Comparison of the chitinolytic properties of *Clostridium* sp. strain 9.1 and a chitin-degrading bacterium from the intestinal tract of the plaice, *Pleuronectes platessa* (L.)

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The chitinolytic properties of a facultatively anaerobic bacterium isolated from the hindgut of plaice were compared with those of *Clostridium* sp. strain 9.1, a bacterium isolated from anoxic estuarine sediment. The chitinolytic enzyme systems of the gut isolate and strain 9.1 both released *N,N'*-diacetylchitobiose (NAG₂) as the major hydrolysis end-product. During the hydrolysis of chitin, there was transient accumulation of a non-sedimentary chitin fraction which was not detectable by high-performance liquid chromatography. Growth on NAG₂ repressed chitinase synthesis in the gut isolate but not in the *Clostridium* species. Thiol reagents were strongly inhibitory to the chitinase of the strict anaerobe but did not affect the hydrolytic enzymes of the gut isolate. When the two bacteria were cocultured with chitin as the sole carbon and energy source, *Clostridium* sp. strain 9.1 was always outcompeted. Experiments with batch and phauxostat cultures showed that the competitiveness of strain 9.1 could be improved dramatically by the inclusion in the cocultures of a non-chitinolytic bacterium capable of fermenting chitin oligomers. The cooperation between the oligomer-fermenting species and the *Clostridium* sp. is discussed in relation to the regulation of chitinolytic activity in the latter organism.

Introduction

Recently we reported the isolation of eight strains of obligately anaerobic chitin-degrading bacteria, all belonging to the genus *Clostridium* (Pel & Gottschal, 1986). The bacteria were isolated from a chitinolytic community, which had been enriched from the upper layer of a tidal mud flat in the Eems-Dollard estuary, The Netherlands. One of these isolates, *Clostridium* sp. strain 9.1, has been examined in detail. Pure culture studies demonstrated poor growth of this highly specialized chitinolytic anaerobe (Pel & Gottschal, 1986). The influence of strong reductants and thiol reagents on the apparently redox-sensitive chitinolytic enzyme system has been reported (Pel & Gottschal, 1986, 1987). In the presence of non-hydrolytic sugar-fermenting or sulphate-

reducing bacteria a strong enhancement of the rate of chitin degradation was observed, due to the release of growth factors by the secondary populations (Pel *et al.*, 1989; Pel & Gottschal, 1989). One of these factors was a thermostable high-molecular-mass compound, probably identical to the redox-active enzyme thioredoxin (Pel & Gottschal, 1989). Some additional, not yet fully identified, low-molecular-mass factors have also been shown to stimulate growth (Pel *et al.*, 1989).

Previously we have argued that the flow of organic carbon in the Eems-Dollard estuary is not dominated by cellulose but that chitinous particulate matter from crustacean exuviae probably represents a major source of organic carbon in the sediments. However, although no detailed information is available on the quantity of chitin entering the sediments in this estuary (or other estuarine ecosystems), the flux of this polysaccharide will be low in comparison with fluxes existing in the digestive tracts of marine fish that daily consume crustaceans. For example, in the plaice *Pleuronectes platessa* (L.), known to feed primarily on small crustacea such as *Corophium volutator* (Stam, 1984), a residence time in the intestinal tract of 10–24 h has been reported (Fänge & Grove, 1979). Therefore, the digestive tract of this fish, especially the anoxic intestine with a pH of 7.4–8.5 (Barrington, 1957),

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Abbreviations: DNS, dinitrosalicylic acid; DP, degree of polymerization; HPLC, high-performance liquid chromatography; IoAc, iodoacetate; NAG, *N*-acetylglucosamine; NAG₂, *N,N'*-diacetylchitobiose; NEM, *N*-ethylmaleimide; pCMB, 4-chloromercuribenzoic acid; pCMPS, 4-chloromercuriphenyl sulphonic acid; TOC, total organic carbon.

is likely to harbour (facultatively) anaerobic species capable of rapid degradation of chitinous material.

Studies on the occurrence of chitinolytic bacteria in the digestive tracts of other marine fish have been restricted to the total chitinase activity *in situ* and the assessment of the number of bacteria capable of aerobic growth on chitin-agar plates. The number of chitin decomposers reported by Seki & Taga (1963), Goodrich & Morita (1977) and Danulat (1986) for the marine fish *Canthigaster rivulatus*, *Enophris bison* and *Gadus morhua*, respectively, were in the range of 10^7 – 10^9 per ml of intestinal contents. The chitin-degrading potential of the predominant chitinolytic bacteria from these marine fish has never been studied in pure culture, and its importance for chitin degradation in the intestinal tract has been questioned by Danulat (1986).

