Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions

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To optimize bacteriocin production processes, the relationships between growth, bacteriocin production and factors affecting the occurrence and intensity of the activity peak during the growth cycle must be understood. Amylovorin L471, a bacteriocin produced by *Lactobacillus amylovorus* DCE 471, displays primary metabolite kinetics with a peak activity during the mid-exponential phase. Because of this growth association, only conditions favouring a drastic increase in biomass improve the volumetric bacteriocin titre. Specific bacteriocin production is enhanced under unfavourable growth conditions such as low temperatures (30 °C), and the presence of potentially toxic compounds such as ethanol (1.0%, v/v) and oxygen (80%, v/v, air saturation). Whereas volumetric biomass formation and growth-associated bacteriocin production are dependent on the amount of glucose and nitrogen supplied, slow growth rates stimulate specific bacteriocin production. Bacteriocin inactivation can be ascribed to protein aggregation and adsorption phenomena. It may be overcome by switching the pH to 2.0 during the fermentation run after having reached the peak activity. Thus, manipulation of the cell environment can stimulate bacteriocin production. The latter can be induced by unfavourable growth conditions, so-called stress factors. The specific growth rate seems to play an important role in the control of bacteriocin production.

Keywords: *Lactobacillus amylovorus*, lactic acid bacteria, bacteriocins, amylovorin L471

INTRODUCTION

Lactic acid bacteria produce a wide variety of antagonistic factors that include primary and secondary metabolites (Piard & Desmazeaud, 1991; De Vuyst *et al.*, 1994b). Bacteriocins constitute an important group of antimicrobials from lactic acid bacteria (Piard & Desmazeaud, 1992; Hoover & Steenson, 1993; De Vuyst & Vandamme, 1994a, b; Dodd & Gasson, 1994). Lactic bacteriocins are bioactive peptides with a bactericidal mode of action towards other Gram-positive bacteria (Klaenhammer, 1988). Based on their physicochemical

and genetic properties, they can be subdivided into four classes (Klaenhammer, 1993; De Vuyst *et al.*, 1994b). Since many destroy food spoilage and food-borne pathogenic bacteria, the bacteriocins or the producer organisms (applied as starters or adjunct cultures) could be utilized as natural additives for food preservation in future (Ray & Daeschel, 1992; Smith, 1993). Whether these antimicrobial proteins are primary metabolites or whether they behave as secondary metabolites is not very clear.

Primary metabolites are produced during the growth phase of an organism and play a vital role in active growth. Secondary metabolites can be described as compounds that are produced after active growth has taken place and which perform no vital function for the producing organism (Kleinkauf *et al.*, 1986). Some secondary metabolites may however play a specific role in

Abbreviations: AU, activity units; B, bacteriocin titre; CDM, cell dry mass; DO, dissolved oxygen level; S, glucose concentration; X, biomass concentration; $Y_{X/S}$, cell yield coefficient; $Y_{P/X}$, bacteriocin yield coefficient.

the life-cycle of a microorganism, for example during sporulation (Vandamme, 1981), mycelium formation (Kondo *et al.*, 1988) or to defend the organism in its typical ecosystem (Hurst, 1981; Harris *et al.*, 1992a). The two types of metabolites require different fermentation conditions and processes for maximal production.

For effective commercial application of bacteriocins, optimization of their production by environmental and technological control of the process will be necessary. Such a detailed physiological and technological study may further explain the low bacteriocinogenic activity often observed when bacteriocin-producing strains are added to food systems. The use of *in situ* bacteriocin production is indeed promising for a widespread and safe application of bacteriocins in future food preservation.

The effects of growth conditions have been studied for the production of nisin (Hirsch, 1951; Hurst, 1966; Egorov *et al.*, 1971; Kozlova *et al.*, 1972; De Vuyst & Vandamme, 1992, 1993; De Vuyst, 1995), pediocin (Biswas *et al.*, 1991), enterocin 1146 (Parente & Hill, 1992; Parente & Ricciardi, 1994), lactocin S (Mørtvedt-Abildgaard *et al.*, 1995) and a number of other bacteriocins (Barefoot & Klaenhammer, 1984; Joerger & Klaenhammer, 1986; Muriana & Klaenhammer, 1987; Daba *et al.*, 1993; Kaiser & Montville, 1993; Parente *et al.*, 1994; Quadri *et al.*, 1994). Maximum bacteriocin production usually requires complex media and well-controlled physical factors such as temperature and pH. Unfortunately, most bacteriocins show a more or less sharp decrease in activity at the end of the exponential growth phase (Barefoot & Klaenhammer, 1984; Joerger & Klaenhammer, 1986; Muriana & Klaenhammer, 1987; De Vuyst & Vandamme, 1992; Daba *et al.*, 1993; Parente *et al.*, 1994). However, a decrease in activity has not been observed for all bacteriocins from lactic acid bacteria, even after prolonged incubation. Therefore it is necessary to understand the relationships between growth, bacteriocin production and factors affecting the occurrence and intensity of the activity peak during the growth cycle of the producing strains. Consequently, metabolic and process control of bacteriocin biosynthesis to enhance their production, activity and stability may have economic significance for bacteriocin production processes.

