The Effect of Proflavin, Ethidium Bromide and an Elevated Temperature on the Appearance of Nisin-negative Clones in Nisin-producing Strains of *Streptococcus lactis*

By WITOLD KOZAK, MARIANNA RAJCHERT-TRZPIL AND
WŁADYSŁAW T. DOBRZAŃSKI

*Department of Molecular Genetics, Institute of Biostructure,
Medical Academy, 02-004 Warsaw, Poland*

(Received 17 December 1973; revised 21 February 1974)

SUMMARY

In some nisin-producing strains of *Streptococcus lactis*, clones which were permanently incapable of producing nisin (Nis⁻) appeared spontaneously with a frequency of 0.03 to 0.82 %. Treatment with proflavin (Pro), ethidium bromide (EB) or at a temperature of 40 °C increased the number of such clones. In one strain 78 % of cells became Nis⁻ after treatment with Pro.

Treatment with nitrosoguanidine did not reverse the effect of Pro or EB. There was no evidence that Pro or EB selected spontaneously-occurring Nis⁻ clones.

These observations suggest that the gene(s) responsible for nisin production in some *S. lactis* strains may be located on a plasmid, but further investigation is required to confirm this.

INTRODUCTION

Nisin is a polypeptide antibiotic produced by some strains of *Streptococcus lactis* (Mattick & Hirsch, 1944). Genetic and biochemical information on the synthesis of nisin is lacking (Hurst & Paterson, 1971). Some strains are known to lose the Nis⁺ character temporarily or permanently for unknown reasons.

The aim of the present work was to check the effect of proflavin, ethidium bromide and an elevated temperature on the appearance in populations of nisin-producing *S. lactis* strains of nisin-negative clones. In one strain the effect of rifamycin was also checked. These substances are used to 'cure' bacteria from plasmids (Courvalin, Carlier & Chabbert, 1972; Novick, 1969; Riva, Fietta, Silvestri & Romero, 1972).

METHODS

Bacterial strains. There were 12 nisin-producing *Streptococcus lactis* strains and a test strain of *Streptococcus cremoris* 1P5 for nisin detection. The strains were obtained from the Institute of Milk Industry, Warsaw, Poland; the Department of Pure Milk Cultures, Olsztyn, Poland and the Department of Technical Microbiology of the Łódź Institute of Technology, Łódź, Poland. The strains were kept in a 10 % suspension of powdered skim milk in water at 4 °C and transferred weekly in this medium.

Media. Peptonized milk (Difco) supplemented with 10 g glucose/l; pH before autoclaving 7.2; either liquid medium (PM), or medium solidified by the addition of 1.5 % agar (PMA) was used.

Chemicals. Ethidium bromide (EB), Boots Pure Drug Co.; proflavin (Pro), Loba Chemie,

Austria; rifamycin (Rif), Calbiochem; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), K & K Laboratories, USA; streptomycin (Str), Polfa, Poland.

The detection of nisin production. Colonies were replicated to Petri dishes containing PMA which had previously been flooded with an 18 h culture of the test strain, *S. cremoris* 1P5, diluted 1:10. After 18 h of incubation the appearance of inhibition zones round the replicated colonies was recorded.

Apparent Nis⁻ colonies were isolated and grown for 18 h in PM medium. Total nisin (endo- and exocellular) was assayed by the method of Hurst (1966). As a rule each assay was made twice.

Sensitivity of strains to proflavin, ethidium bromide and rifamycin. This was tested by (i) the serial dilution method in PM medium – after 18 h incubation the minimal inhibitory concentration (m.i.c.) of these substances was determined; (ii) after 18 h incubation the growth of strains was compared on PMA medium without any additions with the growth of these strains on PMA with Pro or EB. The growth on PMA medium was scored + + +, on PMA with Pro or EB + + +, + + or +.

The treatment of strains with proflavin, ethidium bromide, rifamycin or elevated temperature. (i) Incubation with Pro, EB or Rif (Courvalin *et al.* 1972): 18 h cultures of streptococci in PM medium were diluted 1:50 and plated (0.2 ml/Petri dish) on PMA medium with or without various concentrations of the above-mentioned compounds. After 18 h incubation, the colonies were washed off with saline (0.85 % NaCl) and the suspensions appropriately diluted with the same solution and plated on PMA medium to obtain single colonies. (ii) The strain was incubated in PM medium for 18 h at 40 °C and subsequently, after appropriate dilution in saline, plated on PMA medium to obtain single colonies. The ability to produce nisin was checked in individual colonies after incubation.

