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Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria

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Abstract

The mode of antimicrobial action of chitosan (polymeric β-1,4-*N*-acetylglucosamine) on Gram-negative bacteria was studied with special emphasis on its ability to bind to and weaken the barrier function of the outer membrane (OM). Chitosan (250 ppm) at pH 5.3 induced significant uptake of the hydrophobic probe 1-*N*-phenylnaphthylamine (NPN) in *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*. The effect was reduced (*E. coli*, salmonellae) or abolished (*P. aeruginosa*) by $MgCl_2$. No NPN uptake was observed during exposure of the salmonellae to chitosan at pH 7.2. Chitosan also sensitized *P. aeruginosa* and the salmonellae to the lytic effect of sodium dodecyl sulfate (SDS); such sensitization was not blocked by $MgCl_2$ and was reversible by washing chitosan-treated cells prior to SDS exposure. Chemical and electrophoretic analyses of cell-free supernatants of chitosan-treated cell suspensions showed that interaction of chitosan with *E. coli* and the salmonellae involved no release of lipopolysaccharide (LPS) or other membrane lipids. However, chitosan rendered *E. coli* more sensitive to the inhibitory action of dyes and bile acids used in selective media. Highly cationic mutants of *S. typhimurium* were more resistant to chitosan than the parent strains. Electron microscopy showed that chitosan caused extensive cell surface alterations and covered the OM with vesicular structures. Chitosan thus appeared to bind to the outer membrane, explaining the loss of the barrier function. This property makes chitosan a potentially useful indirect antimicrobial for food protection.

Keywords: Chitosan; Antibacterial; Outer membrane; Gram-negative bacteria

1. Introduction

Chitosan, the deacylated derivative of chitin, is a versatile biopolymer with a number of food applications (Shahidi et al., 1999). Chitosan consists of polymeric $1 \rightarrow 4$-linked 2-amino-2-deoxy-β-D-glucose, but preparations and batches vary with respect to their degree of deacetylation and polymerisation, and many derivatives such as *N*-sulfonyl chitosan or salts such as chitosan lactate with altered physicochemical properties can be prepared.

Since chitosans exhibit antibacterial and antifungal activity, they have received attention as potential food preservatives of natural origin (Chen et al., 1998; Shahidi et al., 1999; Rhoades and Roller, 2000; Roller and Covill, 2000; Tsai et al., 2000). Although more active against spoilage yeasts, chitosan has also been shown to inhibit some Gram-

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negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Vibrio* spp. and *Salmonella typhimurium*. However, the reported Minimum Inhibitory Concentrations (MICs) for Gram-negative bacteria vary widely from 100 to 10,000 ppm (Sudarshan et al., 1992; Wang, 1992; Chen et al., 1998; Tsai and Su, 1999; Rhoades and Roller, 2000; Tsai et al., 2000). Growth of some Gram-negative bacteria such as *Erwinia* spp., *Klebsiella pneumoniae*, and *S. enteritidis* PT4 has been reported as unaffected by chitosan concentrations as high as 5000 ppm (Seo et al., 1992; Chen et al., 1998; Roller and Covill, 2000).

The inhibitory activity of chitosan towards Gram-negative bacteria should be considered in terms of its chemical and structural properties. As a polymeric macromolecule, chitosan is unable to pass the outer membrane (OM) of Gram-negative bacteria, since this membrane functions as an efficient outer permeability barrier against macromolecules (Nikaido, 1996). Therefore, direct access to the intracellular parts of the cells by chitosan is unlikely. A key feature of chitosan is its positive charge of the amino group at C-2 below its pK_a (pH 6.3). This creates a polycationic structure, which can be expected to interact with the predominantly anionic components (lipopolysaccharides, proteins) of the Gram-negative surface (Nikaido, 1996).

Binding of polycationic molecules has been shown to disrupt the integrity of the OM resulting in loss of the barrier function but lacking direct bactericidal activity. Compounds active in this manner include the aliphatic and cationic polymer substance polyethyleneimine (PEI), which binds to and functionally weakens the OM of Gram-negative bacteria, sensitizing the organisms to detergent-induced lysis and to hydrophobic antibiotics or probes (Helander et al., 1997). Polymyxin B nonapeptide (PMBN) acts similarly (Vaara and Vaara, 1983); the binding of these agents to the OM causes considerable structural alterations visualized by electron microscopy (Vaara and Vaara, 1983; Helander et al., 1998a,b). Other polycationic substances (protamine and certain polylysines) are known that disrupt the OM with concomitant release of major proportions of LPS from the cells (Vaara and Vaara, 1983). EDTA also releases considerable proportions of LPS; this is generally assigned to the metal-chelating action of EDTA and removal of divalent cations that are required to stabilize the OM (Hukari et al., 1986).