Amylovorin L471 is a bacteriocin that is produced by a *Lactobacillus amylovorus* strain (L. De Vuyst, R. Callewaert & B. Pot, unpublished results). It is a small, thermostable and extremely hydrophobic antimicrobial protein displaying antagonistic activity towards closely related strains such as *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901T (the most sensitive strain), *L. delbrueckii* subsp. *delbrueckii* ATCC 6949 and *Lactobacillus helveticus* ATCC 15009. Under native conditions, the bacteriocin forms aggregates, the molecular mass of which exceeds 30 kDa. Under reduced conditions, two peptide bands can be visualized by tricine-SDS-PAGE, both with a molecular mass of less than 6000 Da.

In this paper the primary metabolite kinetics of amylovorin L471 biosynthesis are described under different

physiological conditions. Furthermore, it is shown that bacteriocin production can be induced by unfavourable growth conditions, so-called stress factors, and that the specific microbial growth rate seems to play an important role in the control of bacteriocin production.

METHODS

Bacterial strains and media. The bacteriocin-producing strain used in this study was *L. amylovorus* DCE 471. *L. delbrueckii* subsp. *bulgaricus* LMG 6901T was used as the amylovorin-sensitive indicator organism. The strains were stored at -80°C in MRS broth plus 25% (v/v) glycerol. Before experimental use, the cultures were propagated twice in MRS (Oxoid) at 37°C ; the transfer inoculum was 1% (v/v). MRS, TGS (Tryptone Glucose Salts) and LHGS (Lactalbumin Hydrolysate Glucose Salts) broth were used for bacteriocin production. TGS broth contains (g l^{-1}): glucose, 20; tryptone (Oxoid), 20; Na_2HPO_4 , 6; KH_2PO_4 , 3; NH_4Cl , 1; MgSO_4 , 0.245; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.04; CaCl_2 , 0.015; plus 1 ml Tween 80 l^{-1} . LHGS has the same composition as TGS except that tryptone is replaced by an equal amount of lactalbumin hydrolysate (Oxoid). Agar media were prepared by the addition of 1.5% (w/v) granulated agar (Oxoid) to the broth medium; overlay agar contained 0.7% granulated agar.

Fermentation experiments. A 6.4 l Biostat E fermenter (B. Braun Biotech International) containing 5.5 l MRS broth was used to study the kinetics of amylovorin production. The growth medium was adjusted to pH 6.5 and sterilized at 121°C . The carbon source (glucose) was sterilized separately and aseptically added to the fermenter. The fermenter was inoculated with 50 ml (approx. 1%, v/v) of an exponentially growing culture of the amylovorin-producing strain *L. amylovorus* DCE 471. The inoculum was obtained by propagating a fresh culture twice at 37°C for 12 h. The fermenter was operated at 37°C (or the temperature indicated) without aeration. Slow agitation (50 r.p.m.) was maintained to keep the fermentation broth homogeneous. Experiments in which growth and bacteriocin production were examined under aerobic conditions were performed in a stainless steel, *in-situ*-sterilizable Braun Biostat C fermenter (working volume, 12 l) with pH and dissolved oxygen (DO) control. Oxygen was supplied by adding sterile filtered air; constant saturation was achieved by automatic control of the air flow rate. Experiments in Erlenmeyer flasks (250 ml) involved the addition of sterile ethanol (final concentration 0.0, 1.0, 2.0 or 3.0%, v/v) or sodium chloride (final concentration 0.0, 0.5, 1.0, 2.0 or 3.0%, w/v) solutions to 100 ml MRS broth, before inoculation with *L. amylovorus* DCE 471. All fermentations were done in duplicate.

Sampling. Over a period of 24 h or more, samples were aseptically withdrawn from the fermentation vessel to determine cell number (c.f.u.), cell dry mass (CDM), bacteriocin activity (B) and residual glucose concentration (S). DO, pH and base supply were monitored on line. Cells were removed from samples to be assayed for bacteriocin activity and residual glucose concentration by centrifugation (5000 g, 20 min, 4°C). Adsorption and desorption of the bacteriocin molecules from the cells of the producing organism were studied using the technique of Yang *et al.* (1992). Proteolytic activity of cells and cell-free supernatants during fermentation was tested using azocasein as described by Parente *et al.* (1994).

Growth determination. CDM determinations were performed by filtering 50 ml fermentation broth through $0.45\text{ }\mu\text{m}$ HA-filters (Millipore) and drying at 105°C for 24 h. Cell numbers were estimated by plate counts on MRS agar. The maximal

specific growth rate ($\mu_{\max}$) was calculated by linear regression from plots of $\ln X$ versus t (where X is the biomass concentration in g CDM l^{-1} and t the time) at points during the exponential growth phase. Cell yields ($Y_{x/s}$) were calculated by linear regression from plots of $X-X_0$ versus S_0-S (where X is the biomass concentration in g CDM l^{-1} and S the residual glucose concentration in g l^{-1}).

