Cell surface characteristics of *Lactococcus lactis* harbouring pCI528, a 46 kb plasmid encoding inhibition of bacteriophage adsorption

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pCI528 is a 46 kb plasmid, originally isolated from *Lactococcus lactis* subsp. *cremoris* UC503, which causes a decrease in bacteriophage adsorption in lactococcal strains. Cell surface characteristics of two pCI528-containing strains, *L. lactis* subsp. *cremoris* UC503 and *L. lactis* subsp. *lactis* UC505, were compared with those of their isogenic pCI528-cured derivatives, UC563 and MG1363, respectively. Following centrifugation, strains containing pCI528 produced a loose fluffy pellet. The presence of pCI528 also caused a 'hard' colony morphology on agar media. The fluffy pellet effect mediated by pCI528 could be eliminated by washing cells in 0.05 M-NaOH, which also restored full sensitivity to phage in the *L. lactis* subsp. *lactis* UC505 background. GLC analysis of cell wall material indicated that pCI528-containing hosts had significantly elevated levels of both galactose and rhamnose. Marked alterations in the cell surface of strains harbouring pCI528 were observed in electron micrographs.

Introduction

Bacteriophage infection of lactococcal starter cultures is a major problem in cheese fermentations and can result in partial or even complete inhibition of acid production. The milk fermentation industry has adopted a number of approaches to curtail phage proliferation, including efforts to physically exclude phage through the use of strict hygiene and sanitization practices and phage-inhibitory media (Coffey *et al.*, 1989; Everson, 1991). Improved culture systems have also evolved and defined starters consisting of phage-unrelated strains are used in many countries (Lawrence, 1978; Daly & Fitzgerald, 1987; Sanders, 1988; Cogan *et al.*, 1991). More recently, advances in genetic studies of lactococci have led to the discovery of a number of plasmid-encoded phage resistance mechanisms which may be exploited for the construction of strains with enhanced phage resistance properties. These act at the level of adsorption inhibition (Sanders & Klaenhammer, 1983; deVos *et al.*, 1984; Costello, 1988), restriction/modification (Sanders & Klaenhammer, 1981; Chopin *et al.*, 1984; Hill *et al.*, 1989) and abortive infection (Sanders, 1988; Klaenhammer, 1989).

In this laboratory, a 46 kb plasmid designated pCI528

which encodes two phage resistance mechanisms, adsorption blocking and abortive infection, was identified in *L. lactis* subsp. *cremoris* UC503 (Costello, 1988; Coffey *et al.*, 1991). In this host, reduced adsorption was observed with some phages while for others, an additional mechanism resembling abortive infection operates, in that the host remains partially resistant to phages which can adsorb but which produce only small, hazy plaques. The abortive-infection-like phenotype is expressed only in the native UC503 strain. When introduced into *L. lactis* subsp. *lactis* MG1363, yielding *L. lactis* subsp. *lactis* UC505, pCI528 confers total resistance to both prolate- and small isometric-headed phages. Costello (1988) has shown that the adsorption inhibition effect may be a consequence of an alteration in the cell surface of strains containing pCI528, manifested by features such as a soft, easily disturbed pellet following centrifugation and a more hydrophilic cell surface.

Little information is available regarding the molecular nature of the gene(s) or mechanisms elaborated by adsorption inhibition plasmids. The plasmid-associated mechanism described in most detail is that encoded by pSK112 (53 kb), isolated from *L. lactis* subsp. *cremoris* SK110 (deVos *et al.*, 1984; Sijtsma *et al.*, 1988, 1990a, b). This strain was less hydrophobic than its pSK112-cured derivative, SK112. Alkali treatment restored the ability of phages to adsorb to SK110, and cell surface analysis indicated that galactose-containing lipoteichoic acid was

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involved in masking phage receptor sites. The object of this study was to examine how pCI528 prevents adsorption of phage and to analyse the cell surface components of pCI528-containing hosts.

