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Growth and Production Kinetics of a Teicoplanin Producing Strain of *Actinoplanes teichomyceticus*

ARNE HEYDORN[a,*], TRINE SUHR-JESSEN[b] and JENS NIELSEN[a]

[a] Center for Process Biotechnology, Technical University of Denmark, block 223, DK-2800 Lyngby, Denmark
[b] Alpharma, Dalslandsgade 11, DK-2300 Copenhagen, Denmark

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The growth and production kinetics of a teicoplanin producing strain of *Actinoplanes teichomyceticus* (ATCC 31121) was investigated during batch cultivations on defined media. The growth was characterised by two exponential growth phases (EGPs), with a higher specific growth rate in the first than in the second phase. Also the specific rate of formation of teicoplanin was significantly lower in the second phase than in the first phase. This two-phased growth pattern was suggested to be caused by inhibition of growth by teicoplanin accumulated. Furthermore high concentrations of ammonia or phosphate reduced both the specific growth rate in the first EGP and the total production of teicoplanin.

Teicoplanin is a group of closely related antibiotics produced by *Actinoplanes teichomyceticus*, which belongs to the vancomycin-ristocetin family of glycopeptide antibiotics[1]. It acts by blocking cell wall biosynthesis[2] and it is used to fight Gram-positive pathogens resistant to established antibiotics, such as methicillin-resistant *Staphylococcus aureus* (MRSA), coagulase-negative staphylococci, clostridia and enterococci[3]. The worldwide problems with MRSA (see Table 1) have resulted in an increased use of vancomycin and teicoplanin, the only agents that effectively treat these infections.

Many antibiotic producing microorganisms are sensitive towards their own antibiotic. Traditionally it was believed that microorganisms deal with this by not producing the antibiotic until they have passed through part or all of their growth phase. However, there are several reports which show that secondary metabolites may also be produced by actively growing cells, especially when chemically defined media are used. Examples include the production of streptomycin by *Streptomyces griseus* ATCC 12475[7], undecylprodigiosin by *Streptomyces coelicolor* A3(2)[8], and erythromycin by *Saccharopolyspora erythraeus*[9].

Here we studied the growth and production kinetics of an industrial teicoplanin producing strain of *A. teichomyceticus* cultured on defined media, and it is demonstrated that growth is characterised by two exponential growth phases (EGPs) presumably caused by inhibition of growth and product formation by teicoplanin.

Materials and Methods

The strain is an industrial strain used at Alpharma A/S. The strain was chosen by screening for high teicoplanin production after treatments with *N*-methyl-*N'*-nitro-nitrosoguanidine.

The cultures were grown in 4 liter batch bioreactors. The media for the batch cultivations (BC) BC-1 to BC-5 (see Table 2) were developed in a series of shake flask

Table 1. Prevalence (%) of MRSA among clinical *S. aureus* isolates 1990～1991[4～6].

Country	MRSA
Denmark	0.1%
Netherlands	1.5%
Germany	5.5%
Belgium	25%
USA	29%
Spain	30%
France	34%
Italy	34%
Japan	60%

Table 2. Media for the batch cultivations BC-1 to BC-5.

	BC-1, BC-4, BC-5	BC-2	BC-3
Glucose·$1H_2O$	20 g/liter	20 g/liter	20 g/liter
KH_2PO_4	0.88 g/liter	0.88 g/liter	3.00 g/liter
$Na_2HPO_4 \cdot 2H_2O$	—	—	9.00 g/liter
NH_4Cl	2.00 g/liter	4.00 g/liter	2.00 g/liter
NaCl	1.00 g/liter	1.00 g/liter	1.00 g/liter
$MgSO_4 \cdot 7H_2O$	0.40 g/liter	0.40 g/liter	0.40 g/liter
$CaCl_2 \cdot 2H_2O$	0.020 g/liter	0.020 g/liter	0.020 g/liter
Ferric ammonium citrate	0.010 g/liter	0.010 g/liter	0.010 g/liter
Trace metal solution[a]	3 ml/liter	3 ml/liter	3 ml/liter

[a] The trace metal solution contained the following ingredients: $ZnSO_4 \cdot 7H_2O$ (100 mg/liter), $MnCl_2 \cdot 4H_2O$ (30 mg/liter), H_3BO_3 (300 mg/liter), $CoCl_2 \cdot 6H_2O$ (200 mg/liter), $CuCl_2 \cdot 2H_2O$ (5.6 mg/liter), $NiCl_2 \cdot 6H_2O$ (20 mg/liter), $Na_2MoO_4 \cdot 2H_2O$ (30 mg/liter).