Quantitative determination of bacteriocin titres. Amylovorin L471 activity was determined by an adaptation of the critical dilution method currently used for the assay of bacteriocins (Mayr-Harting *et al.*, 1972). Serial twofold dilutions of filter-sterilized cell-free culture supernatant containing bacteriocin (10 μ l) were spotted onto fresh indicator lawns of *L. delbrueckii* subsp. *bulgaricus* LMG 6901T. These lawns were prepared by propagating fresh cultures to OD₆₀₀ 0.45 and adding 100 μ l cell suspension to 3.5 ml overlay agar. Overlaid agar plates were incubated for at least 24 h at 37 °C. Activity was defined as the reciprocal of the highest dilution demonstrating complete inhibitory activity of the indicator lawn and was expressed in activity units (AU) (ml culture medium)⁻¹. Bacteriocin yields ($Y_{p/x}$) were calculated by linear regression from plots of $B-B_0$ versus $X-X_0$ (where B is the bacteriocin titre in AU (g CDM)⁻¹ and X the biomass concentration in g CDM l^{-1}).

Glucose concentration determination. Residual glucose levels were determined using a YSI model 27 biosensor (Yellow Springs Instrument Company).

RESULTS

Dynamics of amylovorin L471 production in batch cultivation

To determine the relationship between growth and bacteriocin production, and also the optimal temperature for production of amylovorin L471, *L. amylovorus* DCE 471 was grown in a fermenter at temperatures of 30, 37 and 45 °C at controlled pH 5.0. The time course of growth, bacteriocin production and substrate consumption are shown in Fig. 1. Amylovorin L471 was first detected early in the exponential growth phase and was produced continuously during this phase supporting the proposal that bacteriocins display primary metabolite kinetics (De Vuyst & Vandamme, 1992). Bacteriocin activity showed a maximum level during the mid-exponential phase and then decreased rapidly. Relatively sharp maxima occurred after 20 h at 30 °C (3200 AU ml⁻¹), after 12 h at 37 °C (6400 AU ml⁻¹) and after 6 h at 45 °C (3200 AU ml⁻¹). Biomass yields based on the amount of NaOH required to neutralize the lactic acid accumulated and hence the amount of glucose consumed (homofermentation) were estimated by linear regression from the plots of $X-X_0$ versus S_0-S (where X is the biomass concentration in g CDM l^{-1} and S the residual glucose concentration in g l^{-1} ; Fig. 2). Yields were constant and independent of time for a given temperature and increased from 0.12 ± 0.01 g g⁻¹ at 30 °C (r^2 0.993) and 45 °C (r^2 0.982) to 0.15 ± 0.01 g g⁻¹ at 37 °C (r^2 0.995). The highest final biomass (2.05 g CDM l^{-1}) was obtained at 37 °C (Fig. 1). That the bacteriocin was produced as a primary metabolite was confirmed by plots of the bacteriocin product yield showing the change in bacteriocin activity ($B-B_0$) as a function of biomass formation ($X-X_0$) (Fig. 3). The straight lines with positive slope

indicated an increasing yield of bacteriocin per unit of cell mass during the active growth phase. Although the highest volumetric activity level was found during fermentation at 37 °C, the specific bacteriocin production was experimentally determined as $4.5 \pm 1.4 \times 10^6$, $3.0 \pm 0.3 \times 10^6$ and $3.8 \pm 0.9 \times 10^6$ AU (g CDM)⁻¹ at 30, 37 and 45 °C, respectively. The maximal specific growth rates were calculated as 0.14, 0.54 and 0.72 h⁻¹ at 30, 37 and 45 °C, respectively (Fig. 1). This indicates that bacteriocin production – in particular the specific bacteriocin production – was stimulated by temperatures unfavourable for growth, particularly low temperatures that are characterized by a very slow growth rate. Slow growth seems to induce a stress response in the bacteria, resulting in more efficient bacteriocin production. Similar results were found in batch cultivations without pH control. In addition, under uncontrolled pH conditions it was observed that a lower temperature coincided with a higher maximum bacteriocin titre achieved during the cell growth cycle (12800, 3200 and 800 AU ml⁻¹ at 30, 37 and 45 °C, respectively).