Methods

Bacterial strains and phages. These are listed in Table 1. Lactococcal strains were grown at 30 °C in M17 medium (Terzaghi & Sandine, 1975) supplemented with 0.5% glucose where necessary.

Bacteriophage plaque assays. Plaque assays were performed at 30 °C. A 0.1 ml volume of an overnight culture, 0.1 ml of 0.185 M-CaCl₂ and 1 ml of the appropriate phage dilution were added to 3 ml of sloppy agar (0.7%) and the contents of the test tube were mixed and poured onto M17 agar. Plates were incubated at 30 °C.

Phage adsorption. A 0.75 ml volume of phage (10⁵ p.f.u. ml⁻¹) and 37.5 µl of 0.185 M-CaCl₂ were added to 0.75 ml of the appropriate culture which had been grown for 18 h at 30 °C. Samples were incubated at 30 °C for 15 min and then centrifuged for 3 min to remove cells and adsorbed phages. The supernatant was assayed for residual phages by standard plaque assay on a sensitive host. Phage adsorption was calculated as follows:

$$\text{Percentage adsorption} = \frac{(\text{Control titre} - \text{residual titre})}{\text{Control titre}} \times 100$$

Chemical treatment of cells. Portions (1.5 ml) of suspensions of whole cells (approximately 10⁹ c.f.u./ml⁻¹) of *L. lactis* subsp. *lactis* UC505 and MG1363 were washed and resuspended in 1.5 ml Triton X-100 (1%, v/v, in distilled H₂O, 30 min, 45 °C), SDS (1%, w/v, in distilled H₂O, 30 min, 45 °C), HCl (0.1 M, 30 min, 21 °C), NaOH (0.05 M, 30 min, 21 °C), pronase E (1.5 mg ml⁻¹ in distilled H₂O, 30 min, 37 °C) or trypsin (1.5 mg ml⁻¹ in distilled H₂O, 30 min, 37 °C) (Sijtsma *et al.*, 1988). Following treatment, cells were washed and resuspended in M17, and assayed for phage sensitivity by plaque assay.

Isolation of cell surface polysaccharides for GLC analysis. Cells were resuspended in 2 ml (approximately 10⁹ c.f.u. ml⁻¹) 30% NaOH and boiled for 3 h. Samples were then ethanol-precipitated as described by Sijtsma *et al.* (1988) and Wallace (1980) and hydrolysed in 1 M-H₂SO₄ at 100 °C for 2.5 h. Hydrolysates were neutralized with Ba(OH)₂ followed by reduction with NaBH₄. Pyridine and acetic anhydride were added and samples were treated at 120 °C for 20 min and finally dissolved in 200 µl acetone and injected into a GLC column (Carbo Erba Strumentazione HRGC 5160 GC with a 15 mm capillary OV-225, temperature 190 °C).

Resuspension test. Volumes (1 ml) of fully grown cultures were pelleted in a bench centrifuge at 13000 g for 30 s. Pellets were resuspended in M17 broth by vortexing for specific time periods. The OD₆₀₀ of the suspension was measured as a function of time.

Hydrophobicity test. An 18 h culture was centrifuged at 13000 g for 10 min and resuspended in PUM buffer (22.2 g K₂HPO₄·3H₂O, 7.26 g KH₂PO₄, 1.8 g urea, 0.2 g MgSO₄·7H₂O per litre, pH 7.1) to give an OD₆₀₀ of 0.5. A 4 ml portion of each cell suspension was added to test tubes containing various volumes of octane (0, 0.5, 1, 1.5 and 2 ml). Samples were vortexed thoroughly for 2 min and allowed to stand at room temperature for 30 min for separation of the aqueous and hydrocarbon layer (Rosenberg *et al.*, 1980). The OD₆₀₀ of the aqueous layer was then determined.