In general, substances lacking inherent toxicity yet causing increases in OM permeability by various mechanisms are termed permeabilizers (Vaara and Vaara, 1983). Food-grade permeabilizers could find applications in food protection, as they would sensitize harmful Gram-negative bacteria to other potentially inhibitory substances by facilitating their entry into the bacterial cells. For example, the resistance of Gram-negative bacteria to the biocidal action of lysozyme has been shown to diminish when the OM has been perturbed with EDTA (Hughey and Johnson, 1987).

On the basis of the above, it can reasonably be postulated that the mode of antimicrobial action of chitosan on Gram-negative bacteria involves binding of the cationic chitosan to the anionic cell surface resulting in changes in permeability. However, apart from several reports of leakage of cell constituents such as enzymes and glucose following exposure to chitosan, there has been little direct experimental evidence to support this hypothesis (Sudarshan et al., 1992; Tsai and Su, 1999). In this paper, we have sought to elucidate the mode of action of chitosan on Gram-negative bacteria important in food processing and hygiene using a range of both direct and indirect techniques for assessing the barrier properties of the OM.

2. Materials and methods

2.1. Materials

Chitosan from crab shells was supplied by Sigma-Aldrich (Steinheim, Germany; minimum 85% deacetylated; Cat. No. C-3646) and Pronova Biopolymer, (Drammen, Norway; 83% deacetylated chitosan chloride CL210). Stock solutions of chitosan (1 mg/ml) were prepared in 10 mM acetic acid (pH-adjusted to 5.3 ± 0.1 with NaOH). HEPES, *n*-heptadecanoic acid methyl ester, 1-*N*-phenylnaphthylamine (NPN) and sodium dodecyl sulfate (SDS) were from Sigma-Aldrich and EDTA from Riedel-de-Haen (Seelze, Germany). Triton X-100 was from BDH (Poole, UK), and glutaraldehyde and proteinase K from Merck (Darmstadt, Germany). Micro-

biological media and diluents were from Oxoid (Basingstoke, UK).

2.2. Microbial strains and culture conditions

E. coli ATCC 11775, *P. aeruginosa* ATCC 9027, and *S. typhimurium* strains ATCC 13311, SH9178 and SH7426 were used. The latter two strains were of the rough chemotype Rb2 (Vaara, 1981). *S. typhimurium* SH7426 was a *pmrA* mutant strain with an abnormally cationic lipopolysaccharide, SH9178 was the corresponding parent strain ($pmrA^+$) with a normal anionic lipopolysaccharide (Helander et al., 1994). The cultivation medium for the above strains for permeability assays was Luria–Bertani broth at 37 °C; further details of cultivations are given below under various experimental settings. *E. coli* O157:H7 (attenuated strain), *P. fragi* and *S. enteritidis* PT4 were original isolates from foods obtained from Unilever Research, Leatherhead Food Research Association and the University of Bath (UK), respectively. These three species were grown in Nutrient Broth in shake flasks for 24 h prior to harvesting. *E. coli* and *S. enteritidis* were grown at 37 °C whilst *P. fragi* was grown at 30 °C.

2.3. Permeability assays

Two distinct methods were utilized to determine permeability properties of the OM: (i) NPN uptake and (ii) sensitization to detergent-induced bacteriolysis. NPN uptake by bacterial suspensions was measured using microtitre plates and the automated fluorometer Fluoroskan Ascent FL (Labsystems, Helsinki, Finland) as described recently (Helander and Mattila-Sandholm, 2000). Black fluoroplates (Cat. No. 9502 867, Labsystems) were used. Bacteria were cultivated to A_{630} of 0.5 ± 0.02, harvested by centrifugation at room temperature for 10 min at $1000 \times g$, and suspended into a half volume of HEPES buffer at pH 5.3 ± 0.1. Aliquots (100 μl) of this cell suspension were pipetted onto fluoroplate wells, which contained NPN (10 μM), and as test substances either chitosan (100 and 250 ppm), EDTA (1 mM) or buffer (control) to make up a total volume of 200 μl. In experiments involving $MgCl_2$, the salt (5 mM) was included in the buffer; controls for these assays included $MgCl_2$ as well. In one experiment the NPN uptake assay was performed at neutral pH; in this assay the pH of both the HEPES buffer and the chitosan solution was adjusted to 7.2. Fluorescence was monitored within 3 min from four parallel wells per sample (excitation, 355 nm, half bandwidth 38 ± 3 nm; emission, 405 nm, half bandwidth 50 ± 5 nm). Each assay was performed at least three times.

