Presence of Choline in Teichoic acid of *Clostridium acetobutylicum* N1-4 and Choline Inhibition of Autolytic Functions

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Addition of choline to growing *Clostridium acetobutylicum* led to abnormal cell septation, lack of cell separation and the consequent formation of chains. Similar results were obtained with the wild-type strain N1-4 and its autolysin-deficient mutant N1-4081. With strain N1-4, addition of choline at 1 to 2 mg ml⁻¹ resulted in inhibition of autolysis assessed as autoplast formation in 0.6 M-sucrose, lysis by 0.3 M-NaCl/0.03 M-sodium citrate, lysis by 0.1% Triton X-100 and lysis by penicillin G. *In vitro* data confirmed the inhibition by choline of wall-degrading activity, using N1-4 cell walls as substrate. Choline was shown to be a component of the teichoic acid of *C. acetobutylicum* N1-4.

INTRODUCTION

Recently, there has been renewed interest in butyl-acetone producing clostridia, since they may soon be used as biocatalysts in transformations for fuels and chemical feedstock. Before developing a programme of genetic engineering we need first to improve our basic knowledge of the physiology of these bacteria, and second to develop efficient gene-transfer systems which can be achieved by PEG-mediated protoplast transformation. It has already been described for *Bacillus subtilis* (De Castro-Costa & Landman, 1977) that difficulties in protoplast regeneration are due to the production of an autolysin. We succeeded in protoplast regeneration in *Clostridium acetobutylicum* N1-4, which produces an *N*-acetylmuramidase as lytic enzyme (Yoshino *et al.*, 1982), both by using an autolytic deficient mutant N1-4081, and by adding to the regeneration medium sodium polyanethole sulphonate (Reysset *et al.*, 1987) or choline (unpublished results), which is shown here to inhibit the autolytic activity.

We report here both *in vivo* and *in vitro* evidence for inhibition by choline of autolysin in *C. acetobutylicum* N1-4, together with evidence that choline is a component of the teichoic acid of this bacterium.

METHODS

Organisms. *Clostridium acetobutylicum* N1-4 (Hongo, 1960) and the autolysin-deficient mutant N1-4081 were used throughout. Mutant N1-4081 was obtained from N1-4080, a N1-4 mutant auxotrophic for proline (Reysset *et al.*, 1987).

Media and growth conditions. Organisms were routinely grown at 35 °C in TYA medium (Ogata & Hongo, 1973). The pH was adjusted to 6.5 with NaOH, and glucose was added to a final concentration of 1% (w/v). Choline was added from a stock solution (500 mg ml⁻¹) that was filter-sterilized (0.45 µm pore size, Millipore). All manipulations and culture incubations were done under conditions of strict anaerobiosis in an anaerobic glove box (Celster Isotechnic) with a controlled atmosphere: N₂/H₂/CO₂ (90:5:5, by vol.). Lysis and growth of bacterial cells were followed by measuring the optical density at 570 nm with a photoelectric colorimeter (Chemtrix, type 24).

Induction of autoplast formation by sucrose. N1-4 cells were grown in TYA until the culture reached an OD₅₇₀ of about 0.6 and then adjusted with pre-weighed solid-sterilized sucrose to 0.6 M, followed by incubation at 35 °C for 180 min. The percentage of autoplasts was determined in a Petroff-Hausser chamber.

Induction of bacterial lysis by NaCl/sodium citrate. Cells were grown in 200 ml TYA and, at various times, 10 ml samples of culture broth were withdrawn and 1 ml 3 M-NaCl/0.3 M-sodium citrate (pH unadjusted) was added (Ogata & Hongo, 1973) together with different concentrations of choline (0 to 2 mg ml⁻¹). Cultures were incubated at 35 °C and OD₅₇₀ readings were made at 10 min intervals. The final percentage lysis was estimated from the change in OD₅₇₀ 100 min after addition of the lytic agent.

Lysis by Triton X-100. Various concentrations of choline (0 to 10 mg ml⁻¹) were added to 10 ml samples of culture in mid-exponential phase (OD₅₇₀ 0.5) before addition of 0.1% (w/v) Triton X-100. Lysis was followed as described above.

Penicillin treatment. Cells were grown in TYA medium to an OD₅₇₀ of about 0.6 and penicillin (Specillin G, Specia, Rhône Poulenc) was added at a concentration of 100 µg ml⁻¹ together with various concentrations of choline (0 to 2 mg ml⁻¹). Growth and lysis of bacterial cells at 35 °C were followed as above.

Cell wall preparation. This was done as described by Ogata *et al.* (1974).