Electron microscopy of cell surfaces. A 1.5 ml volume of an 18 h culture was centrifuged, washed twice and resuspended in quarter-strength

Table 1. *Bacterial strains and bacteriophages*

Strain/phage	Plasmid content (kb)	Comment/source
<i>L. lactis</i> subsp. <i>cremoris</i> UC503	70.7, 54.2, 46, 27.9, 11, 7, 2.3	Wild-type pCI528- containing host (Costello, 1988)
UC563	70.7, 54.2, 27.9, 11, 7, 2.3	UC503 cured of pCI528 by acriflavin treatment (Costello, 1988)
<i>L. lactis</i> subsp. <i>lactis</i> MG1363	Plasmid-free	Derivative of <i>L. lactis</i> 712 (Gasson, 1983)
UC505	46	MG1363 harbouring pCI528
Phages φc2		Prolate-headed, lytic phage for MG1363 and UC563
φ712		Small isometric-headed, lytic phage for MG1363

Ringer's solution. The sample was mixed with 100 µl 2% (w/v) phosphotungstic acid and placed on a Formvar-coated copper grid. Cells were examined using a JEM 1200EX TESCAN scanning electron microscope at an accelerating voltage of 80 kV.

Results

Preliminary studies of the phenotypic effects mediated by the phage resistance plasmid pCI528 have been described previously (Costello, 1988; Coffey *et al.*, 1991) and can be summarized as follows:

(a) pCI528 was originally identified in *L. lactis* subsp. *cremoris* UC503.

(b) Curing of the plasmid by acriflavin treatment allowed some phages to adsorb with increased efficiency (e.g. increase from 0% to 64% for phage c2).

(c) Introduction of the plasmid into *L. lactis* subsp. *lactis* MG1363 by conjugation, generating UC505, resulted in this strain exhibiting complete resistance (e.o.p. <10⁻¹⁰) to both prolate-headed (ml3, c2) and small isometric-headed (712, jj50) phages at 30 and 37 °C. For all phages, the adsorption efficiency decreased from greater than 90% to less than 40% following acquisition of the plasmid.

Phenotypic characteristics of pCI528-containing lactococcal hosts

Lactococcal hosts harbouring pCI528, including *L. lactis* subsp. *lactis* UC505 and *L. lactis* subsp. *cremoris* UC503,

Fig. 1. Resuspension rates of pelleted cells of *L. lactis* subsp. *lactis* UC505 (□) and MG1363 (■) following centrifugation at 13000 *g* for 30 s. These results are the means of at least three independent experiments.

produced a soft, easily suspended, fluffy pellet following centrifugation at 13000 *g*, while strains cured of pCI528 demonstrated a normal hard pellet. This property was measured quantitatively in resuspension assays where *L. lactis* subsp. *lactis* MG1363 and the corresponding pCI528-containing strain, UC505, were pelleted by centrifugation for 30 s and the times required for resuspension compared. UC505 resuspended fully after vortexing for 5 s while MG1363 required vortexing for 20 s before complete resuspension occurred (Fig. 1).

Costello (1988) reported that pCI528-containing strains are less hydrophobic than strains not containing the plasmid and in this study it was observed that hosts harbouring pCI528 produced a hard, compact colony morphology unlike plasmid-cured derivatives, which exhibited a normal, soft colony type. These observations suggested that pCI528 mediated the production of a component that altered the cell surface of its host which may result in masking of phage receptors, leading to inhibition of phage adsorption. Evidence to substantiate this hypothesis was obtained when the cell surfaces of *L. lactis* subsp. *lactis* UC505 and MG1363 were examined by electron microscopy (Fig. 2). The pCI528-containing cells were covered by a thick unevenly distributed structure which spread out over surrounding cells, while the surface of MG1363 cells was smooth and regular.

Chemical treatment of pCI528-containing cells

To investigate the nature of the pCI528-determined component, cells harbouring the plasmid were treated with a number of chemicals, after which phage adsorption and plaque-forming ability were monitored. The host used was UC505, since any effect observed could be attributed solely to pCI528. Treatment of whole cells with SDS, Triton X-100 and HCl did not affect adsorption of ϕ c2 to UC505. Similarly, incubation with

Fig. 2. Electron micrographs of *L. lactis* subsp. *lactis* strains demonstrating the presence of an extracellular substance on the surface of UC505, containing pCI528 (a) which contrasts with the smooth, regular surface of MG1363, which lacks the plasmid (b). Bars, 100 nm.