In this paper we report the isolation of the dominant chitinolytic bacterium from the hind gut of *P. platessa*. Growth characteristics and some properties of the chitinolytic enzyme system of this organism are presented and compared with those of *Clostridium* sp. strain 9.1. The apparent absence of these chitinolytic intestinal bacteria from the upper layer of the sediment in the Eems-Dollard estuary is discussed in relation to data obtained from competition experiments conducted in batch and phauxostat cultures.

Methods

Fish and diet. *Pleuronectes platessa* (L.), age group 1 to 2 (length approx. 17 cm), were obtained from the Department of Marine Biology, University of Groningen. The fish had been caught as breed (fingerlings) in the Marsdiep (outlet of the Waddensea, in the north of the Netherlands) and were kept in a tank containing sea water at 15 °C. They were fed live shrimps (*Crangon crangon*) every 2 d.

Intestinal sampling and enumeration of chitinolytic bacteria. The intestinal tract of the fish was dissected aseptically under a flow of N_2 and cut into four pieces of approximately equal length. Each piece was transferred to a screw-cap Hungate tube (13 ml) containing Na_2S -reduced medium and six glass beads (3 mm diameter). The tubes were then closed and the head-spaces were flushed with N_2/CO_2 (80:20, v/v). The tubes were thoroughly shaken until most of the gut contents had been released from the lumen. The suspended intestinal contents were then serially diluted into Hungate tubes containing liquid medium amended with ball-milled chitin (0.1%, w/v). The wet weight of lumen contents of each intestinal piece was estimated by weighing the tubes after the addition, and again after the empty gut pieces had been removed from the tubes (when the dilutions had been made). After incubation of the dilution series for 2 weeks at 25 °C, the tubes in which chitin degradation had occurred were scored, thus providing a rough estimate of the number of chitinolytic anaerobes in each intestinal section.

Isolation of chitinolytic bacteria. The highest positive dilution from the series inoculated with the hindgut section was used for the isolation of the predominant chitinolytic bacterium. A 1 ml sample was serially diluted into pre-reduced chitin-agar medium as described previously (Pel & Gottschal, 1986). The isolate obtained was designated as strain AW.D2.

Organisms and cultivation. The chitinolytic *Clostridium* sp. strain 9.1 is only capable of utilizing chitin and chitin oligomers (not *N*-acetylglucosamine, NAG), with acetate, ethanol, H_2 and CO_2 as the major fermentation products. Further growth characteristics of this bacterium have been described in earlier papers (Pel & Gottschal, 1986, 1987).

Non-chitinolytic NAG-(oligomer)-fermenting bacteria used in mixed-culture fermentations of chitin with either one of the chitin-degrading species were: a wild-type strain of *Escherichia coli* (Pel & Gottschal, 1989), '*Klebsiella aerogenes*' (*K. pneumoniae*) ATCC 15380, and the facultative anaerobes strain HA 8.1 and strain GH 8.2. These latter two bacteria were isolated from the chitinolytic community containing *Clostridium* sp. strain 9.1. Data on the growth of these bacteria on NAG-(oligomers) and the construction of cocultures are presented elsewhere (Pel *et al.*, 1989).

Pure- and mixed-culture fermentations of chitin (initial concentration 1 mg ml⁻¹) in batch culture were conducted using a mineral brackish medium as described previously (Pel *et al.*, 1989). Cultures were maintained in 17 ml screw-cap Hungate tubes containing 13 ml liquid medium (N_2/CO_2 , 80:20, v/v, in the head-space) and incubated at 25 °C on a rotary shaker run at 80 r.p.m.

For studies conducted in a phauxostat (Martin & Hempfling, 1976) the bicarbonate buffer in the medium was replaced by a 5 mM-phosphate buffer. The pH of the medium was adjusted to 7.2 with NaOH. Medium and chitin were delivered separately by two independent peristaltic metering pumps (Vario Perspex 12000, LKB Produkter). The pumps were turned on when the culture pH fell below 6.6. The buffer capacity of the medium and the chosen pH set-point value of 6.6 allowed a fermentation of approximately 0.4 mg chitin per ml culture fluid. The culture vessel was gassed with O_2 -free N_2 delivered by a third pump, also triggered by the culture pH. Especially at low dilution rates, a controlled and small flow of N_2 over the culture vessel appeared necessary to ensure that gassing with N_2 affected the concentration of dissolved volatile acid-equivalents (CO_2 and acetate) to the same extent at all flow rates of the medium. The ratio of the supply rates of N_2 gas and fresh medium approximated the head-space/liquid-volume ratio of the culture vessel.