The permanence of the inability to produce nisin in nisin-negative clones. (i) The Nis⁻ clones were passaged ten times and the ability to produce nisin checked after each passage. (ii) The Nis⁻ clones unable to produce nisin after ten passages were treated with NTG (20 or 200 µg/ml) for 30 min in PM medium. After removal of the NTG by washing the cells twice, the suspension of bacteria was plated on PMA medium to obtain single colonies and the ability to produce nisin was examined.

With the exception of the cases where it is stated that a temperature of 40 °C was used, all incubations were at 30 °C.

RESULTS

Initial observations on the occurrence of nisin-negative clones in S. lactis strains spontaneously and after treatment with proflavin or ethidium bromide

The occurrence of Nis⁻ clones and the influence of Pro and EB on their appearance was checked in 12 nisin-producing strains. The sensitivity of these strains to the two compounds was first determined, so that appropriate doses could be chosen. For the various strains the m.i.c. for proflavin was 4 to 12 µg/ml and for ethidium bromide 0.5–2.5 µg/ml. In the experiments Pro was used at 7.5 µg/ml and EB at 2.0 µg/ml. Since no selection method for the ability to produce nisin is available only a limited number of clones could be examined in the experiments. The results are presented in Table 1.

Some 300 to 1450 colonies were examined for each strain. Among colonies from control cultures Nis⁻ clones were isolated from only three strains, one from each. After Pro or EB treatment such clones were isolated from eight strains. In two of these strains (45, 51) Pro and/or EB treatment possibly increased the frequency of occurrence of Nis⁻ strains; in a third strain (49) this effect was very pronounced.

Table 1. Occurrence of *Nis*⁻ clones in 12 nisin-producing strains of *S. lactis*, untreated or treated with Pro or EB

<i>S. lactis</i> strain no.	Untreated strains		Treated strains			
			Pro (7.5 µg/ml)		EB (2.0 µg/ml)	
	No. colonies examined	No. <i>Nis</i> ⁻ colonies	No. colonies examined	No. <i>Nis</i> ⁻ colonies	No. colonies examined	No. <i>Nis</i> ⁻ colonies
2	1441	0	1169	0	621	0
27	796	0	1055	1	429	1
28	656	0	1003	0	340	0
29	516	0	1032	0	432	0
38	326	0	523	1	479	0
45	1448	1	527	2	632	2
49	345	1	914	188	321	20
50	914	0	1323	0	361	1
51	623	0	923	3	735	2
52	298	1	814	0	451	1
53	372	0	765	0	388	0
54	346	0	761	4	607	0

Table 2. Occurrence of *Nis*⁻ clones in *S. lactis* strain 49/IV, untreated or treated with various concentrations of Pro and EB or a temperature of 40 °C

Treatment	No. clones examined		Increase in no. <i>Nis</i> ⁻ clones*
	Total	<i>Nis</i> ⁻	
Untreated (control)	4248	35 (0.82 %)	1
Pro (µg/ml)			
5.0	1660	75 (4.40 %)	5.37 ×
7.5	1879	344 (18.47 %)	22.50 ×
10.0	2398	1859 (77.52 %)	94.78 ×
EB (µg/ml)			
0.5	742	38 (5.13 %)	6.26 ×
1.0	385	38 (9.90 %)	12.10 ×
2.5	183	16 (8.74 %)	10.66 ×
Temperature of 40 °C	1248	14 (1.12 %)	1.37 ×

* Control = 1.

More detailed observations were made on strains 45, 49 and 51. In our previous investigations (Kozak, Rajchert-Trzpił, Zajdel & Dobrzański, 1973) no lysogeny was detected in strains 49 or 51, while strain 45 was lysogenic.

Before the investigations, the strains were passaged 4 or 5 times, with plating to obtain single colonies. For each subsequent passage a colony producing substantial amounts of nisin was taken. The clones obtained from these passages were designated 45/V, 49/IV and 51/V.

The occurrence of nisin-negative clones in S. lactis 45/V, 49/IV and 51/V spontaneously and after treatment with proflavin, ethidium bromide and at 40 °C

Strains 45/V, 49/IV and 51/V were subjected to various concentrations of Pro and EB and to a temperature of 40 °C. Strain 51/V was also treated with Rif (the m.i.c. for Rif for this strain was 15 µg/ml). The results are presented in Tables 2, 3 and 4.