Table 2. Effect of incubation in 0.05 M-NaOH on adsorption of ϕ c2 to *L. lactis* subsp. *lactis* UC505 and MG1363 and *L. lactis* subsp. *cremoris* UC503 and UC563

Pre-treatment	Phage adsorption (%)*			
	UC505	MG1363	UC503	UC563
None	35.0 (± 2.3)	96 (± 1.2)	10 (± 1.7)	84 (± 2.0)
0.05 M-NaOH	95.6 (± 1.7)	94 (± 1.8)	86 (± 2.9)	90 (± 1.45)

* The results are means of at least three independent experiments, $\pm$ SE.

pronase E and trypsin did not cause UC505 to become sensitive to either ϕ c2 or ϕ 712. However, following incubation with 0.05 M-NaOH, UC505 allowed adsorption of ϕ c2 at a much higher level than that observed for the untreated cells (Table 2). NaOH treatment of the original pCI528-containing host *L. lactis* subsp. *cremoris* UC503 also had this effect (Table 2). A similar result was observed with the small isometric-headed phage, ϕ 712,

Table 3. Effect of NaOH treatment on the titre of ϕ c2 and ϕ 712 on *L. lactis* subsp. *lactis* UC505 and MG1363 and *L. lactis* subsp. *cremoris* UC503 and UC563

Phage	NaOH pre-treatment	Phage titre (p.f.u. ml ⁻¹)			
		UC505	MG1363	UC503	UC563
ϕ c2	None	<10 ⁻¹⁰	2 × 10 ¹⁰	<10 ⁻¹⁰	2 × 10 ⁴
	0.01 M	ND	ND	*	2 × 10 ⁴
	0.03 M	ND	ND	*	3 × 10 ⁴
	0.05 M	4 × 10 ⁹	2 × 10 ¹⁰	NG	NG
	0.05 M	1 × 10 ⁷	2 × 10 ⁸	†	†
ϕ 712	None	<10 ⁻⁸	3 × 10 ⁸	†	†
	0.05 M	1 × 10 ⁷	2 × 10 ⁸	†	†

ND, Not determined; NG, no growth.

* Poor growth of UC503 at ϕ c2 titres of 10¹⁰ and 10⁹ p.f.u. ml⁻¹.

† ϕ 712 does not plaque on this host.

on UC505 following NaOH treatment (data not shown).

Growth of either UC503 or UC563 was completely inhibited after treatment with 0.05 M-NaOH. Therefore, to evaluate the effect of NaOH treatment on the plaquing ability of phage ϕ c2 on these hosts, the concentration was reduced slightly to 0.03 M. Phage c2 does not normally produce plaques on UC503, but gives a titre of 10⁴ p.f.u. ml⁻¹ on its pCI528-cured derivative, UC563. However, following treatment with 0.03 M-NaOH, high titres (>10⁸ p.f.u. ml⁻¹) of the phage did appear to cause some inhibition of UC503, manifested by poor growth of the host in the overlay (Table 3). The failure of ϕ c2 to produce any plaques on NaOH-treated UC503 may be explained by the fact that this concentration of alkali did not remove all of the cell surface polymer and/or pCI528 also encodes a second phage resistance determinant mediating abortive infection operational in UC503 (Costello, 1988).

Characterization of NaOH-treated pCI528-containing hosts

NaOH treatment of UC505 cells restored the normal hard type of pellet following centrifugation and these cells also displayed resuspension properties typical of hosts not containing pCI528. Similarly, electron microscopic analysis of the cell surface following NaOH treatment indicated that the substance surrounding the cells had been removed (data not shown). In addition, the hydrophobicity of UC503 increased dramatically after the alkali treatment, showing patterns similar to its plasmid-cured derivative, UC563 (Fig. 3a). In the case of MG1363 and UC505, the alkali treatment did not cause any significant change in hydrophobicity (Fig. 3b). This is due to the fact that MG1363 itself is not very hydrophobic and therefore the difference in hydrophobicity in untreated strains is not as obvious.

