The Location of Nisin in the Producer Organism, *Streptococcus lactis*

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SUMMARY

Streptococcus lactis organisms grown in a glucose-containing medium pH maintained at pH 6.8 (neutral cocci) contained three- to seven-fold more nisin/unit dry weight than cocci grown in the same medium without pH control (acid cocci, terminal pH 4.2). After chemical fractionation of acid cocci 57 % of the nisin was found in the fraction soluble in aqueous ethanol and 36 % in a trypsin-insoluble residue. After fractionation of broken cocci (acid and neutral) by differential centrifugation up to 60 % of the nisin was found in the 10,000 g sediment (walls). Nisin was also present in the 30,000 g sediment (membranes) and the 100,000 g sediment (ribosomes). The major difference between acid and neutral cocci was in the 100,000 g supernatant fluid (cell sap); cell sap from neutral cocci was 0.28 % nisin whereas that from acid cocci was 0.04 % nisin. Analysis of the cell wall indicated that it was composed of mucopeptide and polysaccharide. Teichoic acid could not be extracted. The polysaccharide was soluble in hot formamide and accounted for one-third of the dry weight of the wall; it contained rhamnose, galactose, glucose, glucosamine in the molar ratio of 5:1:1:1. Cell walls contributed 31 % to the dry weight of acid cocci and 42 % to the dry weight of neutral cocci.

INTRODUCTION

Many suggestions now exist in the literature that basic polypeptide antibiotics play a role in the bacterial differentiation which occurs at the time of sporulation (e.g. Schaeffer *et al.* 1963, Halvorson, 1965). Recently, further evidence supporting this notion has been obtained with *Bacillus polymixa* (Paulus, 1967). The role of basic proteins in the more overt differentiation processes of eukaryotic systems has been under discussion for several years (de Reuck & Knight, 1966). Recently, for example, Butler & Chipperfield (1967) showed that the inhibition of RNA polymerase by histones was a genuine effect and not an artefact due to decreased solubility. Certain strains of *Streptococcus lactis* produce an antibiotic called nisin, which is active against several Gram-positive bacteria (Mattick & Hirsch, 1947). This antibiotic is a small basic protein, and Hurst (1967) suggested that it may play a regulatory role in the producer organism connected with initiation and halting of growth. Thus the length of lag-phase of growth is directly related to the nisin content of the organism (Hurst & Dring, 1967). Recently Gross & Morell (1967) proposed that the antibiotic

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effect of nisin depends on interception of sulphhydryl groups of metabolically important molecules such as co-enzyme A. Cheeseman & Berridge (1959) proposed that the molecular weight of nisin is 7,000, however Gross & Morell (1967) reported a value of 3,500; our own work (Ingram, Tombs & Hurst, 1967) has confirmed the original estimate of 7,000. Unlike other polypeptide antibiotics so far studied in detail, nisin appears to be synthesized ribosomally (Hurst, 1966*a*). In view of the possible function of nisin as a regulator in the producer organism we attempted in the present work to locate it within the cell by using chemical and physical procedures of cell fractionation.

METHODS

Organisms, media and culture conditions have already been described (Hurst, 1966*a, b*)

Nisin was estimated by bioassay against *Streptococcus cremoris* strain IP5 using the standard of Hurst (1966*a*). Nisin bound to bacteria (cell nisin) was extracted and assayed after washing the cocci in fresh medium. Cell fractions for bioassay were adjusted with 10 N-HCl to give a final concentration of 0.05 N-HCl. HCl was not added to samples containing trichloroacetic acid (TCA). Before assay, samples were heated in a boiling water-bath for 5 min., centrifuged and the clear supernatant fluid used for assay.

Chemical fractionation of bacteria was as described by Park & Hancock (1960) except that the time of digestion with trypsin was increased to 3 hr. Briefly this method consisted of suspending a pellet containing 3 mg. dry wt in 3 ml. cold 5% TCA. The supernatant after centrifuging contained the low molecular weight TCA soluble substances. The residue was extracted at room temperature with 75% aqueous ethanol; the supernatant after centrifuging contained much of the lipid and other alcohol soluble cellular material. The residue was extracted with 5% TCA at 90° for 6 min. to dissolve nucleic acid and the residue was trypsin digested for 3 hr at 37° (1 mg. crystalline trypsin/ml. of 0.05 M-(NH₄)HCO₃ containing 0.005 N-NH₄OH). The final residue after trypsin extraction was resuspended in 3 ml. of 0.05 N-HCl, placed in a boiling water-bath for 5 min., centrifuged and the clear supernatant assayed.