Sensitization of Gram-negative bacteria to the lytic action of the detergents SDS and Triton X-100 by chitosan was investigated according to the method of Helander et al. (1997) with the following modifications. In experiments with *E. coli* ATCC 11775, *P. aeruginosa* and *S. typhimurium*, bacterial suspensions containing 100 ppm chitosan were used directly for the optical monitoring of bacteriolysis, omitting the centrifugation step and resuspension in buffer. In experiments with *E. coli* 0157:H7, *P. fragi* and *S. enteritidis*, cells were exposed to 1000 ppm chitosan for 10 min at room temperature, followed by washing in buffer and exposure to 0.2% SDS.

Results from the permeability assays were analyzed statistically using the two-tailed unpaired Student's *t*-test to determine differences; levels of significance are denoted as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2.4. Release of LPS and glycerophospholipids

The release of LPS in response to external agents was determined by SDS-PAGE of cell-free supernatants after treatment of *E. coli* ATCC 11775 and *S. typhimurium* with 250 ppm chitosan (pH 5.3) or 1 mM EDTA (pH 7.2), as described by Helander et al. (1998a,b). Materials and equipment for SDS-PAGE and silver staining were from Novex (San Diego, CA), including pre-cast 12% polyacrylamide Tris-glycine gels. In addition, such cell-free supernatants were subjected to fatty acid analysis (GC) after methanolysis as described by Helander et al. (1998a,b).

2.5. Electron microscopy

E. coli ATCC 11775 and *S. typhimurium* were grown to A_{630} of 0.4, centrifuged at $1000 \times g$ and washed once with 10 mM Tris–HCl (pH 7.2) and resuspended in 10 mM Tris–HCl (pH 5.3). This

suspension was supplemented with chitosan (250 ppm); the control suspension remained without supplement at pH 5.3. After incubating for 10 min at 37 °C, 1-ml aliquots were centrifuged at 11,000 rpm and the resulting pellets were resuspended in 5 mM HEPES buffer at pH 5.3 containing 2.5% (vol/vol) glutaraldehyde. After 1 h at room temperature, the cells were centrifuged, resuspended in 5 mM HEPES (pH 5.3) and stored at 4 °C. Specimens were prepared for electron microscopy by post-fixing with 1% (wt/vol) OsO_4 in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h at room temperature, washing once with the same buffer, dehydrating in a graded series of ethanol, washing with acetone and embedding in Epon Lx-112. Thin sections were cut with a diamond knife on a Reichert Ultracut ultramicrotome and double-stained with uranyl acetate and lead citrate. The grids were examined with a JEM-1200EX transmission electron microscope (JEOL, Japan) at an operating voltage of 60 kV.

2.6. Recovery of chitosan-treated bacteria on selective agars

E. coli O157:H7 was grown for 18 h in Nutrient Broth at 37 °C, harvested by centrifugation and washed twice in sterile saline (0.9%) solution. The washed cells were added to sterile saline solutions containing 0 or 5000 ppm chitosan at room temperature. After 10 min, viable counts were determined using serial dilution in Maximum Recovery Diluent (MRD) followed by pour-plating on Plate Count Agar (PCA), Violet Red Bile Glucose Agar (VRBGA), and PCA supplemented with 2 ppm Crystal Violet, 30 ppm Neutral Red or 1500 ppm Bile Salts No. 3. Agar plates were incubated at 30 °C and colonies were enumerated after 24 h and again after 4 days.

2.7. Inactivation of mutants with altered surface properties by chitosan

S. typhimurium SH 9178 (wild type) and SH 7426 were grown on Tryptone Soy Agar (TSA) plates at 37 °C for 3 days. Fresh cultures were prepared in 10 ml Tryptone Soy Broth supplemented with 10 g/l glucose (TSBG) incubated at 37°C for 16 h for stationary phase cultures and for 6 h for mid-log phase cultures. The cells were harvested by centrifugation and washed in sterile saline (0.9%) solution. The suspension of cells was added to saline solutions containing 0 or 20,000 ppm chitosan (pH 6.0) at room temperature and samples were removed periodically for up to 24 h for the determination of viable counts on TSA. Agar plates were incubated at 37 °C and colonies were enumerated after 24 h and again after an additional 24 h of incubation.