Fig. 3. Hydrophobicity of *L. lactis* strains with and without pCI528, before and after treatment with 0.05 M-NaOH, as measured by partitioning of cells into the octane phase. (a) *L. lactis* subsp. *cremoris* UC503 (□, ■) and its pCI528-cured derivative UC563 (○, ●) before (□, ○) and after (■, ●) NaOH treatment. (b) *L. lactis* subsp. *lactis* UC505 (□, ■) and its pCI528-cured derivative MG1363 (○, ●) before (□, ○) and after (■, ●) NaOH treatment. A fall in OD₆₀₀ indicates an increase in hydrophobicity. These results are the means of at least three independent experiments.

GLC analysis of cell surface polysaccharides

Preparations of cell surface polysaccharides were obtained from *L. lactis* subsp. *cremoris* UC503 and UC563, and from *L. lactis* subsp. *lactis* UC505 and MG1363, and analysed by GLC. All strains contained glucose and low levels of arabinose and mannose both before and after NaOH treatment. However, differences between the pCI528-containing host UC505 and either MG1363 or NaOH-treated cells of UC505 could be observed with respect to their galactose and rhamnose content. The ratio of both these sugars to arabinose (the level of arabinose was approximately the same in all samples) was much greater in the untreated UC505 sample than in the alkali-treated UC505 or the MG1363 samples (Table 4). When similar calculations were performed with

Table 4. Ratio of arabinose to either galactose or rhamnose following GLC analysis of cell surface polysaccharides of *L. lactis* subsp. *lactis* UC505 and MG1363 and *L. lactis* subsp. *cremoris* UC503 and UC563 before and after NaOH treatment

	UC505	MG1363	UC503	UC563
		<i>Arabinose: galactose ratio</i>		
- NaOH	1:23.4	1:0.3	1:34	1:2.4
+ NaOH	1:2	1:0.05	1:3.7	1:4
		<i>Arabinose: rhamnose ratio</i>		
- NaOH	1:6.3	1:1.6	1:40	1:14
+ NaOH	1:1.1	1:0.94	1:17	1:22

UC503, its pCI528-cured derivative, UC563 and their NaOH treated cells, the ratio of galactose was also greater in the untreated UC503 sample. When the rhamnose content of UC503 and UC563 was examined, the pCI528-containing host had elevated levels, although significant amounts were also detected in UC563, which may explain why there was not as dramatic a reduction in the rhamnose concentration following alkali treatment.

Discussion

The lactococcal plasmid pCI528 mediates phage resistance by an adsorption-blocking mechanism. It had previously been reported that phenotypic traits mediated by pCI528 included decreased hydrophobicity and fluffy pellet production (Costello, 1988). In this study, the nature of these cell surface alterations was examined more closely. Electron microscopy demonstrated the presence of an irregularly distributed substance surrounding the surface of pCI528-containing cells. Cells not containing pCI528 possessed no such extra layer at their surface. This provides clear evidence that pCI528 encodes production of a polymer which resides on the cell surface. This structure probably prevents the specific adsorption of phage to cell surface receptors, except possibly at a small number of sites where the coating may be absent. This would explain how low levels of adsorption of phage to hosts harbouring pCI528 can occur, although non-specific adsorption is also likely (Table 2). Exhaustive attempts to detect a capsule by light microscopy have proved unsuccessful and the slime production phenomenon (Vedamuthu & Neville, 1986), associated with some capsule-producing lactococcal strains when grown in milk, was never observed.

Treatment of the pCI528-containing strain UC505 with pronase E or trypsin did not alter the fluffy morphology of the cell pellet or resistance to phage, suggesting that the shielding agent is not proteinaceous,

even though in other bacteria proteins have been shown to mask phage receptor sites. Virulent strains of *Aeromonas salmonicida* possess an additional cell wall A-layer composed of a 49 kDa protein which apparently shields the phage receptor and impairs adsorption of phage (Ishiguro *et al.*, 1981). Spontaneous attenuated mutants, isolated following growth at a higher than optimal temperature, lacked the A-layer and were phage sensitive.