Chitinase assay. A turbidimetric assay with resting cells was adopted that minimized the chance of enzyme inactivation due to the absence of substrate as indicated by preliminary experiments in the case of strain AW.D2. To this end, cultures were harvested when approximately 25% of the initial amount of chitin had been hydrolysed. The pellets were washed three times with a cold deoxygenated phosphate buffer (0 °C) containing 20 mM-sodium phosphate (pH 7.2) 1 mM- $CaCl_2$ and 210 mM- $NaCl$. Ca^{2+} was added as it could possibly increase the stability of chitinase as shown for some polysaccharases (Johnson *et al.*, 1982; Mackenzie *et al.*, 1985; Skujins *et al.*, 1970). The pellets were then resuspended in 13 ml screw-cap Hungate tubes containing 8 ml of the same buffer to a final concentration of approximately 0.6 mg chitin ml⁻¹. Because of the presence of susceptible thiol groups in the chitinolytic system of strain 9.1 (Pel & Gottschal, 1987), the head-spaces of the tubes were flushed with O_2 -free N_2 and subsequently 1 mM-sulphide was added to ensure strictly anaerobic conditions. Rifampicin was added to a final concentration of 50 µg ml⁻¹ in order to prevent growth. The tubes were incubated horizontally on a rotary shaker at 250 r.p.m. at a temperature of 37 °C. The incubation periods in the assays were approximately 20 and 40 h for strain AW.D2 and strain 9.1, respectively.

Chitinolytic activity resulting in extensive hydrolysis of chitin was monitored by recording the OD_{660} of the chitin suspension in the tubes. Truly soluble products of chitinolysis were determined in the supernatant of the assay fluid after sedimentation of residual chitin by centrifugation at 8500 g for 5 min. Non-sedimentable chitin fragments and dextrans in the supernatant were determined after acid hydrolysis

and a subsequent colorimetric determination of the glucosamine formed. The degree of solubilization obtained in the chitinase assay was calculated from the amount of organic carbon in the supernatants and the total amount of chitin-carbon initially present.

Chemical analyses. Fermentation products (Pel & Gottschal, 1986), residual chitin (Pel & Gottschal, 1989), NAG (Pel & Gottschal, 1986), NAG-oligomers (Pel *et al.*, 1989) and total organic carbon (TOC) (Pel & Gottschal, 1986) were determined as described previously. Total reducing sugar was analysed by the dinitrosalicylic acid (DNS) method as described by Miller (1959). Standard curves for NAG and chitobiose in the DNS assay indicated that both sugars exhibited a nearly equal colorimetric response expressed per mole of (di)saccharide. Non-sedimentable chitin fragments and dextrans were determined as glucosamine. HCl after acid hydrolysis (6 M-HCl) as described by Chen & Johnson (1983).

Chemicals. Chitin (practical grade), glucosamine, NAG, *N,N'*-diacetylchitobiose (NAG₂), 4-chloromercuribenzoic acid (pCMB), 4-chloromercuriphenylsulphonic acid (pCMPS), iodoacetate (IoAc) and *N*-ethylmaleimide (NEM) were purchased from Sigma. Finely dispersed chitin and NAG-oligomers with degrees of polymerization (DP) up to six sugar residues were prepared as described previously (Pel & Gottschal, 1986). All other chemicals used were reagent grade.

Results

Isolation and growth characteristics of strain AW.D2

All dissected parts of the intestinal tract of the plaice contained approximately 10^9 chitinolytic bacteria per ml gut content, except the one comprising the small intestines, which contained approximately 10^6 bacteria ml^{-1} . Strain AW.D2 was isolated as the predominant chitinolytic bacterium from the intestinal part containing the hindgut. It was a facultatively anaerobic, Gram-negative, rod-shaped ($0.5\text{--}0.8 \times 1.5\text{--}2.5 \mu\text{m}$), non-spore-forming bacterium, motile by one or two polar flagella. Acetate, ethanol and formate were the major fermentation products during growth on chitin and NAG (Table 1). The rate of chitin fermentation was influenced by the intensity of agitation of the cultures. Both continuous stirring and static incubations slowed down the rate of chitinolysis. The highest rates were recorded when chitin particles were merely kept in suspension by incubating the cultures in Hungate tubes positioned horizontally on a rotary shaker run at 80 r.p.m.

Fig. 1. shows the time courses of chitin degradation by strain AW.D2 and *Clostridium* sp. strain 9.1 in mono- and coculture. Sometimes a partial loss of chitinolytic activity was observed when strain AW.D2 was maintained on NAG-agar aerobically. Degradation of chitin by strain AW.D2 occurred aerobically at a similar rate as that recorded anaerobically. The temperature range for anaerobic growth was from 5 to 37°C with an optimum at 30°C . The pH optimum for growth of strain AW.D2 ranged from 7.0 to 8.0; no growth occurred at pH 5.0 and 9.0. Supplementation of the growth medium

Table 1. *Products of chitin and NAG fermentation by strain AW.D2 and of chitin fermentation by Clostridium sp. strain 9.1*

Bacteria were grown in a medium buffered with 30 mM-phosphate (pH 7.2). In the headspace, 100% N_2 replaced the N_2/CO_2 mixture usually applied.