3. Results

3.1. Chitosan induces increased NPN uptake

Normally, NPN, which is a hydrophobic probe, is largely excluded by Gram-negative bacteria. Enhanced uptake of NPN occurs in bacterial suspensions containing cells whose outer membrane is damaged and functionally invalid. The uptake is manifested as fluorescence, since the quantum yield of NPN is greatly increased in a glycerophospholipid milieu as opposed to an aqueous one (Träuble and Overath, 1973; Loh et al., 1984; Helander and Mattila-Sandholm, 2000). The NPN uptake by chitosan- and EDTA-treated *E. coli*, *P. aeruginosa*, and *S. typhimurium* is shown in Table 1. Chitosan at pH 5.3 caused significant increases in NPN uptake, compared with control cells; the uptake levels were similar to those yielded by EDTA, a strong permeabilizer acting by chelation of ions from the OM. However, relatively high concentrations of chitosan (250 ppm) were required to obtain significantly increased NPN uptakes. At 100 ppm, chitosan sometimes increased NPN uptake, but such increases were highly variable, leading to statistically non-significant trends. Chitosan concentrations below 100 ppm caused no change in NPN uptake in any of the organisms tested (results not shown). As expected, the addition of 5 mM $MgCl_2$ prevented the increase in NPN uptake caused by EDTA (Table 1). Similarly, the presence of $MgCl_2$ prevented the increase in NPN uptake by chitosan in *P. aeruginosa* but the "protective" effect of magnesium was less pronounced in *E. coli* and *S. typhimurium*. In the case of *E. coli*, the apparent protection from chitosan-induced NPN uptake afforded by $MgCl_2$ was statisti-

Table 1
NPN uptake induced by chitosan and EDTA

Species, strain	$MgCl_2$ (mM)	Relative fluorescence ± S.D.			
		Control	EDTA (1 mM)	Chitosan	
				100 ppm	250 ppm
E. coli ATCC 11775	0	121 ± 14	174 ± 17 *	261 ± 96	244 ± 28 * * *
E. coli ATCC 11775	5	76 ± 9	93 ± 12	136 ± 18 * *	131 ± 6 *
P. aeruginosa ATCC 9027	0	96 ± 12	576 ± 48 * * *	389 ± 243	605 ± 46 * * *
P. aeruginosa ATCC 9027	5	85 ± 11	113 ± 21	111 ± 16	105 ± 14
S. typhimurium ATCC 13311	0	227 ± 29	480 ± 23 * * *	434 ± 149	497 ± 32 * * *
S. typhimurium ATCC 13311	5	79 ± 3	95 ± 9	215 ± 52	164 ± 72
S. typhimurium SH9178	0	117 ± 9	141 ± 7 *	494 ± 50 * * *	ND
S. typhimurium SH7426	0	119 ± 9	132 ± 11	396 ± 28 *	ND

ND, Not determined.
* $P < 0.05$ compared to the control value.
* * $P < 0.01$ compared to the control value.
* * * $P <$ 0.001 compared to the control value.

cally significant but in the case of the salmonellae, it was not (Table 1).

The effect of chitosan on NPN uptake by *S. typhimurium* at pH 7.2 was also investigated. The results (not shown) were negative, indicating that chitosan-induced NPN uptake only takes place at acidic pH.

3.2. Chitosan sensitizes some Gram-negative bacteria to lysis by SDS

Table 2 shows the effect of chitosan on the susceptibility of *E. coli* ATCC 11775, *P. aeruginosa* and *S. typhimurium* to lysis in the presence of the detergents Triton X-100 (nonionic) and SDS (anionic). The results show that chitosan caused no sensitization to Triton X-100 (at concentrations up to 1%) in any of the three organisms studied. By contrast, a marked lysis-promoting effect was observed in *P. aeruginosa* and the salmonellae in the presence of 100 ppm chitosan and both concentrations of SDS tested (0.1 and 1%). The presence of excess Mg^{2+} (10 mM) in the assay system slightly attenuated the lytic effect of chitosan/SDS combinations in these two organisms. However, in *E. coli*, the presence of 100 ppm chitosan had no effect on sensitization to 0.1% SDS and the effect was relatively weak even at the higher concentration of 1%. The presence of

