0.001% w/v) and mercaptoethanol (5% w/v). Solubilized proteins were applied to the gels.

Electrophoresis

Electrophoresis was performed at room temperature and constant voltage. Polypeptides in gels were stained overnight in a solution of Coomassie Blue R250 (0.2% w/v) in 40% (v/v) methanol and 10% (v/v) acetic acid. A Pharmacia Calibration Kit (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) was employed to estimate molecular weights. The Hitchcock and Brown [7] silver stain technique was used to spot LPS.

Pertussis toxin assay

PT antigen in culture supernatant fluid was detected by ELISA using fetuin as a solid phase as reported by Rodriguez et al. [24].

Glutamic acid analysis

The concentration of glutamic acid in the supernatant phases was determined with a glutamic acid test kit (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

Data were subjected to analysis of variance with the Fisher projected least-significant-difference procedure [30] used for comparisons of treatment means.

RESULTS

Kinetics of growth and glutamate utilization

The kinetic behavior of *B. pertussis* was investigated in continuous cultures at steady states over a range of dilution

rates from 0.03 to 0.06 h^{-1}. Table 1 shows that the higher yields obtained from glutamate, the main energy and carbon source used by *B. pertussis* [18] for biomass, LPS and PT production, were achieved at higher dilution rates. The difference between the mean values was highly significant ($P < 0.01$). The energetic parameters, namely [20] theoretical yield of cells on glutamate ($Y'_{x/s}$) and maintenance coefficient (m_s) were obtained from data in Table 1 by performing a regression via the use of the equation:

$$1/Y_{x/s} = 1/Y'_{x/s} + m_s/D \quad (1)$$

$1/Y_{x/s}$ was found to be related to 1/D with a correlation coefficient of 0.998. The value calculated for $Y'_{x/s}$ was 0.33 g cell per g of glutamate and for m_s 0.07 g glutamate per g biomass per h. The value obtained for the maintenance coefficient was greater than that reported by Licari et al. [14] who mentioned a value of 0.01 on the same units. This difference may be ascribed to the different strain and growth condition employed.

The Monod [16] empirical equation was used to describe the relationship between bacterial growth rate and the concentration of a single growth rate-limiting nutrient. Our data fit well with the Monod model of growth kinetics. The determination of Monod substrate saturation constant (Ks) is important in modelling microbial cultures, especially continuous cultures where Ks largely determines the steady-state concentration of the unused growth-limiting nutrient. One of the best methods of analyzing μ vs. S appears to be the direct linear plot of Eisenthal and Cornish-Bowden [4]. In accordance with this, data from the different steady states were plotted in Fig. 1. There was almost no experimental error since there is a single intersection point for all the lines. The coordinates of this point provided the values for Ks and μm which were estimated to be 10 μM and 0.066 h^{-1}, respectively.

PT and LPS release

The release of LPS was analyzed in every case by determining KDO, which was found to be a ubiquitous component of the endotoxins (LPS) of Gram-negative bacteria.

Values for q_{PT} and q_{KDO} were calculated and plotted as a function of growth rate (Figs 2 and 3). From our results,

TABLE 1

Influence of specific growth rate on *Bordetella pertussis* yields of biomass, soluble PT and cell-free KDO[a]

D (h^{-1})	$Y_{x/s}$ (g g^{-1})	$Y_{PT/s}$ (mg g^{-1})	$Y_{KDO/s}$ (μg g^{-1})
0.03	0.18	0.14	0.38
0.04	0.20	0.20	0.49
0.05	0.22	0.26	0.64
0.06	0.23	0.31	0.81

[a]The results are the mean of four determinations.

