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Production and pH-Dependent Bactericidal Activity of Lactocin S, a Lantibiotic from *Lactobacillus sake* L45

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The amount of lactocin S activity in a growing culture depends on the growth stage of the bacteria, the pH of the medium, the presence of ethanol, and the aeration of the culture. We observed the highest levels of bacteriocin activity in the early stationary growth phase of cultures at 30°C. When *Lactobacillus sake* L45 was grown in a fermentor at pH 5, it produced 2,000 to 3,000 bacteriocin units per ml, which represented an 8- to 10-fold increase in bacteriocin production compared with production during batch culture fermentation. Less than 10% of this level of bacteriocin activity was observed during fermentation at pH 6.0. When 1% ethanol was included in the growth medium, a two- to fourfold increase in the bacteriocin yield was observed. Aerating the culture during growth almost completely eliminated the production of active bacteriocin. Our results also showed that lactocin S-mediated killing of target cells depended on the pH of the culture. The pH had to be less than 6 in order to obtain a bactericidal effect with lactocin S-sensitive cells. At pH values greater than 6, lactocin S had no apparent effect on sensitive cells.

Some bacteria produce bacteriocins, which are proteinaceous antimicrobial substances that inhibit the growth of closely related species. Many gram-negative and gram-positive bacteria produce bacteriocin-like compounds (8, 10, 23). Bacteriocins produced by *Lactobacillus sake* strains have been described by various workers (14, 20, 24). Studies have shown that when bacteriocinogenic strains are added to food, the antimicrobial effect of bacteriocins is often less than the effect expected; in fact, in some cases there is no antimicrobial effect (27). The low bacteriocinogenic activity observed in such situations may be due to a decreased rate of production and/or to environmental factors which inhibit bacteriocin activity.

In our initial studies of *L. sake* L45 we observed that the ability to produce lactocin S activity was frequently lost as a result of plasmid instability (14). The unstable plasmid present in this strain was shown to carry the genes required for bacteriocin production (14, 17). The genetic instability of lactocin S production has also been shown to be due to a high frequency of transpositional events caused by an insertion element residing in the bacterial chromosome (22). However, genetic instability by itself cannot explain all of the variations in lactocin S activity that have been observed. Bacterial growth conditions may have profound effects on the yield of active bacteriocin, and it is important to determine how various growth conditions influence bacteriocin production (2). Lactic acid bacteria may prevent growth of spoilage and pathogenic microorganisms more efficiently in food and feed products if the production of active bacteriocin is increased. Hence, optimizing bacteriocin production and enhancing bacteriocin stability and activity may have economic significance. Obtaining greater yields of bacteriocins should also facilitate physical and chemical studies of these compounds.

This study was undertaken to determine how a number of external factors which commonly vary in fermentation processes affect the production of lactocin S and how pH affects the bactericidal activity of lactocin S.

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MATERIALS AND METHODS

Bacterial cultures and media. *L. sake* L45 is the organism which produces lactocin S (14). *Pediococcus acidilactici* Pac1.0 was used as an indicator strain (15). The strains were stored at −80°C in MRS broth (Difco Laboratories, Detroit, Mich.) containing 15% glycerol. The cultures were grown in MRS broth at 30°C (inoculum, 1%). The numbers of CFU per milliliter were determined by plating 10-fold dilutions (diluted in 0.9% NaCl) on MRS medium containing 1.5% (wt/vol) agar.

Preparation of bacteriocin extracts and bacteriocin assay. Bacteriocin extracts were prepared by removing *L. sake* L45 cells by centrifugation, adjusting the pH to 6.5, and filter sterilizing the preparation (15). The level of bacteriocin activity was determined by the microtiter plate assay as previously described (15); 1 bacteriocin unit (BU) was defined as the amount of bacteriocin that resulted in 50% inhibition of the indicator organism under standard assay conditions.

Extraction of bacteriocin activity from *L. sake* cells grown at pH 6.0. The pH of some 5-ml samples removed from a fermentor maintained at pH 6.0 was adjusted to 2.0 with 2 M HCl, and then the preparations were incubated at 4°C for 2 h; after this the cells were removed by centrifugation at 5,000 × *g* for 15 min at 4°C, and the supernatant was frozen until it was assayed. Other 5-ml samples were treated in the same way, except that the cell pellet was resuspended in 5 ml of 20 mM sodium phosphate (pH 5.0); then the pH of one-half of each sample (2.5 ml) was adjusted to 2.0 with 2 N HCl. The two resuspended pellets were incubated at 4°C for 2 h and frozen until the bacteriocin activities of the cell-free supernatants were determined.