The rate of decrease of bacteriocin activity was not temperature-dependent (Fig. 1). However, the decrease in bacteriocin activity levels was much more pronounced in pH-controlled fermentations in comparison with an uncontrolled pH-fermentation profile (unpublished results). Several mechanisms can be responsible for the decrease of bacteriocin activity (De Vuyst & Vandamme, 1992) of which protein aggregation, proteolytic degradation by specific or non-specific proteases, and bacteriocin adsorption to the cells may be the most important ones. Aggregation of individual polypeptide chains as well as formation of complexes with other medium constituents does occur as could be revealed by diafiltration experiments and chromatographic analysis (L. De Vuyst, R. Callewaert & B. Pot, unpublished results). However, denaturing agents such as SDS and urea did not enhance bacteriocin activity (unpublished results), as was shown for helveticin J by Joerger & Klaenhammer (1986). Proteolytic activity was assayed on both cells and cell-free culture supernatants using azocasein as a substrate. No proteolytic activity could be detected with this non-specific substrate. The change in A_{350} was negligible and varied from 0.11 to 0.12 in at least 180 min with cell-free culture supernatant and remained constant (0.08) in the presence of the cells. The controls containing 175, 17.5 or 1.75 U α -chymotrypsin ml⁻¹ (50 mM sodium phosphate buffer, pH 7.5) showed an increase in A_{350} from 0.406 to 0.818, from 0.180 to 0.650, and from 0.093 to 0.360, respectively.

Adsorption/desorption flask experiments using the technique of Yang *et al.* (1992) showed a possible adsorption of the bacteriocin molecules to producing cells at pH > 2.0, although adsorption to the walls of the recipient cannot be excluded. However, after adsorption, almost no bacteriocin activity potentially adsorbed could be released from the cells in flask experiments. In contrast, when fermentations were run at controlled pH 5.0 and at 37 °C, sample culture supernatants both adjusted to pH 2.0 and adjusted to pH 2.0 followed by boiling of the sample,

Fig. 1. Batch fermentation profile of *L. amylovorus* DCE 471 growth and amylovorin L471 production at constant pH 5.0 at 30 °C (a), 37 °C (b) and 45 °C (c). Cells were grown in a Biostat E fermenter containing 5.5 l MRS broth with 20 g glucose l⁻¹ as carbon source (see Methods). ○, Cell numbers (only shown for 37 °C); ▲, cell dry mass; ■, volumetric amylovorin L471 titre; ▽, residual glucose concentration; □, base consumption. Note the differences in the time scale on the x-axis between (a), (b) and (c), and also the variation in the y-axis scale showing bacteriocin activity.

Fig. 2. Relationship between biomass production ($X-X_0$) and substrate consumption (S_0-S) during batch cultivation of *L. amylovorus* DCE 471 in MRS broth with 20 g glucose l⁻¹ at controlled pH 5.0 at 30 °C (a), 37 °C (b) and 45 °C (c).

showed a higher bacteriocin activity level (measured after adjustment to pH 6.5) compared to non-treated samples (Fig. 4a). Furthermore, during fermentations run at 37 °C and at controlled pH 5.0 during the active bacteriocin production phase, followed by a switch to pH 2.0 by

automatic addition of 2 M HCl when bacteriocin production reached its peak, the activity detected in cell-free culture supernatants remained approximately constant. This resulted in a bacteriocin titre plateau at an activity of approximately 3200 AU ml⁻¹ (Fig. 4b).

Fig. 3. Relationship between bacteriocin production ($B-B_0$) and biomass formation ($X-X_0$) during batch cultivation of *L. amylovorus* DCE 471 in MRS broth with 20 g glucose l⁻¹ at controlled pH 5.0 at 30 °C (●), 37 °C (■) and 45 °C (▲).

Fig. 4. Production and activity of amylovorin L471 produced by *L. amylovorus* DCE 471 with time in a Biostat C fermenter containing 12 l MRS broth with 20 g glucose l⁻¹. (a) Samples were taken from a fermentation controlled at a constant pH of 5.0. Bacteriocin activity was assayed on cell-free culture supernatants adjusted to pH 6.5 (■), culture supernatants adjusted to pH 2.0 followed by centrifugation and adjusting the cell-free liquor back to pH 6.5 (△), and culture supernatants adjusted to pH 2.0 followed by boiling for 5 min at 100 °C, centrifuging and adjusting the cell-free liquor back to pH 6.5 (▼). (b) The fermentation was controlled at a constant pH of 5.0 during the phase of active cell growth and bacteriocin production, and at a constant pH of 2.0 after having reached the bacteriocin peak activity. The arrow indicates the switch from pH 5.0 to 2.0 performed by automatic addition of 2 M HCl. ■, Bacteriocin activity.

Decreases in bacteriocin activity were concomitant with decreases in cell numbers (see Figs 1b and 6a), apparently indicating cell death. The OD₆₀₀ did not decrease indicating that no cell lysis occurred.