Product	Amount of product*		
	Strain AW.D2		Strain 9.1†
	Chitin	NAG	Chitin
Acetate	157	137	117
Formate	121	171	10
Lactate	12	17	—
Succinate	5	3	—
Ethanol	69	90	81
CO_2	21	31	150
H_2	—	—	106
Carbon recovery	98%	95%	84%
Redox balance O/R	1.06	1.07	1.04

* Expressed as mmol product per 100 mmol NAG-equivalents fermented.

† Data from Pel & Gottschal (1986).

Fig. 1. Time course of chitinolysis in pure cultures of strain AW.D2 (x) and *Clostridium* sp. strain 9.1 (Δ), and in a mixed culture of *Clostridium* sp. strain 9.1 and strain HA 8.1 (○).

with strong reducing agents (e.g. dithionite, 0.2 mM), did not enhance anaerobic chitinolysis as it did for *Clostridium* sp. strain 9.1 (Pel & Gottschal, 1986).

Anaerobic growth of strain AW.D2 was also supported by NAG-oligomers (tested up to DP = 6), glucosamine, glucose, fructose, arabinose, D-ribose, cellobiose, maltose and peptone. No growth occurred with gluconate, glucosaminic acid, xylose, lactose, saccharose, ethanol, propanol, butanol, glycerol, pyruvate, lactate, fumarate, succinate, chitosan, cellulose, starch, inulin, xylan, polygalactoaraban, polygalactomannan, agar, gelatin, Casamino acids or yeast extract.

Fig. 3. Solubilization of chitin and the formation of hydrolysis products by the chitinase enzyme complexes of strain AW.D2 (a, b) and *Clostridium* sp. strain 9.1 (c, d). Δ , Turbidity of the chitin suspension; $\circ$, NAG; $\times$, NAG₂; $\square$, total reducing sugar; $\blacktriangle$, chitin solubilization; $\bullet$, organic carbon in the supernatant and not accounted for by NAG, NAG₂ and rifampicin. A solubilization of 100% corresponded to the formation of 295 and 275 μg non-sedimentable organic carbon per ml supernatant for strain AW.D2 and strain 9.1, respectively.

Scanning electron microscopy of strain AW.D2 revealed a rough outer surface of the cell wall with globular extensions (Fig. 2a). Negatively stained whole cells and thin sections showed the presence of a glycocalyx (Fig. 2b, d) and the formation and release of numerous vesicles (Fig. 2e, f). The formations of these vesicles was observed in all stages of growth, irrespective of whether chitin, NAG₂, NAG or glucose served as the carbon and energy source. Negatively stained whole cells also demonstrated the presence of a regular spiral-shaped structure in the outer cell envelope (Fig. 2c).

Hydrolytic and physico-chemical properties of chitinases of strain AW.D2 and strain 9.1

Turbidimetric assays, combined with TOC and sugar analyses of the hydrolysis products, demonstrated that

the enzyme complexes of strain AW.D2 and strain 9.1 were capable of extensive solubilization of chitin (Fig. 3a–d). The hydrolysis of chitin by the chitinase of strain AW.D2 was almost complete after 45 h (i.e. about 85% solubilization, Fig. 3b), whereas the chitinolytic activity of the enzyme system of strain 9.1 was lower, resulting in approximately 65% solubilization of the substrate in 140 h (Fig. 3d). Both chitinolytic systems produced NAG₂ as the major hydrolysis product, with an additional formation of small amounts of NAG (Fig. 3a, c). Soluble NAG-oligomers with DP > 2 were not found using HPLC analysis.

The amount of total reducing sugars (DNS method) in both assays matched the sum of the individually determined amounts of chitobiose (HPLC analysis) and NAG, indicating the absence of substantial quantities of other sugars. However, a transient accumulation of non-sedimentable organic carbon was always observed in the

Fig. 2. (a) Scanning electron micrograph of a chitin-grown culture of strain AW.D2 in the stationary phase, showing the globular extensions on the outer surface of the cell wall. Bar, 1 μm . The inset shows a detail at higher magnification. Bar, 0.2 μm . (b) Negatively stained whole cell of strain AW.D2 grown on chitin showing glycocalyx (halo surrounding cell body), polar inserted flagellum (single arrow) and spiral-shaped structure in the cell wall (double arrows). Bar, 1 μm . (c) Detail of cell wall area indicated by the two arrows in (b). Bar 0.1 μm . (d) Ultrathin section of a chitin-grown cell showing the presence of a glycocalyx (arrow). Bar, 1 μm . (e) Ultrathin section of chitin-grown cells showing the formation of vesicles. Bar, 1 μm . (f) Formation of vesicles by a glucose-grown cell. Bar, 0.2 μm .