Preparation of teichoic acid. A cell wall preparation (10 ml) at an OD₄₅₀ of 2 in 0.15 M-NaCl/0.01 M-K₂HPO₄, pH 8.0 was treated with 10 µg DNAase I (Sigma) ml⁻¹ and 50 µg RNAase A (Sigma) ml⁻¹ at 37 °C for 4 h. The pellet was resuspended in 3% (w/v) NaCl and proteins were removed by three treatments with an equal volume of chloroform/isoamyl alcohol (3:1, v/v). After centrifugation at 14000 g for 30 min, the walls were freeze-dried and trichloroacetic acid was used to extract the teichoic acid according to Archibald (1971) with the following modifications: two extractions were made in 5% (w/v) TCA at 100 °C for 1 h, and a third extraction in 10% TCA at 4 °C. The combined acid extracts were precipitated by addition of 5 vols cold ethanol. The crude teichoic acid precipitate was finally washed in cold absolute ethanol.

Detection of choline in teichoic acid. Crude teichoic acid was hydrolysed in a sealed tube with 6 M-HCl at 100 °C for 18 h. The hydrolysate was dried by rotary evaporation under vacuum; residual HCl was removed by 10 successive additions of a small volume of water and evaporating to dryness each time. Ethanol (30 µl) was added to dissolve the hydrolysate which was analysed for the presence of choline by thin-layer chromatography on cellulose sheets (Schleicher & Schüll, F1440) as described by Augustinsson & Grahn (1953). The chromatogram was stained according to Chargaff *et al.*, (1948). The standards run were purchased from Sigma (see below).

Affinity chromatography on choline-Sepharose. Choline-Sepharose was prepared from epoxy-activated Sepharose 6B (Pharmacia). The general conditions were those of Briesse & Hakenbeck (1985), except that the elution buffer was 67 mM-potassium phosphate, pH 6.0, without detergent.

In vitro assay of lytic activity. Lytic activity was measured with cell walls of strain N1-4. The cell wall preparation was pre-incubated for 10 min with choline before adding different lytic enzymes. One unit of lytic activity (1 UA) was defined as a decrease in OD₄₅₀ of 0.001 min⁻¹ (spectrophotometer: DU7, Beckman). Activities were assayed at 35 °C except for lysozyme, which was assayed at room temperature. Preliminary experiments were made to define the cell wall concentration corresponding to a non-limiting concentration of substrate for lytic activity up to 10 UA ml⁻¹. The reaction mixture to assay supernatant activity from stationary phase cells contained: 50 µl of N1-4 cell walls at an OD₄₅₀ of 2; 350 µl 0.1 M-sodium acetate, pH 4.5, containing different concentrations of choline; 60 µl 1 M-sodium acetate, pH 4.5, and 540 µl of stationary phase culture filtrate. The assays with enzymes from a commercial source – lysozyme (EC 3.2.1.17) (Sigma; 59.2 units mg⁻¹) and mutanolysin (Sigma; 303 units mg⁻¹) – were done with lytic activities similar to the N1-4 supernatant activity. The reaction mixture contained: 50 µl of N1-4 cell walls or *Micrococcus lysodeikticus* whole cells (Difco) at an OD₄₅₀ of 2; 850 µl TYA medium containing different concentrations of choline, and 100 µl TYA medium containing the commercial enzyme preparation.

Electron microscopy. Cells were harvested by centrifugation during the exponential growth phase, fixed overnight in 1% (w/v) OsO₄ in 0.25 M-sodium cacodylate, pH 7.2, washed repeatedly with the same buffer, dehydrated in a graded acetone series, and embedded in Epon 812. Sections were cut with an LKB Ultratome Nova using a diamond knife, mounted on copper grids, stained in 2% (w/v) uranyl acetate and 1% (w/v) lead citrate and observed in a Philips CM12 electron microscope.

RESULTS

Cell morphology

In TYA medium, exponentially growing N1-4 cells consisted of short rods 3–6 µm in length (Fig. 1a) with typical septa (Fig. 1c). Addition of choline at concentrations higher than 1 mg ml⁻¹ led to formation of long chains of more than 10 cells. Fig. 1(b) illustrates the cell morphology with 10 mg ml⁻¹ choline. Electron microscopy showed abnormal septa that were thickened with accumulation of cell wall material (Fig. 1d). This abnormal septation did not lead to dissociation of daughter cells. Such chains of cells obtained with 10 mg choline ml⁻¹ sheared when they were centrifuged and suspended in a crude supernatant of strain N1-4 (autolytic titre 4.6 UA ml⁻¹) or in TYA containing 1 µg lysozyme ml⁻¹. In TYA medium, strain

Fig. 1. Effect of choline on *C. acetobutylicum* cell morphology. Late exponential phase N1-4 cells grown in the absence of choline (a) and in the presence of 10 mg choline ml⁻¹ (b). (c, d) Detail of the septa of strain N1-4 in the absence (c) and presence (d) of choline. (e, f) Detail of the septa of strain N1-4081 in the absence (e) and presence (f) of choline. Scale bars are 10 µm (a, b) and 0.1 µm (c, d, e and f).