Table 2
Sensitization of *E. coli*, *P. aeruginosa* and *S. typhimurium* to detergent-induced bacteriolysis in the presence of 100 ppm chitosan at pH 5.3

Lytic medium (concentration)	Relative turbidity (%) at 4 min					
	E. coli		*P. aeruginosa*		*S. typhimurium*	
	Control	Chitosan	Control	Chitosan	Control	Chitosan
Triton X-100 (0.1%)	105 ± 1	109 ± 2	111 ± 3	104 ± 2	104 ± 1	102 ± 2
Triton X-100 (1%)	98 ± 1	97 ± 3	102 ± 1	99 ± 4	96 ± 3	96 ± 1
SDS (0.1%)	54 ± 20	49 ± 10	108 ± 2	20 ± 1 * * *	93 ± 5	17 ± 1 * *
SDS (1%)	42 ± 2	28 ± 7 *	83 ± 2	14 ± 1 * * *	58 ± 3	13 ± 1 * *
SDS (1%) + $MgCl_2$ (20 mM)	28 ± 1	48 ± 3 *	106 ± 2	29 ± 5 * * *	92 ± 4	28 ± 3 * * *

* $P < 0.05$ compared to the control value.
* * $P < 0.01$ compared to the control value.
* * * $P < 0.001$ compared to the control value.

excess Mg^{2+} prevented the weak sensitization observed at 1% SDS.

It is noteworthy that in all of the experiments described above (SDS sensitization and NPN uptake), cells were exposed to chitosan simultaneously with the detergents and NPN probe. By contrast, when similar experiments were carried out using *E. coli* O157:H7, *P. fragi* and *S. enteritidis* PT4 which had been washed twice in buffer after exposure to chitosan (1000 ppm for 10 min at room temperature), no significant changes in SDS (0.2%)-induced lysis were observed (results not shown), suggesting that chitosan-induced permeability effects were reversible.

3.3. Chitosan causes no release of OM lipids

Cell-free supernatants derived from suspensions of *E. coli* and *S. typhimurium* treated with chitosan or EDTA were analyzed for possible lipid components of the OM. The results in Table 3 show that treatment with EDTA resulted in a marked increase in lipid material in the cell-free supernatants whilst chitosan had no such effect. Fatty acids detected in the EDTA supernatants were indicative of both LPS [LPS-specific fatty acids $C_{12:0}$, $C_{14:0}$, $C_{14:0(3OH)}$] and glycerophospholipid (fatty acids $C_{16:0}$, $C_{16:1}$, $C_{18:1}$) release, in accord with the known OM-disruptive property of this chelator (Vaara, 1992; Helander et al., 1998a,b). The quantity and quality of the released LPS in the supernatants were also determined by SDS-PAGE, as shown in Fig. 1. The results show that the EDTA-treated cells released a considerable amount of LPS into the supernatant in a ladder pattern that is typical of the distinct molecular forms of smooth LPS with different numbers of repeating O-specific oligosaccharide units. No such material was detected in the supernatants of chitosan-treated cells. Control supernatants yielded only faint bands conforming to the LPS pattern. These experiments

Table 3
Liberation of lipid material from *E. coli* and *S. typhimurium* by EDTA and chitosan

Strain and fatty acid	Amount (μg) of fatty acid in 8.6 ml of cell-free supernatant after treatment of cells with		
	None (control)	EDTA (1 mM)	Chitosan (250 ppm)
E. coli			
$C_{12:0}$	0.5	1.0	1.1
$C_{14:0}$	0.5	1.3	0.5
$C_{14:0(3\text{-}OH)}$	< 0.3	1.7	< 0.3
$C_{16:0}$	1.1	1.6	1.4
$C_{16:1}$	< 0.3	0.9	< 0.3
$C_{18:1}$	< 0.3	1.9	< 0.3
Total	2.1	8.4	3.0
S. typhimurium			
$C_{12:0}$	0.4	2.8	0.7
$C_{14:0}$	0.4	3.4	0.4
$C_{14:0(3\text{-}OH)}$	< 0.3	4.7	< 0.3
$C_{16:0}$	1.7	4.2	1.0
$C_{16:1}$	0.8	3.3	0.6
$C_{18:1}$	0.7	3.0	0.6
Total	4.0	21.4	3.3