Lactocin S production in broth media. When fermentation studies were performed at a constant pH, a 2-liter working volume of MRS broth was placed in a sterile 3-liter, flat-bottom bioreactor vessel (Applikon model BTS 05 fermentor with automatic pH control). The temperature was maintained at 30°C, and the culture was agitated at 500 rpm. The growth media were adjusted to pH 5.0, 5.6, and 6.0 with 2 N HCl, and during fermentation the pH was maintained at a constant value by adding a 33% NaOH solution. In some experiments ethanol was added to the growth medium before inoculation with *L. sake* L45. Experiments in which we examined growth under anaerobic or aerobic conditions were performed in a Chepman model FZ 3000 fermentor (working volume, 15 liters) with pH control (model 10465 4153 L6701 electrode; Ingold). Anaerobic conditions were maintained by adding N_2 gas at a flow rate of 3 liters/min; the gas flow was controlled with an Ingold O_2 sensor to ensure exclusion of O_2. Under aerobic conditions oxygen was supplied by adding sterile filtered air at a flow rate of 3 liters/min. Continuous growth was monitored at 600 nm with a Cibacoming model 254 colorimeter.

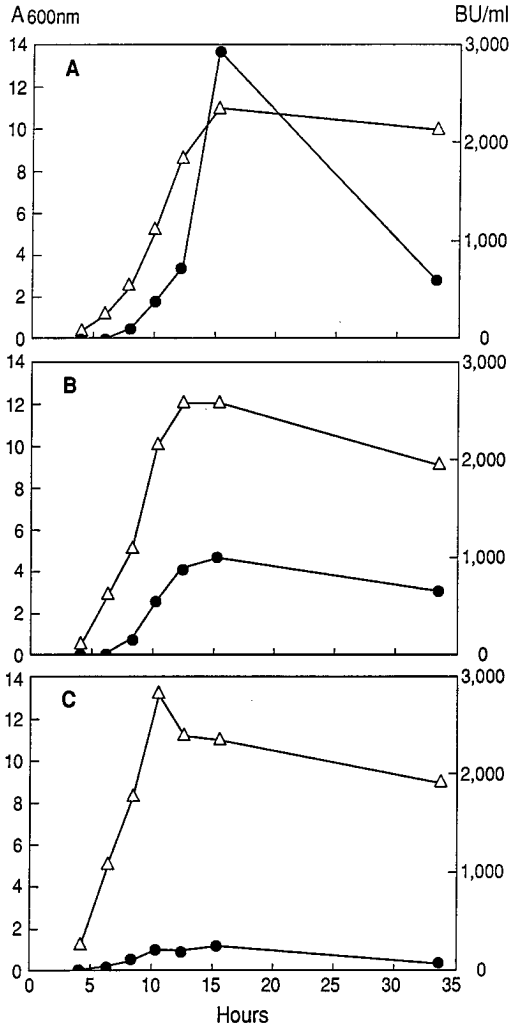

FIG. 1. Production of lactocin S in a fermentor at constant pH values. (A) pH 5.0. (B) pH 5.6. (C) pH 6.0. Symbols: △, growth (A_{600}); ●, bacteriocin activity.

RESULTS

Bacteriocin production in a fermentor at a constant pH. When *L. sake* L45 is grown in static batch cultures at 30°C, it usually produces 200 to 400 BU of lactocin S per ml (13–15), and the bacteriocin activity in the cell-free supernatant is stable during storage at 4°C. To determine the optimal pH for production of lactocin S, *L. sake* L45 was grown in a fermentor at constant pH values of 5.0, 5.6, and 6.0 (Fig. 1). The highest level of lactocin S activity (3,000 BU/ml) was observed in the late exponential and early stationary growth phases at pH 5.0 (Fig. 1A). At pH 5.6 and 6.0 strain L45 produced only 1,000 and 200 BU/ml, respectively (Fig. 1B and C). Compared with the results of the static batch culture growth study, 8- to 10-fold more bacteriocin activity was obtained in the fermentor study at pH 5.0, and approximately 3-fold more activity was obtained at pH 5.6. The bacteriocin activities produced in the fermentor at pH 5.6 and 6.0 appeared to be somewhat more stable upon storage than the activity produced at pH 5.0, although some variations were observed (Fig. 2A). The low level of bacteriocin activity produced at pH 6 may have been due to bacteriocin binding to the producing bacteria, as has been reported previously by Yang et al. (28). In order to test this possibility, bacteria grown in a fermentor at pH 6.0 were collected and extracted at pH 5 and 2 as described in Materials and Methods. No bacteriocin activity was extracted from the cells.