Physical fractionation of bacteria. Washed suspensions of *Streptococcus lactis* strain in water were disrupted in a French pressure cell (American Instrument Co. Inc., Silver Spring, Md., U.S.A.) or a Braun homogenizer (Shandon Instrument Co., London), any intact cocci which survived being removed by twice centrifuging at 3000 g for 10 min., and discarding the sediment. The broken cocci were separated into four fractions by differential centrifugation: (i) 10,000 g sediment; (ii) 30,000 g sediment; (iii) 100,000 g sediment; (iv) 100,000 g supernatant fluid.

Cell walls (10,000 g sediment) were prepared by centrifuging broken cocci at 10,000 g for 10 min., washing the sediment twice with M-NaCl, and twice with distilled water. Cell walls were then digested with trypsin (100 µg./ml. at 37° in 0.01 M-sodium phosphate buffer pH 7.0 for 3 hr), after digestion the walls were washed and resuspended in distilled water.

Membranes (30,000 g sediment) were prepared by centrifuging the 10,000 g supernatant fluid at 30,000 g for 30 min. and washing the sediment twice with M-NaCl and twice with water. Although the 30,000 g sediment was designated membranes it undoubtedly contained other material as well, and was frequently contaminated with cell walls (as judged by amino sugar content); attempts to prepare membranes from spheroplasts of *Streptococcus lactis* were not successful.

Ribosomes (100,000 g sediment) were sedimented from the 30,000 g supernatant fluid by centrifuging at 100,000 g for 3 hr then washed and resuspended in the buffer described by Munro, Jackson & Korner (1964).

When appropriate, the nature of the fractions obtained by differential centrifuging was checked by examining with the electron microscope. Samples were most commonly shadowed, but stained thin sections were also examined.

Hydrolysis of cell walls. 10–20 mg. dry wt of cell walls were hydrolysed with 2 N-H₂SO₄ at 105° for 3 hr. After cooling, the hydrolysate was neutralized with saturated Ba(OH)₂ and solid BaCO₃ and clarified by centrifugation and filtration.

Neutral sugars were estimated by gas chromatography of their alditol acetate derivatives (Sawardeker, Sloneker & Jeanes, 1965), with mannose as an internal standard.

Amino sugar was estimated by the method of Levy & McAllan (1959). Where an estimate of the relative contributions of glucosamine and muramic acid was required, the methods of Randle & Morgan (1955) and Cessi & Piliego (1960) were used, the former method giving a different spectrum for these amino sugars, whilst the latter measures glucosamine only.

Protein was determined by the biuret method (Gornall, Bardawill & David, 1957), with bovine plasma albumin as standard.

Reducing sugar was estimated by the method of Park & Johnson (1949), *phosphate* by the method of Allen (1940), and *nitrogen* by the method of Folin & Farmer (1912).

Ribosomal proteins were examined by polyacrylamide gel electrophoresis (Hurst, 1966b).

Reagents. A.R. grade reagents were used as far as possible. Mannose was from T. G. Gurr Ltd., London, muramic acid from Sigma Chemical Co., St Louis, U.S.A., glucosamine from British Drug Houses Ltd., Poole, England, trypsin from Seravac Laboratories (Pty) Ltd., Maidenhead, England, RNA-ase from Koch-Light and Co. Ltd., Colnbrook, England and lysozyme from Armour Pharmaceutical Co., Eastbourne, England.

RESULTS

Production of nisin by cultures of Streptococcus lactis

Analysis of stationary phase cultures of *Streptococcus lactis* shows that some of the nisin produced is excreted into the culture fluid (medium nisin) and the remainder bound to the cocci (cell nisin; Hurst & Dring, 1967). Table 1 shows the concentration and distribution of nisin in stationary phase cultures after growth with and without pH control. *S. lactis* is a vigorous acid producer and in an uncontrolled fermentation a limiting value of pH 4.2 may be reached (acid culture); when the culture is maintained at pH 6.8 (neutral culture) a higher yield of organism is obtained (2.6 mg. dry wt/ml. as compared with 0.8 mg. dry wt/ml.). It can be seen that not all the nisin originally present (i.e. cells + medium) can be accounted for after separation of culture fluid from cells (i.e. medium nisin and cell nisin). In these experiments 7 % of the nisin was cell bound in the acid culture and 61 % in the neutral culture. The nisin content of cocci was not diminished by prolonged washing with water or 0.01 M-sodium phosphate buffer (pH 6.8).