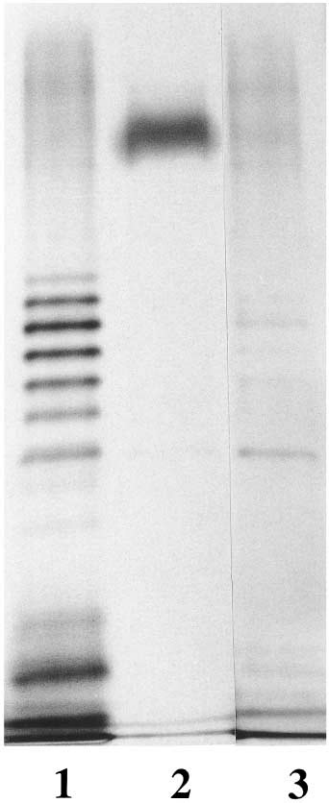

Fig. 1. Silver-stained SDS-polyacrylamide gel (12% acrylamide) of proteinase K-treated cell-free supernatant samples of *E. coli* treated with EDTA (1 mM) (lane 1) and chitosan (250 ppm) (lane 2). Lane 3 shows the control supernatant. Equal volumes of each sample were applied to the gel.

indicated that no release of OM lipids was associated with the action of chitosan on *E. coli* and *S. typhimurium*.

3.4. Binding of chitosan on the cell surface is microscopically evident

Suspensions of *E. coli* and *S. typhimurium* were treated with chitosan at pH 5.3 and examined by electron microscopy, as shown in Fig. 2. Control cells revealed envelopes with the outer membrane (OM) and cell membrane (CM) clearly discernible; the OM appeared as an undulating barrier with few vesicular structures. Chitosan-treated cells exhibited altered OM, the surface of which was covered by numerous vesicular structures and, particularly in *E. coli*, by an additional layer of material, causing the cell envelope to appear considerably thickened. The