Effects of ethanol and oxygen. When ethanol (final concentration, 1%) was included in the growth medium, a two- to fourfold increase in bacteriocin activity was observed. The increase in bacteriocin activity was observed both in batch cultures with no pH control (data not shown) and in the fermentor in which pH was controlled (Fig. 2B). Higher concentrations of ethanol (2 and 3%) resulted in lower bacteriocin yields, presumably because growth of *L. sake* L45 was inhibited at the higher ethanol concentrations (Fig. 2C and D). Judging from the results of a high-performance liquid chromatography analysis, the ethanol was retained in the medium throughout growth and was not metabolized (data not shown). When fermentation was performed under aerobic conditions, low levels of bacteriocin activity were detected in the cultures (<200 BU/ml) compared with the levels of activity observed during anaerobic fermentation (2,000 to 3,000 BU/ml).

Bactericidal effect of lactocin S. In previous studies of the inhibitory action of lactocin S against susceptible cells workers have obtained conflicting results (13). Lactocin S has exhibited both a bacteriostatic effect and a slow bactericidal effect against target organisms (13). Therefore, a more thorough study of the effect of lactocin S on the viability of *P. acidilactici* Pac1.0 was performed. Lactocin S and *P. acidilactici* (the target organism) were added simultaneously to MRS media which were preadjusted to pH 6.8, 6.0, 5.5, or 5.0. The growth of the indicator strain and the pH were monitored during incubation at 30°C (Fig. 3). At pH 6.8, normal growth of the indicator strain was observed for the first 4 h (Fig. 3A). Both growth and pH were normally compared with the control culture without lactocin S. However, when the lactocin S-containing culture reached an optical density of approximately 0.5 and the pH reached 6, bacterial growth was inhibited to some extent. The pH of the culture continued to decline until growth eventually stopped. At pH 5.8 to 5.7 the optical density of the culture started to decline, and after 11 h of incubation the culture had become almost clear (optical density, 0.05). When the same experiment was performed with a medium preadjusted to pH 6.0 (Fig. 3B), some initial growth and metabolic activity of the indicator strain were observed, as indicated by an initial increase in the optical density and a decrease in the pH from 6.0 to 5.8. However, lactocin S seemed to inhibit the target organism at an earlier growth stage. In the experiments performed with media preadjusted to pH 5.5 and 5.0 no significant growth or metabolic activity was observed, as judged by the constant pH and low optical density during the entire incubation period (Fig. 3C and D). The results of these experiments suggest that the bactericidal (bacteriolytic) activity of lactocin S is strongly pH dependent and only affects the target organism at pH values below 6.0.

In order to further substantiate the hypothesis that the bactericidal activity of lactocin S is pH dependent, the number of surviving bacteria was determined by plate counting. Standard growth medium was inoculated with the indicator strain at pH 6.4 and 30°C together with lactocin S. The number of bacteria and the pH of the culture were determined (Fig. 4). During the first 4 h of growth the number of bacteria in the lactocin S-containing culture was comparable to the number of bacteria in the control culture without lactocin S. When the pH reached 5.8 to 5.7, the growth of pediococci in the lactocin S-containing culture ceased; this was followed by a decrease in the number of surviving cells. After approximately 10 h of incubation the