Table 3. Occurrence of *Nis*⁻ clones in *S. lactis* strain 51/v, untreated or treated with various concentrations of Pro, EB and Rif or a temperature 40 °C

Treatment	No. clones examined		Increase in no. <i>Nis</i> ⁻ clones*
	Total	<i>Nis</i> ⁻	
Untreated (control)	6728	2 (0.03 %)	1
Pro† (5-10 µg/ml)	4639	28 (0.6 %)	20 ×
EB (2.5-5.0 µg/ml)	3785	9 (0.24 %)	8 ×
Temperature of 40 °C	1717	7 (0.41 %)	13.6 ×
Rif (3.75-15.0 µg/ml)	2629	0	0

* Control = 1.

† No differences in the increase of the number of *Nis*⁻ clones caused by varying the dose (within the examined range) were observed.Table 4. Occurrence of *Nis*⁻ clones in *S. lactis* strain 45/v, untreated or treated with various concentrations of Pro, EB or a temperature of 40 °C

Treatment	No. clones examined		Increase in no. <i>Nis</i> ⁻ clones*
	Total	<i>Nis</i> ⁻	
Untreated (control)	2448	1 (0.04 %)	1
Pro† (1-5 µg/ml)	1055	6 (0.57 %)	14.2 ×
EB† (0.5-2.5 µg/ml)	1225	4 (0.33 %)	8.2 ×
Temperature of 40 °C	854	7 (0.83 %)	20.7 ×

* Control = 1.

† No differences in the increase of the number of *Nis*⁻ clones due to varying the dose (within the examined range) were observed.

In strain 49/iv, in untreated populations, 0.82 % of clones were *Nis*⁻. In populations previously treated with Pro or EB, depending on the agent and its concentration, 4.0 to 78.0 % of such clones were found. No definite increase of *Nis*⁻ clones was observed in populations grown at 40 °C.

In strains 45/v and 51/v the frequency of occurrence of *Nis*⁻ clones both in treated and untreated populations was considerable, but distinctly lower than in strain 49/iv.

In these strains 0.03 and 0.04 % of *Nis*⁻ clones respectively were found in untreated cultures. In strain 51/v 20-, 14- and 8-fold increases in the frequency of nisin-negative clones were observed as a result of the action of Pro, a temperature of 40 °C and EB, respectively. After Rif treatment among 2629 colonies, no *Nis*⁻ clone was found. Similar results were obtained for strain 45/v.

In strains 45/v and 51/v no dependence (within the examined range) of the number of *Nis*⁻ clones on the dose of applied factors was observed.

Possible causes of the increase in the frequency of occurrence of *Nis*⁻ clones in strains 49/iv, 45/v and 51/v after treatment with Pro, EB or at a temperature of 40 °C include non-

Table 5. *Sensitivity to proflavin of S. lactis 49/IV clones previously either treated or untreated with proflavin*

Clones	No. clones examined	Growth on PMA medium with Pro concn of (μ g/ml):			
		0	5	7.5	10
Nis ⁻ /Pro	10	+++	+++	+++	++
Nis ⁺ /Pro	10	+++	+++	+++	++
Nis ⁻ /C	5	+++	+++	+++	Some clones + +, some clones +
Nis ⁺ /C	7	+++	+++	+++	Some clones + +, some clones +

Nis⁻/Pro and Nis⁺/Pro, clones isolated after incubation of the strain with proflavin. Nis⁻/C and Nis⁺/C, clones isolated from cultures untreated with proflavin. + + +, + +, +, Range of intensity of growth of strains.

Table 6. *Sensitivity to ethidium bromide of S. lactis 49/IV clones previously either treated or untreated with ethidium bromide*

Clones	No. clones examined	Growth on PMA medium with EB concn of (μ g/ml):			
		0	1	1.5	2.5
Nis ⁻ /EB	10	+++	+++	+++	+++
Nis ⁺ /EB	9	+++	+++	+++	+++
Nis ⁻ /C	4	+++	+++	+++	Some clones + + +, some clones + +
Nis ⁺ /C	8	+++	+++	+++	Some clones + + +, some clones + +

Nis⁻/EB and Nis⁺/EB, clones isolated after incubation of the strain with ethidium bromide. Nis⁻/C and Nis⁺/C, clones isolated from cultures untreated with ethidium bromide. + + +, + +, +, Range of intensity of growth of strains.

hereditary modification, selection of Nis⁻ clones as more resistant to these agents or as having a shorter generation time, mutagenic activity of the applied factors, and elimination of a plasmid carrying the determinant(s) for nisin production. Further experiments were undertaken to check these possibilities.