Fig. 4. Thermal stability (a) and pH optima (b) of the chitinase complexes of strain AW.D2 (x) and *Clostridium* sp. strain 9.1 (O). Thermal stability was determined by recording the residual hydrolytic activity following preincubation for 10 min at the indicated temperature. An activity of 100% corresponded to the formation of 300 (a) and 250 (b) μM -NAG₂ in 40 h in the assay of strain 9.1 chitinase, and to 680 (a) and 660 (b) μM -NAG₂ in 26 h for strain AW.D2, under standard assay conditions.

Table 2. Effect of thiol and reducing agents on the activity of the chitinase complexes of strain AW.D2 and *Clostridium* sp. strain 9.1

Reagent	Concn (μM)	Residual activity (%)		
		Strain AW.D2*		Strain 9.1†
		26 h	20 h	50 h
pCMB	200	103	0	0
pCMPS	200	104	0	12
IoAc	200	101	113	109
NEM	800	102	47	32
Fe(CN) ₆	300	101	59	29
H ₂ O ₂	200	102	88	76
O ₂	240	94	80	53
Dithionite	1000	97	ND	110
Dithiothreitol	2000	103	ND	113

ND, Not determined.

* 100% activity corresponds to the formation of 710 μM -chitobiose in 26 h.

† 100% activity corresponds to the formation of 115 and 230 μM -chitobiose in 20 and 50 h, respectively.

course of the enzyme assays (Fig. 3b, d). This surplus amounted to approximately 60 $\mu\text{g C ml}^{-1}$ in the enzyme assays; after acid hydrolysis it was found to consist of at least 50% glucosamine-positive material.

There was no detectable hydrolytic activity in the cell-free culture fluid of strain 9.1 and strain AW.D2 during the active growth phase. Release of soluble chitinase

activity did occur when the cultures entered the stationary phase, but the activities were always lower than the sedimentable activity observed in the early growth phase, especially in the case of strain AW.D2. HPLC analyses indicated that for the cell-free enzyme activity NAG₂ was again the major hydrolysis product (>90%).

The difference in thermal stability of the enzyme complexes of strain AW.D2 and strain 9.1 is shown in Fig. 4(a). Pretreatment for 10 min at 50 °C and lower did not affect the chitinolytic activity of strain AW.D2, whereas 10 min at 45 °C resulted in a large drop in the chitinolytic activity of strain 9.1. The activity of both chitinolytic systems displayed a broad pH range, with a slightly more acidic optimum for strain 9.1 (Fig. 4b).

Thiol reagents and oxygen did not significantly affect the hydrolytic activity of the enzyme complex of strain AW.D2 (Table 2). However, all thiol reagents tested, except IoAc, strongly inhibited the chitinase of strain 9.1 (Table 2), as indicated previously (Pel & Gottschal, 1987).

Regulation of chitinase synthesis

Strain AW.D2 exhibited a diauxic growth pattern when grown in a medium containing both chitobiose and chitin. Chitobiose was always consumed first. This was independent of whether the inoculum was pre-grown with chitin, chitobiose or NAG as the carbon and energy source. Only after depletion of chitobiose, and lag periods of 24 to 48 h, was chitinase activity observed, but at a level five times lower than in cultures with chitin as the sole carbon and energy source (see Fig. 1). Upon transfer of cells from these cultures into fresh chitin-containing medium (without NAG₂) the usual high rate of chitinolysis was observed. Scanning electron micrographs of the retarded cultures showed that chitin fibres were embedded in a mucous material. No chitinolytic activity was detected in supernatants or associated with cells of cultures of strain AW.D2 grown on glucose, glucosamine, NAG, NAG₂, NAG₃ or NAG₅ (initial concn approx. 1 mg ml⁻¹).

When strain 9.1 was grown in a medium containing both chitin and chitobiose the pattern of substrate utilization was quite different from that observed with strain AW.D2 (Fig. 5a): both substrates were consumed simultaneously, though the initiation of chitin hydrolysis preceded the utilization of NAG₂. Lowering the initial amount of chitin from 0.5 to 0.1 mg ml⁻¹ did not change this pattern. The presence of thioredoxin (Pel & Gottschal, 1989) stimulated chitin hydrolysis, and markedly enhanced the consumption of NAG₂ (Fig 5b). The addition of NAG (0.5 mM) or NAG-oligomers (DP 2–6, 0.5 mM each) to chitin-grown cultures of strain 9.1 in

Fig. 5. Time course of the fermentation by *Clostridium* sp. strain 9.1 of chitin and NAG₂ in the absence (a) and the presence (b) of thioredoxin (0.5 µg protein per ml culture fluid). ×, Chitin; △, NAG₂; ○, acetate.

the early growth phase (i.e. when 10% of the initial amount of chitin had been fermented) did not cause detectable changes in the rate of chitinolysis or fermentation (data not shown). NAG-oligomers with DP ≥ 4 disappeared from the culture fluid within 6 h of their addition, with a concomitant transient accumulation of only NAG₂ (up to 1 mM) in the case of NAG₄ and NAG₆, and of NAG₂ plus NAG₃ (about 0.5 mM each) in the case of NAG₅. The hydrolysis of NAG₃ took almost 2 d.