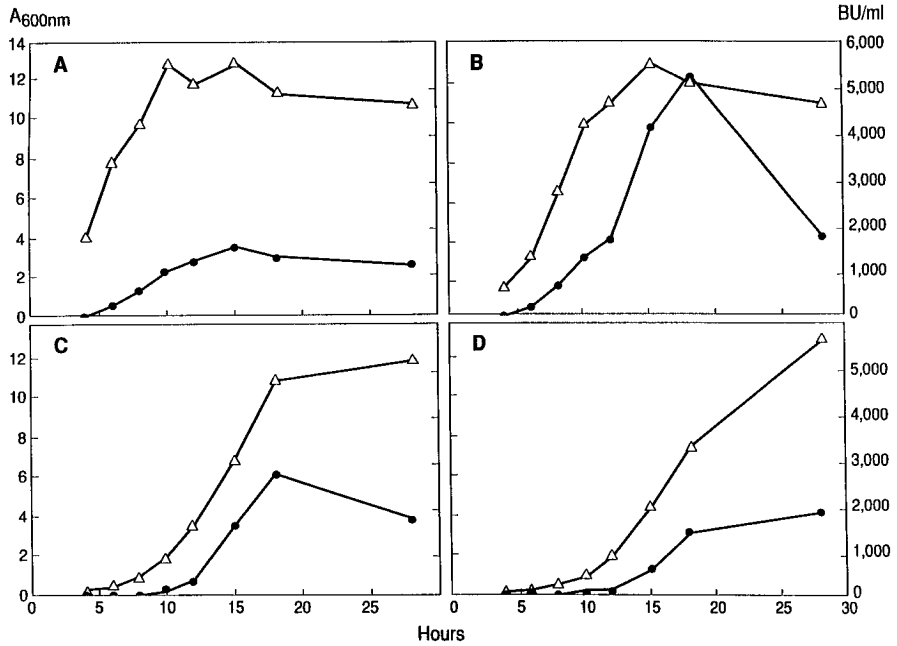

FIG. 2. Effect of ethanol on bacteriocin yield when *L. sake* L45 was grown in a fermentor at pH 5 and 30°C. (A) No ethanol in the growth medium (control). (B) 1% ethanol. (C) 2% ethanol. (D) 3% ethanol. Symbols: △, growth (A_{600}); ●, bacteriocin activity.

number of viable pediococci had decreased more than 99%, which strongly suggests that lactosin S was bactericidal.

DISCUSSION

Our results clearly show the importance of choosing the proper growth conditions for production of active lactocin S. In particular, pH appears to be a crucial factor for production of active bacteriocin. Whereas large amounts of lactocin S activity were released from cells cultured at pH 5.0, less than 10% of the pH 5.0 activity was obtained when the cells were cultured at pH 6.0. Differences in biomass production could not account for the increased levels of bacteriocin activity. Binding of bacteriocin to producer cells has been described previously (28). However, it is unlikely that the low yield of lactocin S at pH 6.0 was due to binding of bacteriocin to the cells, since washing the cells at pH 2 or 5 did not release any bacteriocin activity. It also seems unlikely that the low yield at pH 6.0 was due to inactivation caused by prolonged storage (24 h) at this pH since the pH (between pH 2 and 9) does not affect the stability of lactocin S during storage (16).

Lactocin S belongs to a group of bacteriocins called lantibiotics (4, 7, 17). The production of lantibiotics is complex and depends on the expression of a number of biosynthetic genes,

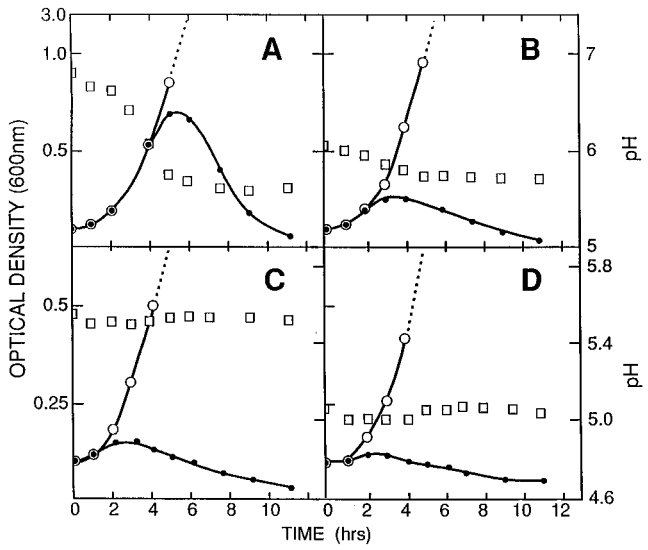

FIG. 3. Effect of lactocin S on the growth of *P. acidilactici* Pac1.0 in MRS media adjusted to initial pH values of 6.8 (A), 6.05 (B), 5.55 (C), and 5.05 (D). Symbols: ○, growth (optical density at 600 nm) in the absence of lactocin S; ●, growth in the presence of 128 BU of lactocin S per ml; □, pH.