Nutritional control of amylovorin L471 production in batch fermentations

Influence of initial glucose levels. When *L. amylovorus* DCE 471 was grown in MRS broth (containing 20 g glucose l⁻¹) without pH control, amylovorin L471 production was limited by the low pH, although glucose seemed to be sufficiently available. Under pH-controlled fermentation conditions (pH 5.0 or 5.4) higher cell densities and hence amylovorin titres were reached. However, growth ceased after 12 h of fermentation, because no glucose remained (L. De Vuyst, R. Callewaert & B. Pot, unpublished results). In order to prolong the exponential growth phase and hence the maximal cell density and concomitantly the amylovorin titre, fermentations were carried out at 37 °C and constant pH (5.0) with increasing initial glucose concentrations of 5, 20, 30, 40 and 60 g l⁻¹. The data are shown in Table 1. The bacteriocin activity seemed to reach a plateau value with an activity of 6400 AU ml⁻¹, except for the fermentation with an initial glucose concentration of 30 g l⁻¹. This may indicate carbon source regulation (De Vuyst & Vandamme, 1992), end product inhibition (Ten Brink *et al.*, 1994) and/or deficient bacteriocin biosynthesis owing to lack of a nutrient other than glucose. After about 10 h of growth the fermentation liquor contained maximal bioactivities in most cases. Upon further fermentation bacteriocin production stopped in those initially containing 30, 40 and 60 g glucose l⁻¹ despite the high residual glucose levels. Similar growth rates (calculated as 0.54 h⁻¹) were observed for all initial glucose concentrations. However, the specific bacteriocin production was significantly higher at 60 g initial glucose l⁻¹ [$8.9 \pm 3.8 \times 10^6$ AU (g CDM)⁻¹] compared to the other glucose levels [$3.0 \pm 1.2 \times 10^6$, $2.5 \pm 0.3 \times 10^6$ and $2.9 \pm 0.4 \times 10^6$ AU (g CDM)⁻¹ for 0.5, 3.0 and 4.0 % (w/v) glucose, respectively], as calculated from the plots of the change in bacteriocin activity ($B-B_0$) as a function of ($X-X_0$) (Fig. 5). This high glucose level may have caused a stress condition for the producing cells. Whereas the total biomass increased from 0.95 g CDM l⁻¹ at 5 g initial glucose l⁻¹ to 2.92 g CDM l⁻¹ at 60 g initial glucose l⁻¹, the maximal bioactivity always corresponded with a biomass of approximately 1.50 g CDM l⁻¹ (except for 5 g glucose l⁻¹ where the maximum biomass level did not reach this value owing to the limited glucose supply). During the stationary phase the culture consumed glucose and produced lactic acid at a constant rate (data not shown), indicating that exhaustion of some other nutrient was responsible for the ending of growth and its associated amylovorin L471 production.

Influence of C/N ratio. To determine whether nitrogen was a limiting factor, amylovorin production was studied with varying C/N ratios in the fermentation broth using the semisynthetic TGS medium (Table 2). Because the biomass concentration did not double with an increasing glucose concentration, no improved bacteriocin yield

Table 1. Influence of initial glucose concentration on *L. amylovorus* DCE 471 growth and amylovorin L471 production in fermentations at controlled pH 5.0

Fermentations were run in a fermenter containing 5.5 l MRS broth (see Methods).

Glucose concn (g l ⁻¹)	$\mu_{\max}$ (h ⁻¹)*	Length of exponential phase (h)	X (g CDM l ⁻¹)†	$B_{\max}$ (AU ml ⁻¹)	Time of $B_{\max}$ (h)	$\times 10^{-3} Y_{p/x}$ [AU (g CDM) ⁻¹]
5.0	0.53	12	0.95	1600	7.5	3000
20.0	0.54	12	2.05	6400	11–12	3000
30.0	0.45‡	14	2.35	4500	10–12.5	2600
40.0	0.39‡	14	2.60	6400	10–12	2900
60.0	0.54	18	2.92	6400	8–9	8900

* Calculated from CDM determinations during the exponential growth phase.

† Biomass concentration at the end of the exponential phase.

‡ No linearity was observed in the plot of $\ln X$ versus t .

Fig. 5. Relationship between bacteriocin production ($B-B_0$) and biomass formation ($X-X_0$) during batch cultivation of *L. amylovorus* DCE 471 in MRS broth at controlled pH 5.0 and 37 °C with increasing initial glucose concentrations of 5.0 (○), 20.0 (■), 30.0 (▲), 40.0 (●) and 60.0 (□) g l⁻¹.

could be detected by the bacteriocin assay (powers of two) used. Only conditions favouring a drastic increase in biomass concomitantly improved bacteriocin titres. As shown in Table 2, biomass production is dependent on glucose supply and, because of its association with growth, so too is bacteriocin production.

Influence of initial nitrogen concentration and other growth factors. In an attempt to further increase growth and bacteriocin production, *L. amylovorus* DCE 471 was cultivated at pH 5.0 in MRS broth containing a double amount of total nitrogen (4.4%) and hence unknown growth factors (e.g. vitamins and minerals derived from yeast extract). No higher bioactivity (6400 AU ml⁻¹) could be detected (Table 2); the total biomass yield was 1.3-fold higher than before. The maximal bioactivity was reached after about 10 h of fermentation and corresponded with a biomass of approximately 2.10 g CDM l⁻¹ (data not shown). Fermentation in MRS broth containing fourfold higher total initial nitrogen concentration (8.8%)

Table 2. Influence of medium composition on *L. amylovorus* DCE 471 growth and amylovorin L471 production at controlled pH 5.0

Fermentations were run in a fermenter containing 5.5 (MRS) or 12 (TGS, LHGS) l culture broth (see Methods).