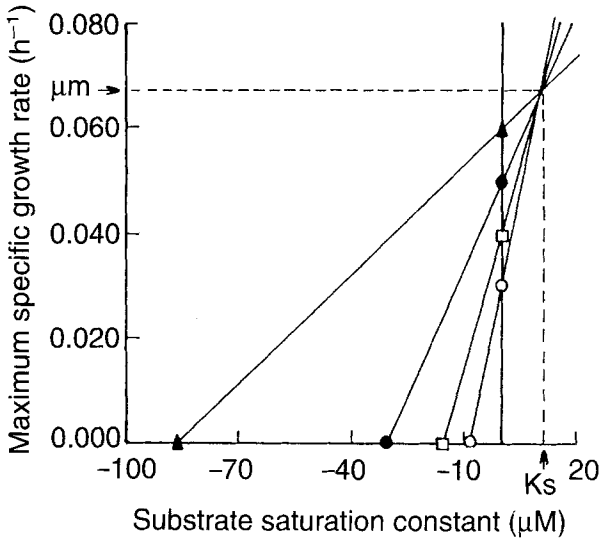

Fig. 1. Determination of *B. pertussis* Monod glutamate saturation constant for growth (Ks) and maximum specific growth rate (μm) by Eisenthal and Cornish-Bowden direct linear plot method [4]. Each line represents one observation of S and μ, plotted as intercept $-S$ on the Ks axis and intercept μ on the μm axis. The point of intersection of the lines gives the coordinates for Ks and μm.

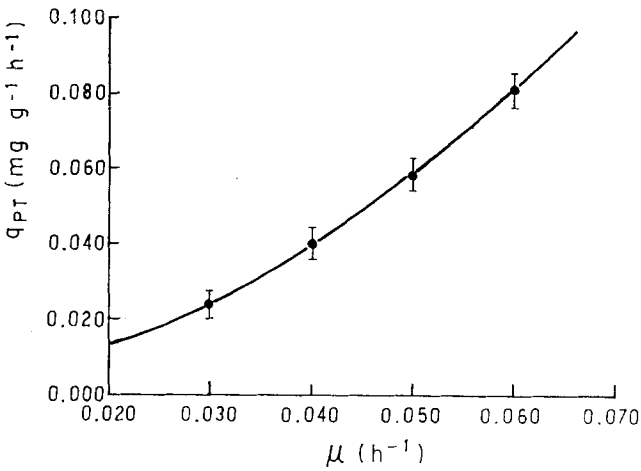

Fig. 2. Effect of the specific growth rate on the specific soluble pertussis toxin production rate.

the formation kinetics of these products can be characterized by:

$$q_{PT} = 0.00 + 0.26\,\mu + 17.02\,\mu^2 \quad (2)$$

$$q_{KDO} = 0.01 + 0.12\,\mu + 52.85\,\mu^2 \quad (3)$$

In both cases, fitness of the regression line was highly significant ($P < 0.01$). Regression removed 90–97% of the data variance.

Both q_{PT} and q_{KDO} showed a similar behavior in relation to the specific growth rate. Fig. 4 shows the correlation

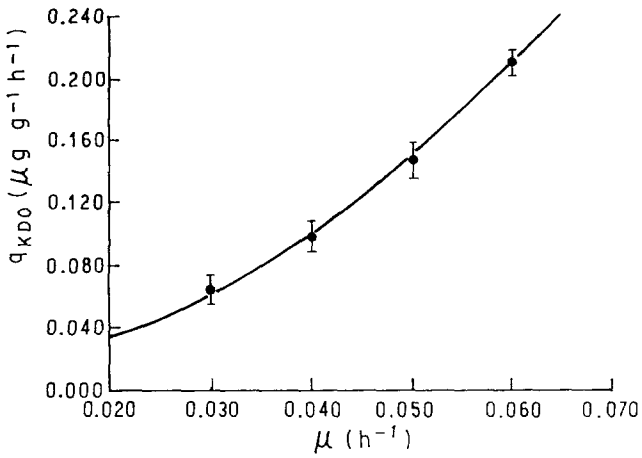

Fig. 3. Effect of the specific growth rate on the specific cell-free KDO production rate.

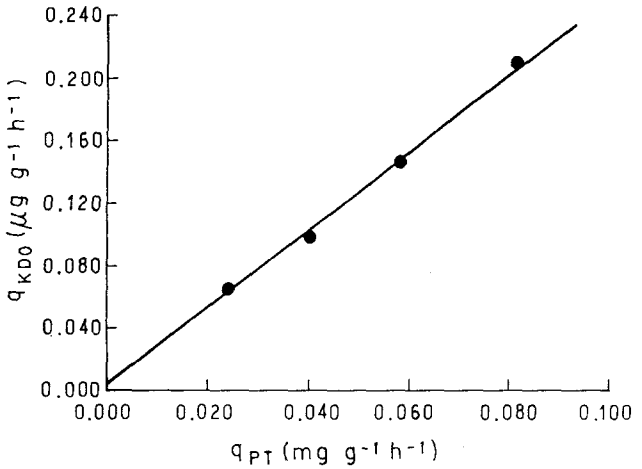

Fig. 4. Correlation between soluble PT and cell-free KDO specific production rates: 0.998.

between these two parameters. A correlation coefficient of 0.998 indicates a direct association between q_{PT} and q_{KDO}.