By increasing the amount of yeast extract in dithionite-supplemented cultures of strain 9.1 (Pel & Gottschal, 1986, 1987) the rate of fermentation could be increased (Fig. 6b) without proportionally enhancing the rate of chitin hydrolysis (Fig. 6a). As a result, decreasing amounts of NAG₂ accumulated transiently in the culture fluid when the concentration of yeast extract was raised from 0.005 to 0.04% (w/v) (Fig. 6c). If dithionite was omitted the rate of chitinolysis decreased considerably (Fig. 6a).

Competition for chitin in mixed cultures

Unlike the growth of *Clostridium* sp. strain 9.1 (Pel *et al.*, 1989; Pel & Gottschal, 1989), growth of strain AW.D2 was not significantly affected by the presence of NAG (-oligomer)-fermenting bacteria such as strain HA 8.1, strain GH 8.2, *E. coli* and *K. pneumoniae*. Moreover, only very low numbers of saccharolytic bacteria could be sustained in cultures of strain AW.D2.

Fig. 6. Combined effects of dithionite and yeast extract concentration on chitin fermentation by *Clostridium* sp. strain 9.1. ○, △, ×, Addition of 0.005% (○), 0.02% (△) or 0.04% (×) yeast extract plus 0.2 mM-dithionite; □, addition of 0.005% yeast extract, dithionite omitted. (a) Solubilization of chitin recorded as the decrease in OD₆₆₀ of the culture; (b) acetate formation; (c) NAG₂ accumulation.

When strain AW.D2 and *Clostridium* sp. strain 9.1 were cocultured with chitin as the sole carbon and energy source, co-existence was not established. The latter organism was always completely outcompeted after one or two transfers (inoculum 1%, v/v) in fresh medium. A similar result was obtained when the pH of the culture medium was lowered to a more favourable value for strain 9.1 (i.e. 6.0) or when the inoculum size of strain AW.D2 was reduced 100-fold. However, the introduction of a third organism, strain HA 8.1, improved the competitiveness of strain 9.1 dramatically. Thus, in the presence of strain HA 8.1, strain 9.1 outcompeted strain AW.D2 after two transfers in batch culture. This was indicated by the decrease in numbers of strain AW.D2 cells (Table 3). Further support for a transition from the three-membered culture to a two-membered strain 9.1 plus strain HA 8.1 culture comes from the increasing concentration of propionate in the stationary phase, as propionate is a characteristic fermentation product of strain HA 8.1, but is formed neither by strain 9.1 nor by strain AW.D2 (Table 3).

Table 3. Competition for chitin in batch and phauxostat cultures between strain AW.D2 and *Clostridium* sp. strain 9.1

	Products and number of cells detected in stationary phase (batch*) or steady state (phauxostat†):						
	Batch cultures					Phauxostat cultures	
	Pure culture of AW.D2	Coculture: 9.1 + HA 8.1	Mixed culture: 9.1 + HA 8.1 + AW.D2			Mixed culture: 9.1 + HA 8.1 + AW.D2	
			No. of batch transfers:			$D = 0.04 \text{ h}^{-1}$	$D = 0.08 \text{ h}^{-1}$
			0	1	2		
Acetate (mM)	7.2	5.8	6.6	6.2	5.9	2.4	2.7
Propionate (mM)	—	1.2	0.6	0.9	1.0	1.0	1.0
Ethanol (mM)	3.2	1.3	ND	ND	ND	ND	ND
$10^{-6} \times \text{AW.D2 cells ml}^{-1} \ddagger$	230 ± 20	—	90 ± 20	44 ± 20	0.3 ± 0.61	1 ± 0.8	2 ± 0.5
$10^{-6} \times \text{HA 8.1 cells ml}^{-1} \ddagger$	—	750 ± 210	ND	ND	ND	250 ± 7	280 ± 58

ND, Not determined; —, not detected.

* The initial amount of chitin in batch culture experiments was 1 mg ml^{-1} .

† Steady-state chitin concentrations in the culture were 0.19 and 0.24 mg ml^{-1} at $D = 0.04$ and 0.08 h^{-1} , respectively. The amount of chitin solubilized in the phauxostat was approximately 0.4 mg ml^{-1} .

‡ Mean of two or three determinations $\pm$ standard deviation.