N1-4081 grew as short rods with normal septa (Fig. 1e) presumably due to residual autolytic activity (Reysset *et al.*, 1987). In the same medium with 10 mg choline ml⁻¹, long chains of cells with abnormal septa were formed (Fig. 1f). Moreover, resuspension of these chains in TYA containing lysozyme or N1-4 crude filtrate of equivalent autolytic activity led to cell separation.

Autoplast formation by 0.6 M-sucrose

Maximum sucrose-induced autoplast formation was obtained with cells from the exponential phase (OD₅₇₀ 0.5) (Reysset *et al.*, 1987). Under these conditions, we observed an inhibition of autoplast formation by the addition of choline; complete inhibition was observed at 1.5 mg choline ml⁻¹ (Fig. 2).

Fig. 2. Inhibition by various concentrations of choline of N1-4 autolysis effects. Autoplast formation in 0.6 M-sucrose (○), lysis by 0.3 M-NaCl/0.03 M-sodium citrate (●), and lysis by 0.1% Triton X-100 (▲) are shown. Autoplast formation and lysis were basically assayed as described in Methods. The extent of lysis was taken to be 100% after 100 min incubation with Triton X-100 or NaCl/sodium citrate in the absence of choline.

Fig. 3. Autolytic effect of NaCl/sodium citrate on (a) the wild-type strain N1-4 and (b) the autolytic deficient mutant N1-4081. Cells were grown in TYA medium (●). At various times during growth (indicated by arrows), samples were added to 0.3 M-NaCl/0.03 M-sodium citrate, after which the OD₅₇₀ was followed for 120 min (○).

With strain N1-4081, autoplasts did not appear in 0.6 M-sucrose and we were therefore unable to test the effect of choline. However, choline inhibited protoplast formation by lysozyme (100 µg ml⁻¹) and penicillin (20 µg ml⁻¹) (results not shown).

NaCl/sodium citrate and Triton X-100 induced lysis

Ogata & Hongo (1973) showed that strain N1-4 lysed in the presence of univalent cations, with rapid lysis in NaCl at 0.3 to 0.5 M; moreover, the rate of lysis was increased by sodium citrate. Under our experimental conditions, 0.3 M-NaCl/0.03 M-sodium citrate had a maximum lytic effect at the end of the exponential growth of strain N1-4 (Fig. 3a) with 83% lysis in 100 min.

Preliminary experiments on strain N1-4 showed optimal lysis with cells in the exponential phase, with 60% lysis after 100 min in the presence of 0.1% Triton X-100. The effect of various

Fig. 4. Inhibition by choline of penicillin-induced lysis. At zero-time, mid-exponential phase cultures were treated with penicillin ($100 \mu\text{g ml}^{-1}$) and a range of concentrations of choline. No addition (●); addition of penicillin alone (■); addition of penicillin with choline at 0.5 mg ml^{-1} (□), 0.75 mg ml^{-1} (○), 1 mg ml^{-1} (△) or 2 mg ml^{-1} (▽).

concentrations of choline on lysis by $0.3 \text{ M-NaCl}/0.03 \text{ M-sodium citrate}$ and by 0.1% Triton X-100 is shown in Fig. 2. In both cases complete inhibition of lysis was observed at $2 \text{ mg choline ml}^{-1}$.

No lysis was evident with the autolysin-deficient mutant N1-4081 under similar conditions; this is illustrated by the lack of lysis in the presence of $0.3 \text{ M-NaCl}/0.03 \text{ M-sodium citrate}$ (Fig. 3b).

Lysis by penicillin G

Autolytic enzymes should be involved in the bacteriolytic effect of β -lactam antibiotics (Rogers *et al.*, 1980), as directly demonstrated in *Streptococcus pneumoniae* (Ronda *et al.*, 1987). Strain N1-4 was shown to be sensitive to penicillin G at $100 \mu\text{g ml}^{-1}$ (Ogata *et al.*, 1980). When penicillin was added at this concentration to exponential cells of strain N1-4, lysis started 3.5 h after penicillin addition (Fig. 4). Addition of various concentrations of choline at the same time as penicillin (Fig. 4), or 15 min before (data not shown), resulted in inhibition of lysis, except at the lowest concentration of choline tested (0.5 mg ml^{-1}). No significant lysis was observed in strain N1-4081 at the same concentration of penicillin.