Outer membrane polypeptides and cell-bound LPS

OMPs from cells grown at different dilution rates exhibited similar patterns in polyacrylamide gels (Fig. 5). In the same way, the dilution rate had no effect on LPS pattern, as can be observed in Fig. 6. In all cases the profile of LPS preparations extracted from *B. pertussis* consisted of two bands (rough-type of LPS), namely an upper one, LPS_a, and a lower one, LPS_b.

DISCUSSION

Spontaneous release of LPS during exponential and even stationary growth phase in Gram-negative bacteria cultures is well established. In previous studies we demonstrated that during batch cultures of *B. pertussis* both PT and LPS levels in the supernatant medium increase [9,24] but no conclusion could be drawn from these results as to whether PT and LPS production were dependent on specific growth rate or on the nutrient composition.

In this study it was demonstrated that under glutamate limitation the specific growth rate affects release of both PT and LPS. Our results indicate that specific yields of soluble PT and cell-free LPS were not constant but a function of the specific growth rate (i.e. $y_{p/x} = k\ \mu$).

By employing the chemostat it was possible to define a relationship between these products and the growth rate (Eqns 2 and 3). Both PT and LPS could be considered growth-associated products. However, although PT production is entirely growth-associated (the non-growth associated term is zero) there was some LPS release without growth. This finding is in agreement with that of Hozbor et al. (1993) [9] who showed a release of LPS into the surrounding medium during the stationary growth phase of *B. pertussis* in batch culture.

The linear relationship between PT and LPS productivities at different dilution rates (Fig. 4) is in agreement with earlier reports which show that some enzymes and virulence factors of pathogenic bacteria are released in association with outer membrane material [1,5,22]. An association of PT with small micellar forms of outer membrane components was particularly suggested for *B. pertussis* [19].

The influence of the specific growth rate on the pathogenesis associated with OMPs and cell-bound LPS composition for other Gram-negative bacteria is well documented [3,6,12]. In this respect, the SDS-PAGE pattern of OMP and cell-bound LPS were analyzed. There was no difference between OMP profiles of cells grown under different dilution rates. In the same way, the specific growth rate had no effect on LPS pattern. In all cases, the profile of LPS preparation extracted was the same. These results indicate that the increase in extracellular PT and LPS levels with the specific growth rate would be basically concerned with adjustment of the microorganisms's metabolic regulation under the different specific growth rates rather than with an outer membrane alteration, an observation which deserves further research.

The results obtained show (Table 1) that $Y_{x/s}$ depends on the specific growth rate. Conversely, the maintenance coefficient is independent of the dilution rate which is in accordance with that expected for an energy-limited culture [21]. Consequently, the higher the growth rate, the smaller the relative amount of energy source needed for maintenance. Taking into account these considerations and that PT production is strongly growth-associated, continuous culture at high dilution rate seems to be an effective means for producing PT, as mentioned by Licari et al. [14] for other strains and culture conditions, and confirmed in this study for Tohama phase I grown under energy- and carbon-limitation. However, during the selection of the best culture condition for soluble PT production, it must be taken into account that the release of LPS, an undesirable constituent of an acellular vaccine, is also a growth-associated phenomenon. At present, we are investigating whether the extracellu-