Medium composition	$\mu_{\max}$ (h ⁻¹)	$X_{\max}$ (g CDM l ⁻¹)	$B_{\max}$ (AU ml ⁻¹)
TGS broth (1.0% glucose, 2.0% tryptone)	0.26	0.63	1600
TGS broth (2.0% glucose, 2.0% tryptone)	ND	0.78	1600
TGS broth (2.0% glucose, 4.0% tryptone)	ND	0.99	1600
LHGS broth (2.0% glucose, 2.0% lactalbumin hydrolysate)	ND	0.16	800
MRS broth (2.0% glucose, 2.2% nitrogen)	0.54	2.05	6400
MRS broth (2.0% glucose, 4.4% nitrogen)	0.52	2.59	6400
MRS broth (4.0% glucose, 8.8% nitrogen)	0.47	5.74	11200

ND, Not determined.

Fig. 6. Batch fermentation profile of *L. amylovorus* DCE 471 growth and amylovorin L471 production at constant pH of 5.0 and 37 °C with aeration at 40 % (a) and 80 % (b) air saturation levels. Cells were grown in a Biostat C fermenter containing 12 l MRS broth with 20 g glucose l⁻¹ as carbon source (see Methods). ○, Cell numbers (only shown for 40 % air saturation); ▲, cell dry mass; ■, volumetric amylovorin L471 titre; ▽, residual glucose concentration; □, base consumption.

Table 3. Influence of the addition of sodium chloride and ethanol on *L. amylovorus* DCE 471 growth and amylovorin L471 production

Fermentations were carried out in 250 ml Erlenmeyer flasks containing 100 ml MRS broth. Samples were withdrawn after 12 h incubation.

Component added to standard MRS broth	X (g CDM l ⁻¹)	B (AU ml ⁻¹)	$\times 10^{-6} Y_{p/x}$ [AU (g CDM) ⁻¹]
None	2.19	400	0.18
NaCl (5 g l ⁻¹)	2.10	400	0.19
NaCl (10 g l ⁻¹)	2.05	400	0.19
NaCl (20 g l ⁻¹)	1.79	300	0.17
NaCl (30 g l ⁻¹)	0.75	100	0.13
Ethanol (10 ml l ⁻¹)	2.16	800	0.37
Ethanol (20 ml l ⁻¹)	2.12	400	0.19
Ethanol (30 ml l ⁻¹)	2.05	400	0.19

and twofold higher initial glucose concentration (4.0 %), hence ensuring a sufficient supply of the energy source (glucose), building blocks (amino acids) and growth factors (e.g. vitamins), resulted in a much higher cell density (5.74 g CDM l⁻¹; Table 2). The maximal amylovorin L471 activity then averaged 11 200 AU ml⁻¹ (Table 2) and was reached after 12 h of growth (data not shown). It corresponded with a biomass of about 4.70 g CDM l⁻¹. The importance of unknown growth factors can also be concluded from the bacteriocin titres obtained with the semisynthetic media TGS and LHGS (Table 2).

Effects of oxygen

To investigate the influence of oxygen on growth and bacteriocin production, fermentations were run at 40 and 80 % (v/v) air saturation levels. The results are shown in Fig. 6. When fermentations were performed under aerobic conditions (40 % air saturation), the cell growth, glucose consumption and bacteriocin production profiles were

similar to those found in unaerated fermentations. Higher air saturation levels (80 %) did not affect either cell growth or glucose consumption. For instance, the cell yield coefficients were calculated as 0.17 and 0.14 g CDM (g glucose)⁻¹ at 40 % and 80 % air saturation, respectively. As with unfavourable growth temperatures, specific bacteriocin production increased with higher air saturation levels [2.9 × 10⁶ and 3.6 × 10⁶ AU (g CDM)⁻¹ at 40 % and 80 % air saturation, respectively], despite the lower maximal bacteriocin titres (3200 and 1600 AU ml⁻¹ at 40 % and 80 % air saturation, respectively). Maximal specific growth rates were calculated as 0.45 and 0.53 h⁻¹ at 40 % and 80 % air saturation, respectively.

Effects of ethanol and NaCl

To determine if lower specific growth rates could cause the cells to display a higher specific bacteriocin production under environmental conditions not favouring microbial growth, the influence of added sodium chloride and

ethanol were tested. The data are shown in Table 3. Increasing concentrations of both sodium chloride and ethanol inhibited cell growth and bacteriocin production. When ethanol (final concentration 10 ml l⁻¹) was included in the growth medium, a twofold increase in the volumetric bacteriocin activity was observed. Specific bacteriocin production was also higher in the presence of ethanol (10 ml l⁻¹). The lower values of *B* and *Y_{p/x}* shown in Table 3 are due to the late time (after peak activity) at which samples were withdrawn.