Incubation in SDS, Triton X-100 or HCl effected no increase in phage adsorption to UC505. However, when a mild alkali treatment was used, the fluffy pellet was eliminated, phage could adsorb and cells showed sensitivity to phage at levels similar to that of the plasmid-free strain. These results suggested that the cell surface component may be polysaccharide in nature and indicated that it may have some similarities to that previously described for pSK112-containing cells (Sijtsma *et al.*, 1988, 1990a).

L. lactis subsp. *cremoris* UC503 was more hydrophilic than its pCI528-cured derivative UC563. After alkali treatment UC503 partitioned readily into octane, indicating that the hydrophilic substance had been removed to expose a hydrophobic cell surface. An increase in hydrophobicity was also reported by Sijtsma *et al.* (1990a) following removal of cell surface polysaccharide from the pSK112-containing strain SK110, also by using mild alkali treatment. However, Watanabe *et al.* (1987) showed that a phage-resistant derivative of *Lactobacillus casei* ATCC 27092, to which phage were unable to adsorb, was more hydrophobic than the wild-type parent strain.

GLC analysis of cell surfaces of lactic acid bacteria has shown that typically they contain rhamnose, galactose and glucose (Valyasevi *et al.*, 1990; Watanabe *et al.*, 1987). Analysis of cell surface polysaccharides examined here showed clearly that the pCI528-containing strains UC503 and UC505 possessed significantly elevated levels of both galactose and rhamnose compared with the corresponding strains lacking this plasmid. NaOH treatment of these cells reduced the concentrations of both these sugars, usually to levels comparable to those obtained in their pCI528-cured derivatives. The reduction in rhamnose concentration in UC503 following NaOH treatment was not as obvious as that obtained for UC505 (due to the fact that UC563 itself contained a relatively high level of this sugar), but it was still a significant reduction and comparable to that of UC563. From these results we conclude that pCI528 directs the production of a component containing not only galactose but also rhamnose. Sijtsma *et al.* (1988, 1990b) proposed that masking of the phage receptor by a galactosyl-containing lipoteichoic acid occurs in the phage-resistant strain SK110 harbouring pSK112. No evidence was

given for the involvement of rhamnose. Interestingly, Valyasevi *et al.* (1990) have shown that the ϕkh receptor on the cell wall of *L. lactis* subsp. *cremoris* KH is rhamnose and that galactose may also be involved as a secondary receptor. A mutant of *Staphylococcus aureus* H, designated *Staphylococcus aureus* T, has been identified which is resistant to phage that attack the parent strain (Wu & Park, 1971). This mutant contained an additional surface polymer composed of *N*-acetylfucosamine and *N*-acetylmannosaminuronic acid. When some of this polymer was removed following an EDTA wash, partial sensitivity to phage was restored.

There appear to be several similarities between the mechanisms encoded by pCI528 and pSK112. There are, however, some differences. For example, both the galactose and rhamnose concentrations are reduced in NaOH-treated cells of pCI528-containing strains, while only galactose was indicated to be reduced in NaOH-treated pSK112-containing cells. Furthermore, the resuspension results observed here are the reverse of those obtained for pSK112, in that the pCI528-containing strain UC505, resuspended much more easily than the plasmid-cured MG1363, while the pSK112-containing strain resisted resuspension much more than the plasmid-cured strain (Sijtsma *et al.*, 1990a).

In conclusion, pCI528 reduces phage adsorption to lactococcal hosts by causing the production of a hydrophilic polymer containing both rhamnose and galactose. This component prevents phage adsorption by blocking phage receptor sites. Weak alkali treatment removes this polymer, thus allowing phages to adsorb to the cell surface and infect the host. Further work will identify the exact nature of the structure involved in adsorption interference. Genetic analysis of pCI528 is under way to determine how many genes are involved in production of this masking agent.

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