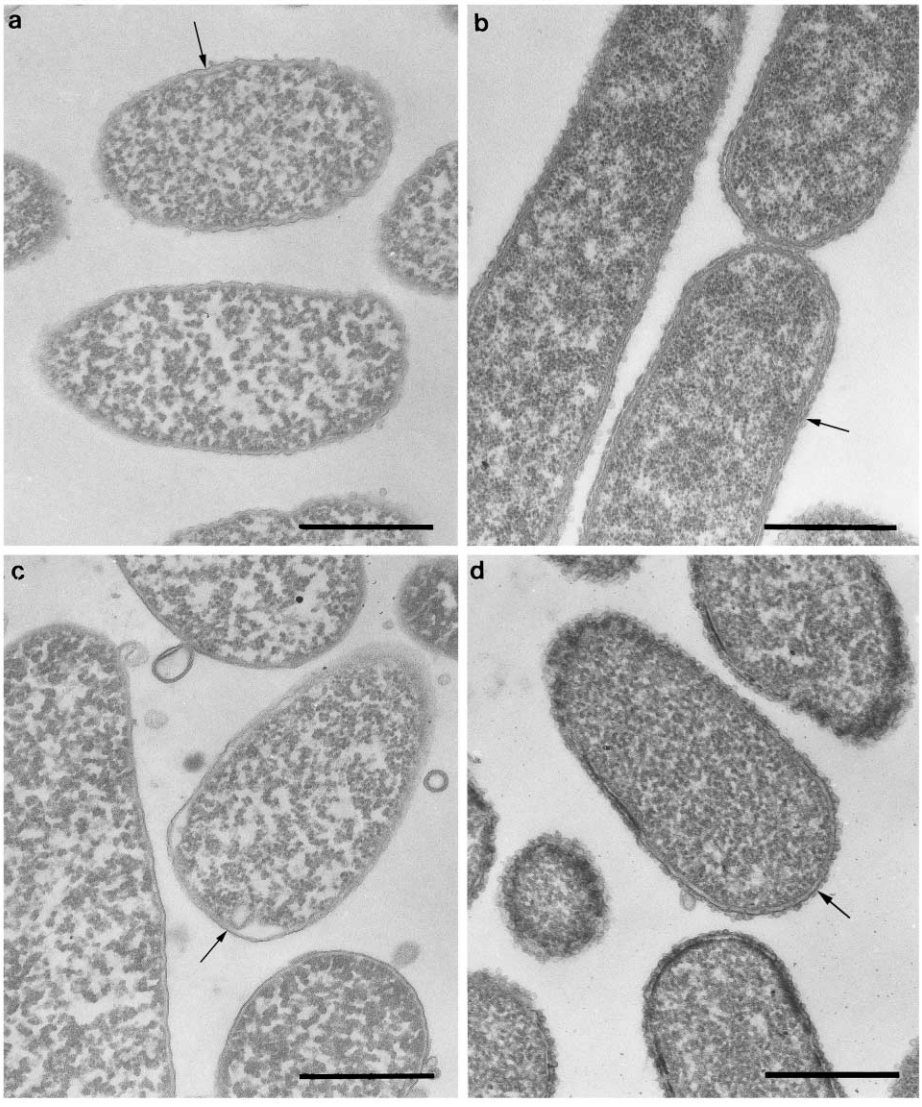

Fig. 2. Electron micrographs of *E. coli* treated with (a) buffer or (b) chitosan (250 ppm) and *S. typhimurium* treated with (c) buffer or (d) chitosan (250 ppm) at pH 5.3. Arrow points to outer membrane (OM). Bar = 0.5 nm.

Table 4
Recovery of viable *E. coli* O157:H7 on agar media after exposure to 5000 ppm chitosan for 10 min at room temperature

Medium composition	Viable count (log CFU/ml)[a]		
	Untreated cells	Chitosan-treated	Difference
PCA	5.1	4.4	−0.7
VRBGA	4.8	1.3	−3.5
PCA	7.9	7.6	−0.3
PCA + Neutral Red	7.8	7.5	−0.3
PCA + Bile Salts	7.8	7.3	−0.5
PCA + Crystal Violet	7.7	7	−0.7
PCA + all of the above	7.7	6.1	−1.6

[a]Results represent means of triplicate counts and were the same after 24 h and 4 days of incubation.

CM appeared to be unaffected in chitosan-treated cells.

3.5. Chitosan sensitizes E. coli to dyes and bile acids

Selective media are used frequently to differentiate between viable counts of microorganisms belonging to different genera. Coliforms are often enumerated on agar media containing inhibitors of yeasts and Gram-positive bacteria. An example of a selective medium is VRBGA, containing Crystal Violet, Neutral Red and Bile Salts with antimicrobial properties to which Enterobacteriaceae such as *E. coli* are normally resistant. Whilst selective media are useful for enumerating organisms from mixed cultures that have not been exposed to stress or sublethal injury, it was postulated that disruption of the barrier properties of *E. coli* by chitosan may render the bacteria more susceptible to inhibition by the dyes and bile salts present in VRBGA. Using different starting counts of *E. coli* O157:H7 (5.1 and 7.9 log CFU/ml), the results in Table 4 show that chitosan at 5000 ppm reduced the viable numbers by only 0.3 and 0.7 log CFU/ml, respectively, when determined on PCA, a complex and nutrient-rich laboratory medium without added inhibitors. However, when chitosan-treated cells were plated out on the commercially prepared VRBGA, there was a 3.5 log CFU/ml reduction in count, compared with that obtained on PCA. When the chitosan-treated cells were plated out on PCA supplemented with Neutral Red, Bile Salts and Crystal Violet (added individually at the same levels as in the commercial medium),

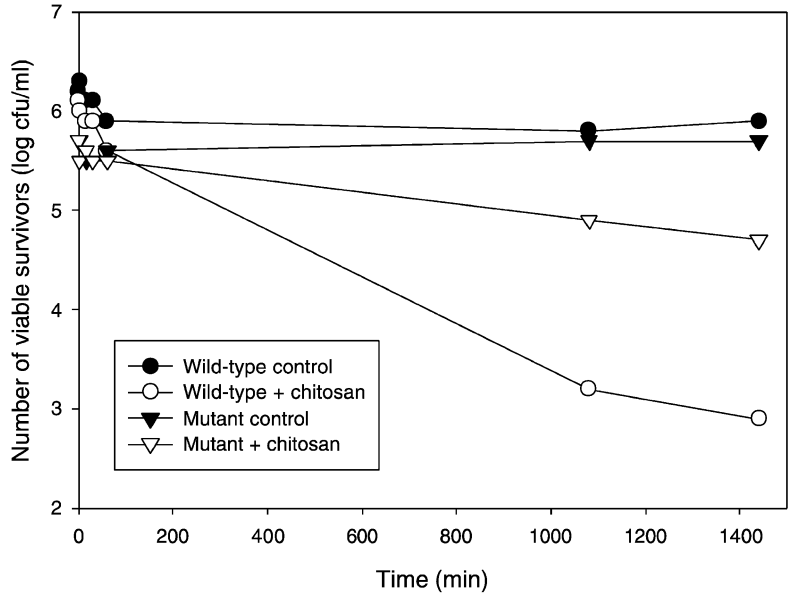

Fig. 3. Survival of parent (circles) and cationic mutants (triangles) of *S. typhimurium* in the presence of 0 (closed symbols) and 20,000 ppm (open symbols) chitosan in saline solution at room temperature. Results represent means of triplicate counts for each data point obtained on two separate occasions.