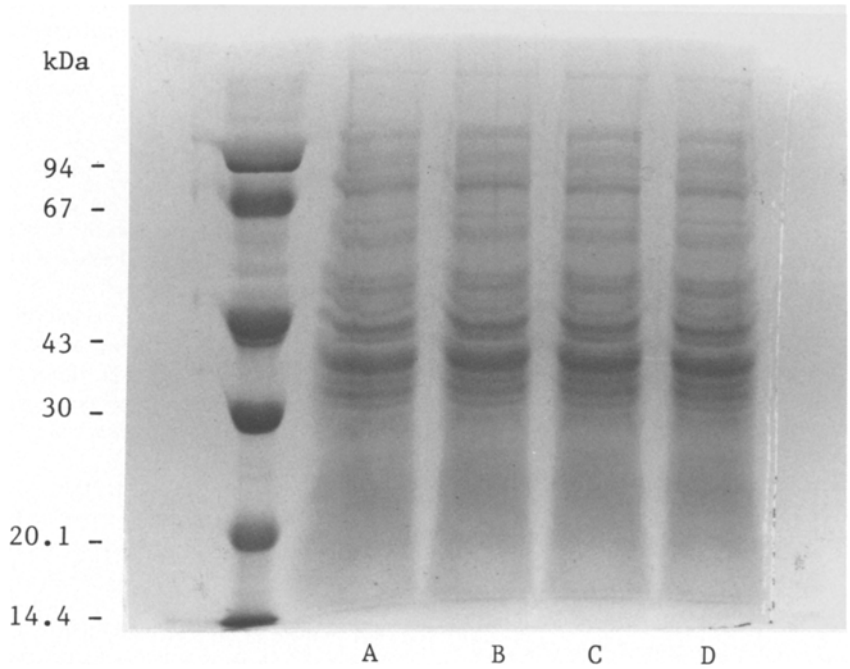

Fig. 5. SDS-PAGE (12.5%) of outer membrane proteins of *B. pertussis* grown under different dilution rates. Lanes: A, 0.03 h^{-1}; B, 0.04 h^{-1}; C, 0.05 h^{-1}; D, 0.06 h^{-1}. Molecular mass markers (kDa) on the left.

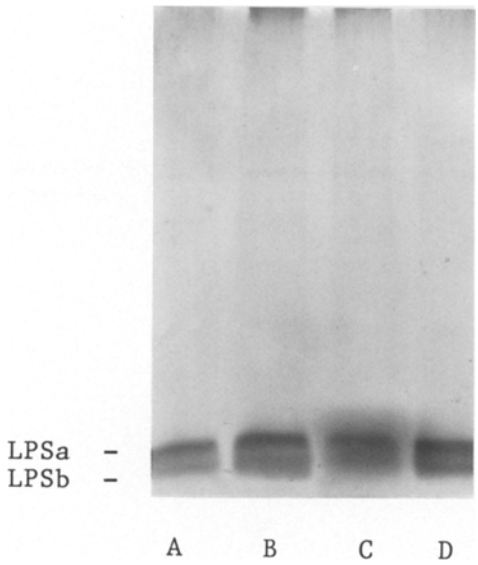

Fig. 6. Effect of dilution rate on the electrophoretic mobility of *B. pertussis* lipopolysaccharide in SDS-PAGE (10%). Lanes: A, 0.03 h^{-1}; B, 0.04 h^{-1}; C, 0.05 h^{-1}; D, 0.06 h^{-1}.

lar LPS:PT ratio is influenced by other environmental parameters.

Further chemostat studies will be of great value in determining the effect of growth rate and environment on the regulation of the other virulence factors as well as for extending the limited knowledge available about the *B. pertussis* physiology. Such information may be useful not only to determine the vaccine production process best suited but also to better understand the disease process, taking into account that colonizing bacteria are likely to grow at lower rates than those in vitro.

ACKNOWLEDGEMENTS

Maria E. Rodriguez and Daniela F. Hozbor were supported by a fellowship from CICPBA. Analia L. Samo is scientific assistant of CICPBA. This work was supported by CONICET and SECYT-CABBIO.

REFERENCES

1 Austin-Prather, S. and S. Booth. 1984. Evidence for a membrane-bound form bacteriocin of *Bacteroides uniformis* Tl-1. Can. J. Microbiol. 30: 268–272.
2 Blennow, M., S. Hedenskog and M. Granström. 1988. Protective effect of acellular pertussis vaccine. Eur. J. Clin. Microbiol. Infect. Dis. 7: 381–383.
3 Davies, R., R. Parton, J. Coote, H. Alison Gibbs and H. Freer. 1992. Outer-membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype A1 under different growth conditions. J. Gen. Microbiol. 138: 909–922.
4 Eisenthal, R. and A. Cornish-Bowden. 1974. The direct linear plot: a new graphical procedure for estimating enzyme kinetic parameters. Biochem. J. 139: 715–720.
5 Gankema, H., J. Wensink, P. Guinee, W. Jansen and B. Witholt. 1980. Some characteristics of the outer membrane material released by growing enterotoxigenic *Escherichia coli*. Infect. Immun. 29: 704–713.
6 Harakeh, S. and A. Matin. 1989. Influence of nutrient-limited growth on pathogenesis-associated outer membrane proteins of *Yersinia enterocolitica*. J. Appl. Bacteriol. 67: 209–212.
7 Hitchcock, P. and T. Brown. 1983. Morphological heterogeneity

among *Salmonella* lipopolysaccharide chemotype in silver-stained polyacrylamide gels. J. Bact. 154: 269–277.