experiments (data not shown). The bioreactors were inoculated with 200 ml of a shake flask culture grown on the same medium as BC-1 for 5 days at 28°C on an orbital shaker at 200 rpm. The pH of the medium was kept at 7.0 and the temperature was kept at 28°C throughout the fermentation. The aeration was 1.0 v/v/minute. Samples of approximately 20 ml were taken from the bioreactor at every point.

For the dry weight measurements approximately 15 ml of culture was filtered through a dried (105°C, 24 hours) preweighed Whatman filter (1PS), washed with 30 ml 0.9% (w/w) NaCl solution, and dried for 24 hours at 105°C. Glucose and ammonia were measured on an automatic analyser (Cobas Miras Plus, Roche Diagnostic Systems) using standard kits from Roche (Unimate 7 GLUC GDH) and Boehringer Mannheim (Ammonia). The partial pressure of CO_2 in the exhaust gas from the bioreactors was measured using a gasanalyser (Servomex® 1410 infrared gasanalyser, Servomex Ltd.).

The MIC (minimum inhibitory concentration) value was determined by an agar dilution technique. 0.1 ml of a dense culture ($>10^9$ CFU/ml) was spread on agar plates (Soytone (Difco) 5 g/liter, Tryptose (Difco) 2 g/liter, yeast extract (Difco) 1 g/liter, soluble starch (Merck) 4.5 g/liter, agar (Difco) 20 g/liter, pH 7.0) containing teicoplanin at concentrations of 100, 50, 25, 12.5 and 6.25 mg/liter respectively. The lowest concentration which completely inhibited growth was defined as the MIC-value.

For HPLC measurements of teicoplanin a Waters instrument with a reversed phase Waters Nova-Pak column (C-18, 4 μm particles, 3.9 × 150 mm) and UV detector was used. Injection: 50 μl (samples), 20 μl (standards). Linear gradients (minutes, %B, ml/minute): (0, 35, 1.2), (11, 50, 1.2), (12, 100, 1.4), (14, 100, 1.4), (15, 35, 1.4), (20, 35, 1.2). The UV-detector was set at 280 nm. Samples were mixed 1 : 1 (v/v) with *N*-methylpyrrolidon, mixed and centrifuged at 100 g for 15 minutes. External standards: Teicoplanin (Targocid from Astra) was dissolved in water at concentrations of about 1 g/liter and 2 g/liter. Buffer A: 10.5 g citric acid + 0.4 g Na_2-EDTA was dissolved in 1 liter milli-Q water. The pH was adjusted to 5.0 with triethylamine. The solution was vacuum filtered through a 0.45 μm Millipore filter (type HV). Buffer B: 400 ml acetonitrile was mixed with 600 ml buffer A.

Results

In order to study the influence of ammonia and phosphate concentrations in the defined medium five batch cultivations were carried out (see Table 2).

Figs. 1 and 2 show the growth and production of teicoplanin in BC-1. BC-2 and BC-3 show the same kind of two-phased growth, and the results are summarised in Table 3. BC-1 had a low concentration of ammonia (37.4 mM) and phosphate (6.47 mM), BC-2 had a high concentration of ammonia (74.8 mM) and a low concentration of phosphate (6.47 mM), whereas BC-3 had a low concentration of ammonia (37.4 mM) and a high concentration of phosphate (72.6 mM).

In all three batch cultivations the two-phased growth

Fig. 1. Growth in batch cultivation BC-1.

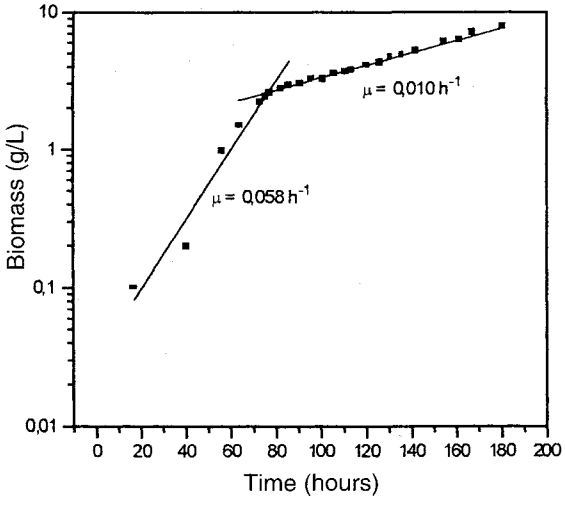


Fig. 2. Production of teicoplanin in batch cultivation BC-1.