The competitiveness of the two chitinolytic species was also tested under chitin limitation in a phauxostat at dilution rates of 0.04 and 0.08 h^{-1} , and steady-state chitin concentrations of 0.19 and 0.24 mg ml^{-1} , respectively. Strain 9.1 and strain HA 8.1 were cocultured until a steady state was reached, prior to the inoculation of strain AW.D2. Strain AW.D2 was not completely washed out but remained present with a constant but very small number of cells (Table 3). The number of cells ($1.2 \times 10^6 \text{ ml}^{-1}$) represented the consumption of approximately 2% of the chitin fermented, based on the results obtained with pure cultures of strain AW.D2.

Discussion

Aerobic plate counts indicated that 10^7 – 10^9 chitinolytic bacteria ml^{-1} occur in the digestive tract of marine fish consuming chitin-containing food (Seki & Taga, 1963; Goodrich & Morita, 1977; Danulat, 1986). Our anaerobic isolation and enumeration procedure suggests that in spite of the anoxic conditions in the intestinal tract a facultatively anaerobic bacterium predominated over strictly anaerobic chitinolytic species in the hindgut of the plaice, *Pleuronectes platessa*.

The pH optimum for growth and chitin hydrolysis of strain AW.D2 was in the range 7.0 – 8.0 , reflecting its adaptation to the prevailing pH values occurring in the intestinal tract of fish [cod, 7 – 8 (Danutat, 1986); plaice, 7.4 – 8.4 (Barrington, 1957)]. The formation of vesicles and blebs during growth on chitin and various sugars could be the result of a continuous synthesis of a glycocalyx-like coat surrounding the cell envelope of

strain AW.D2. The presence of a glycocalyx has been implicated in protection against anti-bacterial agents such as surfactants (e.g. bile salts: Costerton *et al.*, 1981).

The importance of the degradation of chitin in the intestines of marine fish has been questioned because of an apparently low *in situ* chitinolytic activity of the gut micro-organisms (Danutat, 1986; Seki & Taga, 1963). However, considering the high numbers of chitinolytic bacteria in the intestinal tract of *P. platessa*, the strong hydrolytic activity of strain AW.D2 and the residence time of the food in the digestive tract of plaice (12 – 24 h : Fänge & Grove, 1979), extensive degradation of chitinous material passing through the intestines has to be expected. The low *in situ* chitinolytic activities detected in cod by Danulat (1986) were probably severe underestimates, since the activity associated with solid particles in the intestines was not accounted for in the assays.

The extracellular chitinolytic enzyme systems of strain AW.D2 and *Clostridium* sp. strain 9.1 both release NAG_2 as the major hydrolysis product from chitin. The actual uptake of NAG_2 by strain 9.1 is implicated by the organism's very poor growth on NAG and related monosaccharides (Pel & Gottschal, 1986). The absence of an extracellular chitinase activity in chitin-grown cultures of strain AW.D2 also suggests the presence of a transport system for the dimer in this organism. The formation and uptake of disaccharides is a fairly common feature of strictly anaerobic polysaccharide-degrading species (Saddler & Khan, 1979; Hiltner & Dehority, 1983; Patel *et al.*, 1980). This may be due to the more favourable energetics of dimer transport compared with that of monomers via PTS systems, and the ATP gain obtained by the action of intracellular disaccharide-

phosphorylases (Martin & Russell, 1987). However, the uptake of NAG₂ has never been demonstrated, nor suggested, for chitin-degrading (facultatively) aerobic species such as *Serratia marcescens* (Monreal & Reese, 1969), *Beneckea neptuna* and *Beneckea nereida* (gut endosymbionts in the white shrimp) (Hood & Meyers, 1977) and *Streptomyces griseus* (Berger & Reynolds, 1958).

Analyses of the products formed during the solubilization of chitin by the enzyme complexes of strain AW.D2 and strain 9.1 revealed the transient accumulation of a hydrolysis product(s), which could not be identified by HPLC. This intermediate product consisted of glucosamine-containing material but was not pelleted by centrifugation at 8500 g for 5 min. The formation of similar fractions has also been found during the degradation of cellulose by *Clostridium thermocellum* (Weimer & Chou, 1986) and *Trichoderma reesei* (Enari & Niku-Paavola, 1987). Studies on the enzymic degradation of insoluble polysaccharides usually concentrate on residual (= sedimentable) substrate and truly soluble hydrolysis products. So far, little attention has been paid to products of intermediate (colloidal) character. However, a more detailed knowledge of the formation, composition and properties of such intermediate products seems essential for the complete elucidation of the hydrolytic mechanism involved in the degradation of crystalline polysaccharides (Enari & Niku-Paavola, 1987).

The enzyme complexes of strain 9.1 and strain AW.D2 show much resemblance with respect to some general properties (formation of NAG₂ and absence of extracellular chitinase) but differ in temperature stability, pH optima and susceptibility to thiol reagents. The insensitivity of the chitinolytic enzyme system of strain AW.D2 to thiol reagents and strong reductants indicates the absence of accessible and essential thiol or disulphide groups. These results are in agreement with the observation that normally cyst(e)ine is not present in extracellular proteins produced by facultatively anaerobic bacteria (Fahey *et al.*, 1977).