Stability of Nis⁻ clones originating from populations treated with Pro, EB or at 40 °C

The ability to produce nisin was examined during ten passages in 44 Nis⁻ clones from strain 51/V, 17 clones from strain 45/V and 100 clones from strain 49/IV. The clones were obtained after Pro, EB or elevated temperature treatment.

In no case was there a reversion to the ability to produce the antibiotic in the examined clones. It thus seems that these clones are not variants with temporarily changed characters.

Sensitivity to Pro and EB of clones from S. lactis strains previously treated with these substances

To check whether the selection of Nis⁻ clones as more resistant to proflavin and ethidium bromide than Nis⁺ clones occurs, the sensitivity of Nis⁺ and Nis⁻ clones to these substances was compared. These clones originated from populations either untreated or treated with Pro or EB. The experiments were performed as described in Methods.

In strain 49/IV, Nis⁺ and Nis⁻ clones isolated from populations treated with Pro or EB do not differ in their sensitivity to the concentrations of these compounds used in the experiments; similarly Nis⁺ and Nis⁻ clones isolated from populations not treated with Pro or EB do not differ in their sensitivity to the concentrations of these compounds used in the experiments. When the sensitivity to Pro or EB of Nis⁺ and Nis⁻ clones from untreated populations was compared with the sensitivity of such clones from treated populations (Tables 5 and 6), no difference was observed in their sensitivity towards the lower concentrations of Pro or EB used in these experiments, concentrations which caused an increase in the frequency of occurrence of Nis⁻ clones in the population. Only certain clones, but both Nis⁺ and Nis⁻ ones, from treated populations were slightly more resistant to the highest of the Pro and EB concentrations used than the Nis⁺ and Nis⁻ clones from untreated populations; this increase in resistance was essentially the same in Nis⁺ and Nis⁻ clones.

Similar investigations were performed on 39 Nis⁺ and Nis⁻ clones of strain 51/V and 20 clones of strain 45/V from untreated and treated populations. No differences in sensitivity to Pro or EB were observed.

Comparison of the growth of Nis⁺ and Nis⁻ clones

The increased frequency of appearance of Nis⁻ clones after Pro or EB treatment could possibly be due to the greater rate of division of Nis⁻ cells.

The kinetics of growth of populations of two Nis⁻ (obtained after Pro treatment) and two Nis⁺ clones of strain 49/IV in liquid medium were compared. No differences in the rate of growth were observed.

The growth of Nis⁺ and Nis⁻ clones in a mixed culture

The possibility of the domination of Nis⁻ over Nis⁺ clones in a mixed culture was examined.

Cultures of Nis⁺ and Nis⁻ clones (diluted 1:100) of strains 45/V and 51/V were used. The Nis⁻ clones came from populations treated with Pro, the Nis⁺ clones from untreated populations. The Nis⁻ and Nis⁺ clones of the same strain were mixed in the proportions 1:100 and 5:95, respectively. The mixed culture was incubated for 20 h, and samples taken every 4 h were plated on PMA medium and the ratio of viable Nis⁺ to Nis⁻ cells was determined.

In all samples the proportions of Nis⁻ to Nis⁺ cells were close to the initial ratio. Thus no dominance of Nis⁻ over Nis⁺ clones was shown.

The permanence of inability to produce nisin in Nis⁻ clones mutagenized with NTG

One of the characteristic properties of plasmids is the fact that a cell cured of a plasmid cannot regain the feature determined by the plasmid, by means of mutation. It was therefore checked whether NTG treatment could restore the ability to produce nisin in Nis⁻ clones originating from populations treated with Pro, EB or at 40 °C. These investigations were performed on Nis⁻ clones of strains 51/V and 49/IV as described in Methods.

We examined 30730 colonies originating from four Nis⁻ NTG-treated clones of strain 51/V, and 20115 colonies derived from two Nis⁻ NTG-treated clones of strain 49/IV. No Nis⁺ colonies were found.

DISCUSSION

Our results show that in some nisin-producing strains of *S. lactis*, Nis⁻ clones appeared spontaneously with considerable frequency (0.03 to 0.82 %). The treatment of Nis⁺ strains in

appropriate conditions with Pro, EB or elevated temperature (40 °C) increased the frequency of occurrence of Nis⁻ clones in the population. In one of the three strains examined, 78 % of clones became Nis⁻ after treatment with Pro or EB.

Control experiments indicated that the appearance of Nis⁻ clones in populations of the examined strains after treatment with Pro or EB was not due to a temporary modification or to selection of Nis⁻ clones.