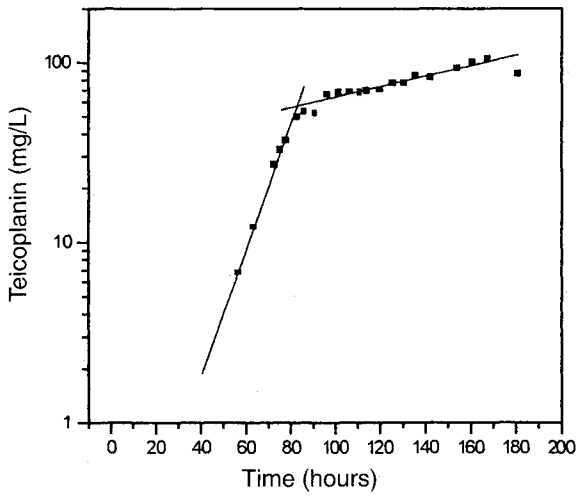


Table 3. Growth and teicoplanin production kinetics in batch cultivations BC-1, BC-2 and BC-3.

Batch cultivation	BC-1	BC-2	BC-3
1. EGP			
Duration	0~77 h	0~115 h	0~117 h
Specific growth rate	0.058 h^{-1}	0.047 h^{-1}	0.047 h^{-1}
Specific rate of formation of teicoplanin[a]	0.80 mg/g DW h	0.12 mg/g DW h	0.14 mg/g DW h
Biomass conc. at end of 1. EGP	2.6 g/liter	4.0 g/liter	4.1 g/liter
Teicoplanin conc. at end of 1. EGP	36 mg/liter	10 mg/liter	12 mg/liter
Glucose conc. at end of 1. EGP	14.2 g/liter	11.0 g/liter	10.9 g/liter
Ammonia conc. at end of 1. EGP	0.44 g/liter	1.05 g/liter	0.30 g/liter
2. EGP			
Duration	77~181 h	115~167 h	117~181 h
Specific growth rate	0.010 h^{-1}	0.017 h^{-1}	0.012 h^{-1}
Specific rate of formation of teicoplanin[a]	0.13 mg/g DW h	0.02 mg/g DW h	0.04 mg/g DW h
Final biomass conc.	7.8 g/liter	8.4 g/liter	7.5 g/liter
Final teicoplanin conc.	105 mg/liter	14 mg/liter	24 mg/liter

[a] The specific rate of formation of teicoplanin is defined as the production rate of teicoplanin [mg/liter/hour] divided by the biomass concentration [g/liter]. The values are averages for the whole phase.

was also reflected by the concentration of CO_2 in the exhaust gas; an example in BC-3 is illustrated in Fig. 3.

The MIC value for teicoplanin against *A. teichomyceticus* grown on agar plates was found to be 25 mg/liter.

Discussion

Two-phased exponential growth in batch cultivations has been reported for *Streptomyces hygroscopicus*[10], *Streptomyces coelicolor*[11] and *Streptomyces griseus*[12]. In these organisms growth is characterised by an initial lag phase, two EGPs separated by a short lag phase of reduced growth and a stationary phase. In *Streptomyces hygroscopicus* producing bialaphos the specific growth rate is higher in the first EGP than in the second EGP and production of bialaphos takes place during the second EGP and the stationary phase[10]. In *Streptomyces griseus* producing streptomycin the specific growth rate is lower in the first EGP than in the second EGP and production of streptomycin is confined to the stationary phase[12]. These findings suggest that *Streptomyces* and possibly other actinomycetes do not have a uniform vegetative growth phase but a more complex one.

In this study it is suggested that the two-phased growth of *A. teichomyceticus* is caused by teicoplanin since:

- In BC-1, BC-2 and BC-3 the transition to the

Fig. 3. Partial pressure of CO_2 (expressed as mole-%) in the exhaust gas from batch cultivation BC-3.