Chemical fractionation of Streptococcus lactis organisms

Cocci from an acid stationary-phase culture were fractionated by the method of Park & Hancock (1960) and the nisin content of the different fractions estimated; the results are shown in Table 2. Most of the nisin was recovered in the aqueous ethanol-soluble and trypsin-insoluble fractions. Only 72% of the nisin originally present in the whole cocci was recovered after this chemical fractionation. In one experiment the alcohol extraction step was repeated four times before passing on to the next extraction

Table 1. *Nisin yield and distribution in cultures of Streptococcus lactis grown with and without pH control*

Samples from stationary phase cultures were assayed for nisin content directly (complete culture), and after separation of cocci from culture fluid by centrifugation. The nisin content of cocci was estimated after washing in fresh medium and resuspending and extracting with an equal volume of hot 0.05 N-HCl.

	Nisin ($\mu\text{g./ml. culture}$)	
	Without pH control (terminal pH 4.2)	With pH control (controlled to pH 6.8)
Complete culture	30	194
Culture fluid	25	52
Cocci	2	82

Table 2. *Distribution of nisin after a chemical fractionation of Streptococcus lactis organisms*

Washed stationary-phase cocci (grown without pH control; 'acid' cocci) were fractionated by the method of Park & Hancock (1960), and the distribution of nisin estimated by bioassay.

Fraction	Nisin content (%) of total recovered
Cold trichloroacetic acid soluble	1
Aqueous ethanol soluble	57
Hot trichloroacetic acid soluble	3
Trypsin-soluble	2
Trypsin-insoluble residue	36

with hot trichloroacetic acid. In this case the first alcohol extraction still contained 57% of the nisin and the subsequent extractions contained negligible amounts. The repeated alcohol extractions did not affect the amount of nisin recovered from the trypsin-insoluble residue which remained 36% as shown in Table 2. When whole cocci were incubated with trypsin (100 $\mu\text{g./ml.}$ in 0.01 M- NaHCO_3 at 37° for 3 hr) there was no decrease in their nisin content.

Physical fractionation of Streptococcus lactis organisms; nature of the fractions

Broken cocci were fractionated by differential centrifugation as described. The 10,000 g pellet material was examined by electron microscopy and showed the classical appearance of cell walls (Salton, 1964). With increasing purification the walls became thinner and less easy to see but even after extraction with hot formamide (200° for 15 min) they retained their original shape.

The 30,000 g deposit was pale yellow and contained irregularly shaped particles, about one-tenth the size of cell walls. Chemical analysis supported the suggestion that the 30,000 g deposit was mostly membraneous material.

Table 3. *Distribution of nisin after physical fractionation of disrupted Streptococcus lactis organisms*

Cocci from stationary phase cultures ('acid' and 'neutral') were harvested, washed, disrupted and fractionated by differential centrifugation. The nisin content of the fractions was estimated by bioassay.

Fraction	Distribution of nisin			
	Acid culture		Neutral culture	
	% dry wt	% of total recovered	% dry wt	% of total recovered
Whole cocci	0.94	100	2.63	100
10,000 g deposit	1.98	65	2.70	43
30,000 g deposit	2.10	9	2.47	4
100,000 g deposit	1.66	18	2.42	7
100,000 g supernatant fluid	0.04	2	0.28	5
Recovery (%) of that in unbroken cells	—	94	—	59

Table 4. *Composition of Streptococcus lactis cell wall*

Cell walls were prepared by differential centrifugation of disrupted 'acid' stationary phase cocci. Intact cocci were removed by discarding the 3000 g deposit. Cell walls were deposited at 10,000 g, washed with 1 M-NaCl, digested with trypsin and washed with water. Neutral sugars, amino sugars and reducing substances were estimated after hydrolysis of 10 mg. of cell walls in 2 N-H₂SO₄ for 3 hr at 105°. Amino sugar is expressed as glucosamine equivalents and reducing substances as glucose equivalents. Protein was estimated by the biuret method, with bovine plasma albumin as standard.