In vitro N1-4 autolytic activity

A cell wall preparation from strain N1-4 was used as the substrate for an *in vitro* wall degradation assay (see Methods). Crude supernatant from a 24 h culture of strain N1-4 showed 4.6 UA ml^{-1} in the assay, and this activity was strongly inhibited by addition of choline (Fig. 5). When commercially supplied mutanolysin and lysozyme were tested in the same assay, inhibition by choline required much higher concentrations. Degradation of *M. lysodeikticus* cells and the N1-4 wall preparation by commercial lysozyme were inhibited by choline to about the same extent (Fig. 5).

Fig. 5. Inhibition of different wall-degrading activities by choline. Activity ($\Delta OD_{450} \text{ min}^{-1}$, expressed as a percentage of the activity measured in the absence of choline) was measured with N1-4 supernatant (■), mutanolysin (□) or lysozyme (○). An N1-4 cell wall preparation was used as substrate. Lysozyme was also tested with *M. lysodeikticus* cells as substrate (●).

Presence of choline in the teichoic acid

By using the technique described in Methods, a blue spot with the same R_F value (0.38) as found for a choline standard was detected in the crude teichoic acid hydrolysate after separation on a thin-layer chromatogram. Various choline derivatives were similarly stained by the colour reagent, but they had different R_F values, viz. choline dichloride (0.49), carbamylcholine chloride (0.32), phosphatidylcholine (0.87) and histamine (0.16).

Affinity chromatography on choline–Sephacrose

A stationary phase culture of strain N1-4 was centrifuged and the supernatant was salted out with $(\text{NH}_4)_2\text{SO}_4$ at 60% saturation. The precipitate was dissolved in 67 mM-potassium phosphate, pH 6.0 and extensively dialysed against the same buffer. After filtration (0.45 μm pore size, Millipore) and concentration by filtration on PM10 membrane (Amicon), the crude autolysin preparation contained 220 mg protein ml^{-1} and 5200 UA autolysin ml^{-1} . The preparation (8 ml) was applied to a choline–Sephacrose column. Most of the enzyme activity was retained on the column during elution by 50 ml 67 mM-phosphate buffer, pH 6.0, followed by 95 ml 0.5 M-NaCl in the same buffer. Autolysin was eluted by 15 ml 2% (w/v) choline in the same buffer. Of the autolytic activity 84.6% was recovered from the eluate after extensive dialysis in 67 mM-phosphate buffer, pH 6.0.

DISCUSSION

In growing cells of *C. acetobutylicum* N1-4, choline inhibited cell separation, lysis by a non-ionic surfactant (Triton X-100) and lysis by penicillin, all of which are related to autolytic activity in bacteria (Cornett & Shockman, 1978; Guidicelli & Tomasz, 1984; Rogers *et al.*, 1980; Ronda *et al.*, 1987). Moreover, choline had a pronounced effect on properties that are known to depend on endogenous autolysin production. Choline prevented sucrose-induced autoplast formation (Ogata *et al.*, 1975), and cellular lysis induced by NaCl/sodium citrate (Ogata & Hongo, 1973). The autolysin-deficient mutant N1-4081 (Reysset *et al.*, 1987) was shown to have properties expected from an autolysin-deficient mutant.

The addition of high concentrations (1–2 mg ml^{-1}) of choline to *C. acetobutylicum* N1-4 had *in vivo* effects basically similar to those described in *S. pneumoniae* (Briese & Hakenbeck, 1985; Guidicelli & Tomasz, 1984).

The concentration of 1 $\text{mg choline ml}^{-1}$ inhibiting the *in vitro* extracellular *N*-acetylmuramidase of strain N1-4 is comparable to the concentration of 2.5 mg ml^{-1} required for 50% inhibition of lysis of *N*-acetylmuramyl-L-alanine amidase from *S. pneumoniae* (Briese & Hakenbeck, 1984; Howard & Gooder, 1974). When using N1-4 cell walls and lysozyme or

mutanolysin, or *M. lysodeikticus* cells and lysozyme, choline concentrations 20 times higher than for the muramidase from *C. acetobutylicum* are required for complete enzyme inhibition. Such high concentrations can hardly be considered to act specifically.

We have shown choline to be a component of the N1-4 teichoic acid. *S. pneumoniae* was previously the only bacterium known to have a choline-containing teichoic acid (Brundish & Baddiley, 1968; Mosser & Tomasz, 1970) and lytic enzymes inhibited by exogenous choline (Höltje & Tomasz, 1974; Sanchez-Puelles *et al.*, 1986). The elution of N1-4 autolysin adsorbed to a choline-Sepharose column by a high choline concentration shows the affinity of the N1-4 autolysin for choline. As in *S. pneumoniae* amidase (Höltje & Tomasz, 1974), choline residues in teichoic acid should be ligands for *C. acetobutylicum* autolysin.

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