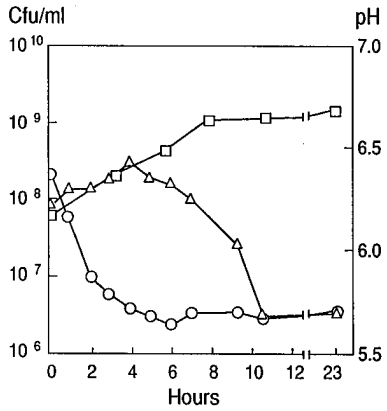

FIG. 4. Effect of lactocin S on the growth *P. acidilactici* Pac1.0. Approximately 9×10^7 CFU of pediococci per ml was incubated together with lactocin S (110 BU/ml) in MRS medium. Symbols: △ and ○, cell density and pH, respectively, of lactocin S-containing culture; □, cell density of culture to which lactocin S was not added.

which take part in the formation of modified amino acids in the polypeptide and regulatory genes (3, 8, 9). Studies of lactacin B produced by *Lactobacillus acidophilus* and helveticin J produced by *Lactobacillus helveticus* 481 have shown that the production of these bacteriocins also is pH dependent (6). Lactacin B is produced at pH 6.0 but not at pH 6.6 or 5.4 (1), while the greatest concentrations of helveticin J are produced at pH 5.0 (5). The strong dependence of lactocin S production on pH suggests that the expression of the biosynthetic genes may be regulated by pH, as has been observed previously for several classes of genes (18). However, DNA sequencing of the lactocin S operon has provided no evidence which supports the hypothesis that regulatory elements are involved in lactocin S production (21).

When growing *L. sake* L45 was exposed to oxygen, no active lactocin S was produced. In addition, O_2 has been shown to affect gene expression (12) and might have a decisive effect on lactocin S biosynthesis. However, it is also possible that the lack of bacteriocin activity is due to chemical degradation or alteration of the chemical structure. The results of a mass spectrometric analysis suggested that a significant fraction of the lactocin S molecules contain an oxidized methionine residue (21). Kühnen et al. (11) also observed a substantial decrease in the activity of an antimicrobial substance, LIQ 4, when the producing strain, *Streptococcus faecalis* subsp. *liquefaciens* K 4, was grown aerobically.

Addition of ethanol resulted in a two- to fourfold increase in the amount of lactocin S activity produced. The effect of ethanol might be the result of (i) prevention of bacteriocin aggregation, (ii) stabilization of the bacteriocin or gene products participating in bacteriocin production, or (iii) enhancement of the expression of genes involved in lactocin S biosynthesis. Lactocin S is a highly hydrophobic bacteriocin and might aggregate in solution (15). Aggregation of bacteriocins is observed commonly (4, 19). Organic solvents such as ethanol may dissociate bacteriocin aggregates, thereby increasing the amount of bacteriocin activity detected. However, when ethanol was added to a bacteriocin assay mixture, no enhancement of bacteriocin activity was detected (16). Apparently, ethanol must be included in the medium during synthesis. Ethanol has been found to induce genes involved in the heat shock response in *Escherichia coli* (25, 26), but only further studies will reveal if ethanol may affect the synthesis of lactocin S on the transcriptional level.

The mode-of-action experiments revealed that lactocin S acts bactericidally in a pH-dependent manner. Lactocin S had a pH threshold of 6, above which no bactericidal activity was observed. With few exceptions, the bacteriocins of lactic acid bacteria are cationic peptides, and some of these compounds have isoelectric points (pI) of more than 10. The cationic nature of these peptides is probably crucial for their biological activity. Lactocin S is one of the very few bacteriocins which have an almost neutral pI; the pI of lactocin S has been calculated to be 7.1 (17). Lactocin S has two positively charged (Lys) and two negatively charged (Glu and Asp) amino acids. In addition, lactocin S contains two histidines which are the two major residues which determine the effective charge. The pK of the imidazole group of histidine is 6.0. The biological activity of lactocin S is expressed at pH values just below 6.0, and it seems likely that positively charged histidines are crucial for the activity of lactocin S.

Our results show that both synthesis of active lactocin S and lactocin S activity may be severely affected by environmental factors. Minor changes in environmental conditions, which apparently do not affect the growth of the bacterium, can have detrimental effects on the production of active lactocin S. In view of the possible future use of bacteriocins as food and feed preservatives, our findings reveal potential obstacles that have to be overcome if these kinds of compounds are to be produced and used in new environments, such as food and feed.

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