	% dry wt of cell wall		% dry wt of cell wall
Amino sugar	9.0	Phosphate	0.6
Rhamnose	20.3	Nitrogen	7.0
Glucose	5.0	Protein	5.0
Galactose	3.3	Nisin	2.2
Ribose	1.7	Ash	9.5
Reducing substances	26.2		

Analysis of the 100,000 g deposit indicated that it was mostly ribosomes (61 % RNA, 39 % protein). Polyacrylamide gel electrophoresis of basic proteins from these ribosomes revealed about 20 distinct bands (see Traut, 1966; Moller & Castleman, 1967) including a band with the same mobility as nisin marker; the presence of nisin was confirmed by bioassay.

Distribution of nisin in physically separated fractions of broken organisms

Acid-grown and neutral-grown cocci were disrupted, fractionated by differential centrifugation and the nisin estimated in the various fractions; the results are given in Table 3.

Gravimetric determinations indicated that the cell wall contributed 30–40 % of

the dry wt of whole organisms, membranes 4%, ribosomes 10%, and supernatant fluid fraction (cell-sap) 46–56%. Complete recovery of fractions was not usually attempted. Although in the experiment quoted the yield of nisin was only threefold higher in the 'neutral' cocci, the results for 'acid' cocci were variable and on occasion they contained very little nisin (e.g. total nisin only 5 µg./ml. medium).

Analysis of cell walls

Table 4 summarizes the results for the composition of cell walls prepared from 'acid' stationary-phase cocci. The purification of cell walls involves a trypsin digestion (see Methods) and during this procedure the protein content decreased from 45% of the

Table 5. *Contribution of cell walls to dry weight of whole Streptococcus lactis organisms*

Cell walls were prepared from exponential (3 hr growth) and stationary phase (overnight growth) as described for Table 4. The amino sugar (as glucosamine equivalents) and rhamnose content of whole organisms and cell walls was estimated after hydrolysis in 2 N-H₂SO₄ for 3 hr at 105°. Calculation of the cell wall's contribution to the dry weight of the whole cocci is based on the assumption that rhamnose and amino sugars occur exclusively in the cell wall (this is supported by analysis of the other fractions).

	Rhamnose (% dry wt)	Amino sugar (% dry wt)	Cell wall as % dry wt of cocci	
			By Rham- nose	By amino sugar
Exponential cocci	7.9	2.8	29.2	31.2
'Exponential' walls	27.0	9.0		
Acid stationary cocci	6.8	2.6	29.6	31.6
'Acid stationary' walls	23.0	8.2		
Neutral stationary cocci	8.4	3.7	41.5	42.0
'Neutral stationary' walls	20.3	9.3		

Table 6. *Comparison of formamide-soluble fraction and formamide-insoluble fraction from cell wall of Streptococcus lactis*

Cell walls were prepared from 'acid' stationary phase cocci as described for Table 4 and heated in formamide at 200° for 15 min. The resultant formamide-insoluble fraction was deposited by centrifuging at 30,000 g for 15 min, and washed twice with water. Rhamnose and amino sugar (as glucosamine equivalents) were estimated after hydrolysis of the formamide soluble and insoluble fractions in 2 N-H₂SO₄ for 3 hr at 105°.

	Mole ratios			
	Amino sugar	Rhamnose	Glucose	Galactose
Formamide-soluble	1.1	5.2	1.0	0.8
Formamide-insoluble	1.0	0.1	0.1	0.1

dry wt of the walls to 5%. The nitrogen content suggested that the protein content of purified walls was somewhat higher. However, it should be remembered that the protein was expressed as bovine serum albumin and this might be the reason for the discrepancy. Nisin was not lost from walls during the trypsin digestion. Attempts to demonstrate the presence of teichoic acid in the walls proved negative, the phosphate content being unchanged after prolonged extraction with trichloroacetic acid (5% trichloroacetic acid at 0° for 72 hr). A comparison of the rhamnose and amino sugar

contents of whole organisms and of cell walls permitted an estimate of the contribution of walls to the dry wt of whole cocci (such a calculation assumes that these carbohydrates occur exclusively in the wall). Figures are given in Table 5 for exponential and stationary-phase organisms.

Formamide extraction of walls

Previous work with group A streptococci (Krause & McCarty, 1961) showed that Fuller's (1938) formamide extraction was an efficient means of separating polysaccharide from mucopeptide. Extraction of *Streptococcus lactis* cell walls with formamide at 200° for 15 min. yielded two fractions: formamide-soluble and formamide-insoluble. During the extraction procedure there was a dramatic decrease in turbidity. The ratio of amino sugar to rhamnose, glucose and galactose in acid hydrolysates of the formamide-soluble and formamide-insoluble fractions was markedly different (see Table 6). The *E* 505/530 *mμ* ratios in the Randle & Morgan (1955) estimation for amino sugar in the two fractions were 0.88 for the formamide-soluble fraction and 1.35 for the formamide-insoluble fraction; in the same assay glucosamine alone gave a ratio of 0.85 whilst an equimolar mixture of muramic acid and glucosamine gave 1.33.