the reduction in count was relatively small at 0.3, 0.5 and 0.7 log CFU/ml, respectively. However, when all three inhibitors were added to PCA together, the viable numbers of the chitosan-treated *E. coli* were reduced by 1.6 log CFU/ml (Table 4). The numbers of viable organisms were the same after 24 h and 4 d of incubation of the agar plates suggesting that recovery from sub-lethal injury had not taken place.

3.6. Cationic cell surfaces render bacteria more resistant to chitosan

The objective of this part of the work was to determine whether changes in cell surface structure affected the sensitivity of Gram-negative bacteria to the antimicrobial action of chitosan. It was hypothesised that the *pmrA* mutant of *S. typhimurium* would be more resistant to chitosan than its parent strain because chitosan, which is highly polycationic at pH values below its pKa (pH 6.3), would be less likely to bind to its unusually cationic cell surface. The results indicated that both parent and mutant strains of *S. typhimurium* grown to stationary phase were resistant to 20,000 ppm of chitosan for up to 60 min at room temperature in saline solution (data not illustrated). However, when the cells were grown to mid-log phase, the highly cationic mutant strain maintained viable numbers for up to 24 h whilst the viable numbers of the parent strain were reduced by about 3 log CFU/ml (Fig. 3).

4. Discussion

The experimental data presented in this paper provide evidence that chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. The electron micrographs demonstrate that the site of action is the OM, which binds chitosan. The perturbation of the OM is reflected in increased permeability to hydrophobic probes such as NPN and increased sensitivity to the biocidal and/or inhibitory action of a range of inimical compounds including anionic detergents, dyes and bile acids. These effects are observed at concentrations of chitosan (100–250 ppm) that are well below the levels needed to achieve substantial kill rates for Gram-negative bacteria (20,000 ppm for *S. typhimurium* in this paper and 2000–5000 ppm reported for Gram-negatives elsewhere (Wang, 1992; Chen et al., 1998). The low levels of chitosan needed to observe effects on the permeability function of the cell membrane agree with those reported previously as causing leakage of intracellular components from *E. coli* (Sudarshan et al. 1992).

The permeabilizing effects were demonstrated at slightly acidic conditions (pH 5.3) in which chitosan is protonated; at this pH the carboxyl and phosphate groups of the bacterial surface are anionic and offer potential sites for electrostatic binding of chitosan. At neutral pH, chitosan is much less protonated (pK_a 6.3) and would not be expected to be attracted by the OM. This expectation was reflected in the lack of enhancement of NPN uptake by chitosan at pH 7.

The mode of permeabilizing action of chitosan resembles that of other polycationic molecules such as PEI and PMBN (Vaara and Vaara, 1983; Helander et al., 1997, 1998a,b). Although the definitive MICs required to achieve permeability increases in different species of Gram-negative bacteria may be difficult to ascertain due to the variability in the degree of deacetylation and polymerization of chitosan batches, it is apparent that chitosan is less active than PEI which has a minimum permeabilizing concentration below 10 ppm. Similarly, EDTA is very active at concentrations as low as 1 mM and additionally induces the release of LPS from the cells. No such release was observed after treatment with chitosan. Furthermore, the permeabilizing effect of chitosan is reversible.

Apart from direct bactericidal or bacteriostatic activity, chitosan's ability to disrupt the permeability barrier of the OM in Gram-negative bacteria expands the applicability of chitosan as an antimicrobial substance in foods. Since opening up of the OM provides access for other substances present in the food materials to the deeper and more vital parts of the bacterial cell, the permeabilizing phenomenon should generally sensitize bacteria to many external agents. This is compatible with the hurdle concept, which refers to incorporating several antimicrobial measures to gain a synergistic antimicrobial net effect (Leistner, 2000). More research is, however, required to evaluate chitosan's antimicrobial potency in combination with other interfering substances in food materials.

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