Chitinase is not a constitutive enzyme in strain AW.D2, as indicated by the growth pattern in media containing both chitin and chitobiose, and because no chitinolytic activity was detected in the culture fluid or associated with the cells when the bacterium was grown on NAG-oligomers (DP ≥ 2). It is not clear whether NAG-oligomers do not induce chitinase synthesis, or carbon and energy limitation is required for its derepression or induction. In contrast to strain AW.D2, no repression of chitinase synthesis by chitobiose occurs in strain 9.1. This has also been reported for other specialized polysaccharide-degrading anaerobes only capable of utilizing the polymer or oligomers with

DP ≥ 2 (Hiltner & Dehority, 1983; Garcia-Martinez *et al.*, 1980). Interestingly, the initiation of chitinolysis in cultures of strain 9.1 occurs 2–4 d prior to the uptake of NAG₂ when chitin and the disaccharide are both present in the culture medium. This phenomenon, perhaps indicating the organisms' initial preference for NAG₂ derived directly from chitin, warrants a more detailed examination of sugar transport across the cell membrane in this bacterium, especially since polymer hydrolysis is generally considered the rate-limiting step in the growth of polysaccharide-degrading bacteria.

In a previous paper, chitinolysis by *Clostridium* sp. strain 9.1 was shown to be relatively unaffected by the consumption of 10–70% of the total hydrolysis products by secondary populations (Pel *et al.*, 1989). It is remarkable that in pure cultures with a greatly decreased rate of fermentation due to low yeast extract concentrations (Fig. 6b), the rate of chitin hydrolysis also remains unaffected if the reducing agent dithionite is present (Fig. 6). In such cultures a considerable transient accumulation of NAG₂ occurred, up to 50% of the quantity of chitin hydrolysed. It is tempting to speculate that in nature the secondary population(s) sustained by strain 9.1 participate directly in maintaining a high chitinolytic activity by releasing stimulatory growth factors (e.g. the redox-active enzyme thioredoxin: see Pel & Gottschal, 1989; Pel *et al.*, 1989), and at the same time increase the competitiveness of strain 9.1 by consuming the hydrolysis products formed by other competing chitin-degrading species. The constitutive nature of the chitinolytic activity of strain 9.1, together with a substantial consumption of the hydrolysis products by strain HA 8.1, is likely to be one of the reasons why the *Clostridium* species outcompeted strain AW.D2. Further, it is of importance that in mixed cultures of strain 9.1 plus strain HA 8.1 plus *Clostridium acetobutylicum* and of strain 9.1 plus *C. acetobutylicum*, *C. acetobutylicum* reached similar final cell densities (unpublished data). This indicates that in both mixed cultures NAG was available equally well for this organism (although *C. acetobutylicum* is only capable of using the chitin monomer, it succeeds in consuming 13–16% of the hydrolysis products: see Pel *et al.*, 1989). This suggests that the strain HA 8.1 does not compete very effectively for the monomer, thus leaving NAG accessible to other organisms, such as strain AW.D2 in the above triculture on chitin. As a result the chitinolytic enzyme system of strain AW.D2 is probably subject to (partial) repression. The combination of these factors represents the first documented example for polysaccharide-degrading cultures of a cooperation between a primary and a secondary species resulting in an increase in the competitiveness of both (Fig. 7).

The question has been raised (Pel & Gottschal, 1986)

Fig. 7. Hypothetical representation of the flow of chitin hydrolysis products and the species interactions in a triculture consisting of the two chitinolytic bacteria, *Clostridium* sp. strain 9.1 and strain AW.D2, and the non-chitinolytic strain HA 8.1. $\Rightarrow$, Formation of hydrolysis products; $\blacktriangleright$, consumption of hydrolysis products. Thickness of the lines represents the quantitative importance of the flow of sugars [see also Pel *et al.* (1989) for sugar consumption in two-membered cocultures]; $\cdots$ —, regulatory interaction; (+), stimulatory (growth) factors (see Pel *et al.*, 1989); (—), repression of chitinase synthesis.

why in anaerobic enrichments on chitin from sediment samples (made on four separate occasions) a rapidly chitin-degrading species like strain AW.D2 never became the dominant organism, in spite of its continuous 'inoculation' of the sediment surface via the faeces of flatfish such as plaice. Apparently the inferior competitiveness of the more versatile chitinolytic species from the fish-gut under conditions of continuous growth on chitin precludes its widespread occurrence in the sediment. This more opportunistic type of organism, with an inducible/repressible chitinolytic system, is clearly far better adapted to the strongly fluctuating food conditions encountered in animal intestines.

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