DISCUSSION

All the characteristics of amylovorin L471 production mentioned above clearly indicate that its biosynthesis displays primary metabolite kinetics. For several bacteriocins reported in the literature, it is indeed clear that the compound is produced during the exponential growth phase with a maximum in the middle or at the end of this phase, or at the beginning of the stationary phase (De Vuyst & Vandamme, 1992; Parente *et al.*, 1994; Mørtvedt-Abildgaard *et al.*, 1995). Only in a few cases has bacteriocin production been shown to occur during the stationary phase (Kozak *et al.*, 1978; Biswas *et al.*, 1991; Jiménez-Díaz *et al.*, 1993). In the case of lactostrepcin 3 (Kozak *et al.*, 1978) and plantaricin T (Jiménez-Díaz *et al.*, 1993), the compound is the second bacteriocin produced during the growth cycle. The production of a second bioactive peptide at a later time in the growth cycle may be the response to an environmental stimulus. Only Biswas *et al.* (1991) suggested that bacteriocins, particularly pediocin and nisin, are secondary metabolites, since they found that 60% of the pediocin AcH from *P. acidilactici* AcH was produced during the first 8 h (exponential phase), and the final 40% was produced during the next 8 h (stationary phase) of fermentation. In addition, Ten Brink *et al.* (1994) demonstrated production of acidocin B by non-growing cells of *Lactobacillus acidophilus* M46. In the latter case the inability of the cells to grow was caused by omitting Tween 80 from the medium. This manipulation may have resulted in a stress condition for the cells and hence overproduction of the bacteriocin (see below).

Whether bacteriocins have a vital function for the producing microorganism as is the case for primary metabolites is not yet known. It is, however, possible that bacteriocins do play some functional role during the growth cycle (Hurst, 1978; De Vuyst & Vandamme, 1992; De Vuyst, 1994). From a physiological point of view, it can be stated that bacteriocin inactivation in the stationary phase is compensated by continuous production of lactic acid at a constant rate. Indeed, lactic acid displays characteristics of both a primary and a secondary metabolite (Luedeking & Piret, 1959). However, it is not clear if the disappearance of bacteriocin during the stationary phase is mainly due to proteolytic degradation. Adsorption of bacteriocin to the cell surface via ionic or hydrophobic interaction at pH values near neutrality and its desorption at low pH values may be an alternative explanation for the observed profile of the bacteriocin titre detected in the cell-free culture supernatant. Ad-

sorption to the cells may for instance be the result of complex formation between the mature bacteriocin molecules and the immunity peptide. The latter is responsible for immunity of the cell to its own product during active growth. Both peptides are hydrophobic and bacteriocin immunity peptides indeed seem to be localized at the outer surface of the cytoplasmic membrane (Reis *et al.*, 1994). Therefore, it may be advisable to follow both cell-bound and extracellular bacteriocin during the fermentation course. Such an approach was followed during the early investigations of nisin production by Hurst (1966). However, a decrease in bacteriocin activity also occurs during fermentation runs at uncontrolled pH (De Vuyst & Vandamme, 1992; L. De Vuyst, R. Callewaert & B. Pot, unpublished results). Indeed, at the end of the fermentation the pH reached values which should favour bacteriocin desorption and hence a higher total bacteriocin activity should be detected in cell-free culture supernatants. However, the opposite was true. Finally, protein aggregation, for example via hydrophobic interactions between different polypeptide chains, cannot be completely ruled out.

Regarding a possible ecological role of bacteriocins, these compounds may offer a competitive advantage to the producing strains and help them in a competing microbial environment. This may underline their future use in mixed starter cultures for a directed and controllable fermentation, for instance in the production of olives, sauerkraut, sausages, etc. (Jiménez-Díaz *et al.*, 1993; Harris *et al.*, 1992b).

To harvest maximum bacteriocin levels it is advisable to collect the fermentation broth as soon as maximum activity is reached (De Vuyst & Vandamme, 1992) or to carry out the fermentation under environmental conditions which prevent adsorption, such as a switch to pH 2.0 (as shown here), or aggregation, for example by adding ethanol to the fermentation broth as suggested by Mørtvedt-Abildgaard *et al.* (1995). An alternative would be to remove the bacteriocin from the culture broth during fermentation by using appropriate adsorbents, either batchwise (Hörner *et al.*, 1990; De Vuyst & Vandamme, 1992) or continuously (L. De Vuyst, R. Callewaert & B. Pot, unpublished results), or using a membrane reactor (Ungermann *et al.*, 1991; De Vuyst & Vandamme, 1992; Champagne *et al.*, 1994). Apparently, the halt in bacteriocin production and the subsequent decrease in bacteriocin activity levels is accompanied by a decrease in cell numbers upon prolonged fermentation. However, based on our experimental data, we suggest that the decrease in cell number is not caused by cell death but has to do with bacteriocin adsorption to the cells. This adsorption phenomenon results in: (i) no further cell division after plating (only the cells whose surfaces are not occupied by bacteriocin molecules can produce new colonies); and (ii) no further bacteriocin biosynthesis by the cells owing to product inhibition. Only washed cells allow further growth and bacteriocin production. The latter has been shown by Ten Brink *et al.* (1994) who used non-growing *L. acidophilus* cells for bacteriocin production.