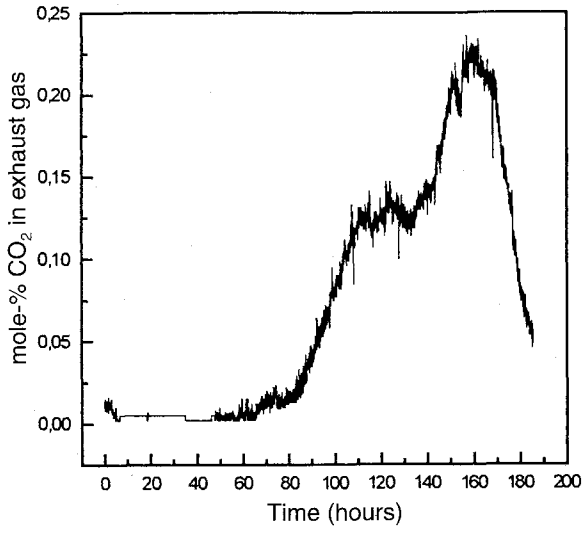
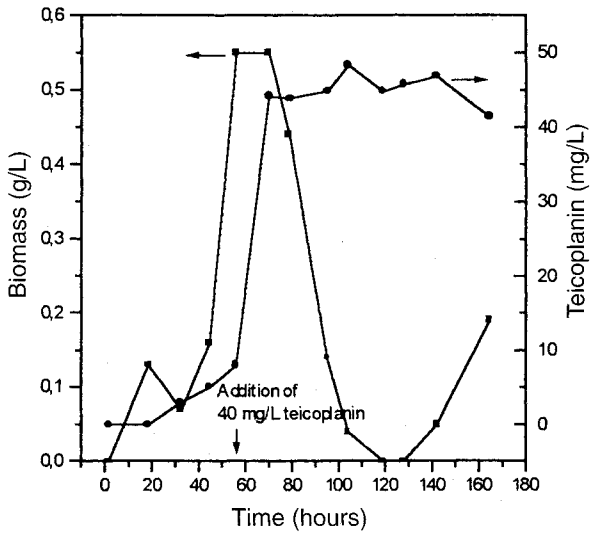


Fig. 4. Biomass and teicoplanin concentration in batch cultivation BC-4.



second EGP occurs at concentrations of teicoplanin close to the MIC value of 25 mg/liter.

• The transition to the second EGP is delayed by high concentrations of ammonia or phosphate (BC-2 and BC-3). This is probably an indirect effect caused by a slower growth and production in the media containing high concentrations of ammonia or phosphate. The concentration of teicoplanin increases more slowly, and the time needed before the inhibitory concentration of teicoplanin is longer. In another batch cultivation (data not shown) with an even higher initial concentration of ammonia (100 mM) than in BC-2 (74.8 mM), the transition to the second EGP occurred after 135 hours at a teicoplanin concentration of 13 mg/liter.

In BC-1 teicoplanin inhibition brought on the second EGP at a concentration of 36 mg/liter, whereas lower concentrations (10 ~ 12 mg/liter) brought it on in BC-2 and BC-3. This is likely to be a consequence of the dynamics in the batch cultivations since it probably takes some time for the bacteria to adapt to the new environment of higher teicoplanin concentrations and since in BC-1 the production of teicoplanin in the first EGP is considerably higher than in BC-2 and BC-3.

In order to study whether the transition from rapid growth to slow growth is due to inhibition by teicoplanin a batch cultivation (BC-4) identical to BC-1 was carried out, but with 40 mg/liter teicoplanin added to the medium after 55 hours of cultivation. At this time the bacteria are in the first EGP and it was anticipated that the addition of teicoplanin at this point would force the bacteria to enter the second EGP earlier than in BC-1. Measurements of the biomass and teicoplanin concentrations are shown in Fig. 4. It is observed that growth is stopped immediately after the addition of teicoplanin, and instead of entering a second growth phase the bacteria lyse.