8 Hitchcock, P., L. Leive, P. Makela, E. Rietshel, W. Strittmatter and D. Morrinson. 1986. Lipopolysaccharide nomenclature—past, present and future. J. Bact. 166: 699–705.

9 Hozbor, D., M.E. Rodriguez, A. Samo, A. Lagares and O. Yantorno. 1993. Release of lipopolysaccharide during *Bordetella pertussis* growth. Res. Microbiol. 144: 201–209.

10 Karkhanis, Y., J. Zeltner, J. Jackson and D. Carlo. 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. Analyt. Biochem. 85: 595–601.

11 Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227: 680–685.

12 Langford, P. and R. Moxon. 1992. Growth of *Haemophilus influenzae* type b in continuous culture: effect of dilution rate on outer-membrane protein and lipopolysaccharide expression. FEMS Microbiol. Letts 93: 43–48.

13 Le Dur, A., M. Caroff, R. Chaby and L. Szabo. 1978. A novel type of endotoxin structure present in *Bordetella pertussis*. Europ. J. Biochem. 84: 579–589.

14 Licari, P., G. Siber and R. Swart. 1991. Production of cell mass and pertussis toxin by *Bordetella pertussis*. J. Bacteriol. 20: 117–130.

15 Lowry, O., N. Rosebrough, A. Farr and J. Randall. 1952. Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265–275.

16 Monod, J. 1942. Récherches sur la Croissance des Cultures Bactériennes. Herman & Cie, Paris.

17 Moreau, M., R. Chabo and L. Szabo. 1984. Structure terminal reducing heptasaccharide of polysaccharide I isolated from *Bordetella pertussis* endotoxin. J. Bact. 159: 611–617.

18 Parker, C. 1976. Role of genetics and physiology of *Bordetella pertussis* in the production of vaccine and the study of the host–parasite relationship in pertussis. Adv. Appl. Microbiol. 20: 27–42.

19 Perera, V., A. Wardlaw and J. Freer. 1987. Release of pertussis toxin and its interaction with outer-membrane antigens. J. Gen. Microbiol. 133: 2427–2435.

20 Pirt, S. 1975. Principles of Microbe and Cell Cultivation. Blackwell Scientific Publications, Oxford, UK.

21 Pirt, S. 1982. Maintenance energy: a general model for energy-limited and energy-sufficient growth. Arch. Microbiol. 133: 300–302.

22 Pugsley, A., C. Chapton and M. Schwartz. 1986. Extracellular pullulanase of *Klebsiella pneumoniae* is a lipoprotein. J. Bacteriol. 166: 1083–1088.

23 Robinson, A., L. Irons and L. Ashworth. 1985. Pertussis vaccine: present status and future prospects. Vaccine 3: 11–21.

24 Rodriguez, M.E., A. Samo, D. Hozbor and O. Yantorno. 1993. Effect of hydrochemical forces on the production of filamentous haemagglutinin and pertussis toxin of *Bordetella pertussis*. J. Ind. Microbiol. 12: 103–108.

25 Rutter, D., L. Ashworth, A. Day, S. Funnell, F. Lovell and A. Robinson. 1988. Trial of a new acellular pertussis vaccine in healthy adult volunteers. Vaccine 6: 29–32.

26 Sato, Y., M. Kimura and H. Fukumi. 1984. Development of a pertussis component vaccine in Japan. Lancet 1: 122–126.

27 Schaintman, C. 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bact. 108: 545–552.

28 Stainer, D. and M. Scholte. 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. 63: 211–220.

29 Steinman, L., S. Sriram, N. Adelman, S. Zanvil, H. McDevitt and S. Urich. 1982. Murine model for pertussis encephalopathy linkage to H-2. Nature (Lond.) 299: 738.

30 Stell, R. and J. Torrie. 1980. Principles and Procedures of Statistics: A Biometrial Approach, 2nd edn, p. 176. McGraw-Hill, Hamburg.

31 Westphal, O. and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further application of procedure. Methods Carbohydr. Chem. 5: 83–91.