In Nis⁻ clones obtained from populations treated with these substances it was not possible to restore the ability to produce nisin by mutagenesis with nitrosoguanidine. The Nis⁻ clones isolated from populations treated with EB or Pro remain highly resistant to nisin (Kozak, Rajchert-Trzpił and Dobrzański, unpublished observations).

It should perhaps also be checked whether Pro and EB are mutagenic in strains 45/v and 51/v. We have performed preliminary experiments to examine the influence of these substances on the frequency of occurrence of streptomycin-resistant mutants. No distinct mutagenic effect was observed. However, because of variations in the results of particular experiments we were unable to determine whether mutations are not induced by these substances, or whether either substance causes a slight increase in the frequency of mutations in the examined gene (unpublished data).

The problem of the possible mutagenic activity of these factors in strain 49/iv is of lesser importance. In this strain, as a result of Pro or EB treatment a considerable proportion of cells in the population permanently lost the ability to produce nisin. The participation of mutations in this phenomenon, if it occurs at all, could only be slight.

Ethidium bromide, proflavin and an elevated temperature are well-known factors in the elimination of plasmids from cells (Courvalin *et al.* 1972; Novick, 1969). Our work thus suggests that the gene(s) responsible for nisin production may be located on plasmids in some *S. lactis* strains.

To support this hypothesis physico-chemical investigations on the presence of non-chromosomal DNA in cells are necessary. We are making such investigations.

Genetic methods are also valuable in demonstrating the presence of plasmids (see Novick, 1969). These methods could not hitherto be applied to *S. lactis*. Recently, the possibility of transduction in *S. lactis* has been reported (McKay, Cords & Baldwin, 1972), and it will thus perhaps be possible to apply transduction to test for the presence of plasmids in *S. lactis*.

The demonstration of the plasmid nature of the determinant of nisin production could have valuable implications in the basic and practical investigations on *S. lactis*, in particular on the choice of strains for industrial nisin production.

The data on the localization of the genes responsible for the production of antibiotics in micro-organisms are so far scant. The plasmid nature of antibiotic determinants has only been shown in the case of some bacteriocins (Novick, 1969). They are not, however, classical antibiotics. Moreover, there are some unconfirmed preliminary data on the plasmid character of determinants of two antibiotics produced by *Streptomyces kasugaensis* (Okanishi, Ohta & Umezawa, 1970).

We thank Mrs Zofia Chada, Mrs Jolanta Sikorska and Mr S. Sikorski for expert technical assistance. This work was supported by the Polish Academy of Sciences within Project 09.3.1.3.4.

REFERENCES

- COURVALIN, P. M., CARLIER, C. & CHABBERT, Y. A. (1972). Plasmid-linked tetracycline and erythromycin resistance in group D Streptococcus. *Annales de l'Institut Pasteur* **123**, 755-759.
- HURST, A. (1966). Biosynthesis of the antibiotic nisin by whole *Streptococcus lactis* organisms. *Journal of General Microbiology* **44**, 209-220.
- HURST, A. & PATERSON, G. M. (1971). Observations on the conversion of an inactive precursor protein to the antibiotic nisin. *Canadian Journal of Microbiology* **17**, 1370-1384.
- KOZAK, W., RAJCHERT-TRZPIL, M., ZAJDEL, J. & DOBRZAŃSKI, W. T. (1973). Lysogeny in lactic Streptococci producing and not producing nisin. *Applied Microbiology* **25**, 305-308.
- MATTICK, A. T. R. & HIRSCH, A. (1944). A powerful inhibitory substance produced by group N streptococci. *Nature, London* **154**, 551-552.
- McKAY, L. L., CORDS, B. R. & BALDWIN, K. A. (1973). Transduction of lactose metabolism in *Streptococcus lactis* c2. *Journal of Bacteriology* **115**, 810-815.
- NOVICK, R. P. (1969). Extrachromosomal inheritance in bacteria. *Bacteriological Reviews* **33**, 210-263.
- OKANISHI, M., OHTA, T. & UMEZAWA, H. (1970). Possible control of formation of aerial mycelium and antibiotic production in Streptomyces by episomic factors. *The Journal of Antibiotics* **23**, 45-47.
- RIVA, S., FIETTA, A. M., SILVESTRI, L. G. & ROMERO, E. (1972). Effect of rifampicin on expression of some episomal genes in *E. coli*. *Nature New Biology* **235**, 78-80.