From the batch cultivations it appears that the effect of teicoplanin on growth depends on how fast the concentration of teicoplanin increases. If the concentration of teicoplanin increases slowly, the bacteria have time to adapt to the new environment probably by activating genes encoding resistance against teicoplanin. If instead the concentration of teicoplanin increases rapidly the bacteria die. This fact was also illustrated by another batch cultivation (BC-5) identical to BC-1 except for the temperature being kept at 30°C instead of 28°C (data not shown). In this cultivation the specific growth rate was higher ($0.091\,h^{-1}$) and there was a faster production of teicoplanin during the first EGP. The growth stopped after only 40 hours and the biomass concentration started to decrease. At this time the concentration of teicoplanin was 90 mg/liter. Apparently the concentration of teicoplanin increased so rapidly that the bacteria did not have time to acquire resistance and instead they died. In some antibiotic producing organisms the genes for resistance against the organism's own antibiotic are regulated so that they are always expressed when the genes for production of the antibiotic are expressed. Hereby antibiotic biosynthesis is always

accompanied by resistance in the host organism. Examples include streptomycin biosynthesis by *Streptomyces griseus* and *Streptomyces glaucescens*[13]. Since the effect of teicoplanin on the growth of *A. teichomyceticus* depends on how fast the concentration of teicoplanin increases this mechanism does not seem to be operating in *A. teichomyceticus*.

References

1) PARENTI, F.; G. BERETTA, M. BERTI & V. ARIOLI: Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov. sp. I. Description of the producer strain, fermentation studies and biological properties. J. Antibiotics 31: 276~283, 1978
2) REYNOLDS, P. E.: Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur. J. Clin. Microbiol. Infect. Dis. 8: 943~950, 1989
3) BROGDEN, R. N. & D. H. PETERS: Teicoplanin. A reappraisal of its antimicrobial activity, pharmacokinetic properties and therapeutic efficacy. Drugs 47: 823~854, 1994
4) VOSS, A.; D. MILATOVIC, C. WALLRAUCH-SCHWARZ; V. T. ROSDAHL & I. BRAVENY: Methicillin-resistant *Staphylococcus aureus* in Europe. Eur. J. Clin. Microbiol. Infect. Dis. 13: 50~55, 1994
5) PANLILIO, A. L.; D. H. CULVER, R. P. GAYNES, S. BANERJEE, T. S. HENDERSON, J. S. TOLSON & W. J. MARTONE: Methicillin-resistant *Staphylococcus aureus* in U.S. hospitals, 1975~1991. Infection Control and Hospital Epidemiology 13: 582~586, 1992
6) KIMURA, A.; H. IGARASHI, H. USHIODA, K. OKUZUMI, H. KOBAYASHI & T. OTSUKA: Epidemiological study of *Staphylococcus aureus* isolated from the Japanese National University and Medical College Hospitals. Kansenshogaku-Zasshi 66: 1543~1549, 1992
7) FAZELI, M. R.; J. H. COVE & S. BAUMBERG: Physiological factors affecting streptomycin production by *Streptomyces griseus* ATCC 12475 in batch and continuous culture. FEMS Microbiol. Lett. 126: 55~62, 1995
8) HOBBS, G.; C. M. FRAZER, D. C. J. GARDNER, F. FLETT & S. G. OLIVER: Pigmented antibiotic production by *Streptomyces coelicolor* A3(2): kinetics and influence of nutrients. J. Gen. Microbiol. 136: 2291~2296, 1990
9) TRILLI, A.; M. V. CROSSLEY & M. KONTAKOU: Relationship between growth rate and erythromycin production in *Streptomyces erythraeus*. Biotechnol. Lett. 9: 765~770, 1987
10) HOLT, T. G.; C. CHANG, C. LAURENT-WINTER, T. MURAKAMI, J. I. GARRELS, J. E. DAVIES & C. J. THOMPSON: Global changes in gene expression related to antibiotic synthesis in *Streptomyces hygroscopicus*. Mol. Microbiol. 6: 969~980, 1992
11) BLANCO, G.; M. R. RODICIO, A. M. PUGLIA, C. MÉNDEZ, C. J. THOMPSON & J. A. SALAS: Synthesis of ribosomal proteins during growth of *Streptomyces coelicolor*. Mol. Microbiol. 12: 375~385, 1994
12) NEUMANN, T.; W. PIEPERSBERG & J. DISTLER: Decision phase regulation of streptomycin production in *Streptomyces griseus*. Microbiology 142: 1953~1963, 1996
13) DISTLER, J.; K. MANSOURI, G. MAYER, M. STOCKMANN & W. PIEPERSBERG: Streptomycin biosynthesis and its regulation in Streptomycetes. Gene 115: 105~111, 1992