Growth at low temperature results in low specific growth rates and high specific productivities. Slow growth can make more energy available for bacteriocin production; this energy is required for both polymerization of building blocks (amino acids) and specific and coordinated control of bacteriocin gene expression. The high ATP requirement for bacteriocin biosynthesis further underlines its primary metabolite nature. It may also explain why, in contrast with secondary metabolites (Martin, 1977), bacteriocin production is not repressed by phosphate, other than at very high inorganic phosphate concentrations, as has been shown for nisin (De Vuyst & Vandamme, 1993). Apparently, bacteriocin production is stimulated by less favourable growth conditions, so-called stress factors, such as low temperatures, low specific growth rates, potentially toxic compounds such as sodium chloride, ethanol and oxygen, and a competing microflora, etc. Slow growth at low temperature indicates a stress condition and may explain the higher specific bacteriocin production. An extremely high concentration of the carbon source may also cause a stress situation owing to a higher osmotic pressure, and hence stimulate specific bacteriocin production. Ecologically, this may be explained by the fact that under unfavourable growth conditions the antagonistic effect of lower acid production is not sufficient for successful competition with other microorganisms. Parente *et al.* (1994) also found that reduction in growth and lactic acid production is more than compensated by the increase in bacteriocin production. Mørtvedt-Abildgaard *et al.* (1995) further suggested that ethanol may induce genes involved in the synthesis of lactocin S. Thus, it is clear that manipulation of the cellular environment may stimulate bacteriocin production, or, more precisely, that lactic acid bacteria may react to physiological inducers. Recent studies indicate that bacteriocin production is controlled by an autoinduction regulatory mechanism through signal transduction. For instance, in the production of the lantibiotic nisin by *Lactococcus lactis* subsp. *lactis*, a two-component regulatory system encoded by the *nisRK* genes is involved in signal transducing (Engelke *et al.*, 1994). The occurrence of a histidine kinase sensor protein and one or more response regulatory proteins has also been shown for subtilin (Klein *et al.*, 1993), plantaricin A (Diep *et al.*, 1994) and sakacin A (Axelsson & Holck, 1995). For bacteriocin production, this cell-cell cooperative interaction or communication system employs either an antimicrobial peptide, e.g. carnobacteriocin (Saucier *et al.*, 1995), a bacteriocin-like peptide, e.g. sakacin P (Tichaczek *et al.*, 1994), or another peptide (Barefoot *et al.*, 1994), as the signalling molecule. Because induction does not occur in the stationary phase, bacteriocins are considered to be primary metabolites of growth from this point of view too (Saucier *et al.*, 1995). Apparently, a small amount of induction factor is necessary, since induction occurs from the early growth phase. On the other hand, glucose concentration and pH have been shown to affect the bacterial two-component signal-transducing system in *Staphylococcus aureus* (Regassa & Betley, 1992; Regassa *et al.*, 1992). In lactic acid bacteria too, nutritional and environmental control seem to play an important role in

bacteriocin production (De Vuyst & Vandamme, 1992, 1993; De Vuyst, 1995; Mørtvedt-Abildgaard *et al.*, 1995). Alternatively, bacteriocin production could be the result of the induction of genes like those involved in the heat shock response in *Escherichia coli* or the cold shock response in psychrophilic microorganisms (Schlessinger *et al.*, 1982; Van Bogelen *et al.*, 1987).

The fact that slower growth favours a higher bacteriocin production is of practical importance on an industrial scale, since metabolic regulatory mechanisms such as catabolite repression and feedback-inhibition may be circumvented by a slow feeding rate of the substrate responsible which in turn results in a slower growth regime. Slow feeding may be controlled by fed-batch and continuous culture fermentations. The stimulation of bacteriocin production by unfavourable growth conditions may be of further importance when bacteriocin-producing lactic acid bacteria are added to foods as starters or protective cultures. However, the specific environment of the food may limit not only the capacity of strains to produce antimicrobials but also the antagonistic effect of secreted or added bacteriocins. The presence of salts and other food ingredients, adsorption to proteins and other food particles, unequal distribution of the bacteriocin molecules in the food water phase, etc. may explain both phenomena.

Our results show that production of active bacteriocin may be severely affected by environmental factors. In view of the possible future use of bacteriocins as food and feed biopreservatives, these factors have to be taken into account in the design of bacteriocin production processes.

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