Sensitivity to lysozyme

Whole organisms of *Streptococcus lactis* and cell walls were incubated with lysozyme (100 μ g./ml. in 0.1 M-sodium phosphate buffer pH 7.0 at 37°) and the extinction at 700 *mμ* followed. There was a very slow decrease in both cases (about 50 % in 24 hr). Extraction of cell walls with formamide (200° for 15 min) or hot 10 % trichloroacetic acid (6 hr at 60°) caused a slight increase in sensitivity to lysozyme (see Schleifer & Kandler, 1967, who solubilized *S. cremoris* by this treatment).

DISCUSSION

Nisin is a strongly basic surface-active molecule (Hirsch, 1954) so that its distribution within the producer organism cannot easily be established. For this reason two different methods of analysis of the *Streptococcus lactis* organisms were used here: chemical and physical. In the chemical separation 57 % of the nisin was recovered in the fraction soluble in aqueous ethanol. This fraction presumably contained most of the membrane material and also any ethanol-soluble protein present elsewhere in the cocci. Centrifugally prepared membranes contained less nisin than the aqueous ethanol soluble fraction (9 % of total as compared with 57 % of total). Thus much of the nisin in the alcohol soluble fraction did not come from membranes. However, in both fractionation procedures a comparable amount of nisin was found associated with the wall material (trypsin-insoluble residue and 10,000 g deposit). The higher nisin content of neutrally-grown cocci was partly due to the increased amount of wall material present and to the higher nisin content of the walls (Tables 3, 5). Although free nisin is ethanol soluble, nisin attached to the trypsin-insoluble residue was not removed by repeated extraction with ethanol or by repeated washing with M-NaCl. The nature of the bond between wall material and nisin remains to be determined. In its association with cell wall, stability to boiling with acid and solubility in ethanol, nisin resembles the M protein of the group A Streptococci (Krause & McCarty, 1961). Physical fractionation of neutrally-grown *S. lactis* organisms gave a poorer recovery of nisin than did acid-

grown cocci; more of the nisin in neutrally grown cocci may have been loosely bound and lost during washing. However, some of the extra nisin in the neutral cocci was found in the cell sap (Table 3). Differences in the concentration of nisin inside and outside the cocci may be caused by different pH values. During growth without pH control the lactic acid produced by fermentation may be causing an acid extraction of the 'acid' cocci. However, in a culture maintained at neutral pH, the inside of the cocci might be at a more acid pH value than that of the medium. Since nisin is more soluble at acid pH values it may distribute itself accordingly. Zarlengo & Abrams (1964) reported that the intracellular pH value of stationary-phase *S. faecalis* might be as low as pH 5.0.

The cell wall of *Streptococcus lactis* contains mucopeptide and a polysaccharide which can be separated by formamide extraction. However, even after 15 min. in boiling formamide, rhamnose was still present in the mucopeptide (insoluble residue). Residual rhamnose probably means incomplete removal of polysaccharide, and this may have caused the poor sensitivity to lysozyme. Although Krause & McCarty (1961) were unable to extract all the rhamnose from group A streptococcal cell walls with formamide, the mucopeptide obtained was completely solubilized by lysozyme. Table 6 suggests that the formamide-soluble polysaccharide of *S. lactis* contains rhamnose, glucose, galactose and glucosamine in the molar ratio of 5:1:1:1, the glucosamine in the polysaccharide being presumably *N*-acetylated, since partial acid-hydrolysates contained *N*-acetyl amino sugar. Polysaccharide contributed about 30% of the dry wt of the *S. lactis* wall and mucopeptide about 10%. Schleifer & Kandler (1967) established the composition and amino acid sequence of mucopeptide from *S. lactis*. Little data is available about the ash content of cell walls but the value found here for *S. lactis* (about 10%) is similar to that quoted by Holdsworth (1952) for *Corynebacterium diphtheria*. Ribose was always present in the purified wall preparations of *S. lactis*, even after treatment with RNA-ase; the wall, membrane and other associated organelles may form a highly integrated structure in this organism.

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