Morphological and Chemical Characteristics of a *Cytophaga* sp. Grown under Conditions of Magnesium Excess and Magnesium Limitation

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Cytophaga sp. NCMB 1314 produces, especially under Mg^{2+} -limited growth conditions, a factor that liberates a cholinesterase from plaice muscle. Just after the onset of Mg^{2+} limitation in a batch culture, there is a period of rapid production of the factor. Changes also occur in the morphology, ATP content and gross chemical composition of the bacteria. The protein and carbohydrate contents are raised by 60 % and 100 %, respectively, while the RNA and ATP contents are reduced to 70 % and 40 % of the original values. Increased amounts of carbohydrate found outside the cells after Mg^{2+} limitation at least partly correlated with extracellular slime observed in electron micrographs.

INTRODUCTION

A bacterium which can liberate structurally bound cholinesterase from plaice muscle was isolated by Lundin & Bovallius (1966). The cholinesterase-solubilizing agent, called the S-factor, could be detected in sonicated bacteria and was found to have enzyme-like characteristics (Lundin, 1968). In a simple medium, the S-factor could be detected in the culture supernatant (Bovallius, 1969). Its activity depended on the Mg^{2+} concentration in the medium; shortly after the onset of Mg^{2+} starvation during growth in a Mg^{2+} -limited medium, a sudden increase in S-factor activity was demonstrated (Bovallius, 1978). The bacterium was classified at Torry Research Station, Aberdeen, Scotland, as a probable *Cytophaga* sp. or a *Flavobacterium* sp. and was catalogued as *Cytophaga* sp. NCMB 1314 (T. G. Mitchell, personal communication).

In the present work, the changes in gross chemical composition, nucleotide pool and ultrastructure of the *Cytophaga* sp. accompanying the change from Mg^{2+} -excess to Mg^{2+} -limited growth were studied in relation to the observed rise in S-factor activity.

METHODS

Cultivation. *Cytophaga* sp. NCMB 1314 was maintained on nutrient agar slopes. Small flasks with Tryptone T (Oxoid; 5 g l⁻¹) were inoculated and incubated for 24 h at 30 °C to provide inocula for the experimental cultures. The basal medium consisted of Tryptone T (5 g l⁻¹) and KCl (0.1 g l⁻¹) supplemented with $MgSO_4 \cdot 7H_2O$ at 0.05 g l⁻¹ (normal medium) or at 0.002 g l⁻¹ (Mg^{2+} -limited medium). Batch and continuous cultures were grown in 1 l fermenters at 30 °C with aeration at 20 l h⁻¹. Growth was measured as increase in bacterial dry weight or as absorbance at 650 nm (A_{650}) as described in detail elsewhere (Bovallius, 1969).

Analyses. Intracellular protein, carbohydrate and RNA were measured using the Folin-Ciocalteu, anthrone and orcinol reagents, respectively, as described by Herbert *et al.* (1971). Extracellular carbohydrate and RNA were determined in the culture supernatant after dialysis against cold distilled water and concentration at low pressure at 30 °C. Bovine serum albumin, glucose and yeast RNA were used as standards. S-factor activity was determined by measuring liberated cholinesterase from plaice muscle as described by Bovallius (1969).

Extracellular and intracellular adenine nucleotides were assayed at different times during growth of *Cytophaga* sp. by the firefly luciferase method (Lundin & Thore, 1975). For extraction of intracellular nucleotides from the bacterial cells, treatment for 90 s with boiling Tris/EDTA was adequate. Thus a portion of the whole culture (or, for determination of extracellular nucleotides, a portion of membrane-filtered culture) was transferred to a boiling solution of Tris/EDTA. After extraction, the levels of ATP, ADP and AMP were analysed in a luminometer using pyruvate kinase and adenylate kinase for the stepwise conversion of ADP and AMP to ATP (Lundin & Thore, 1975).

Preparation for electron microscopy. Samples from the cultures were withdrawn and centrifuged in the cold; the bacteria were washed once in cold 0.9% (w/v) NaCl. They were pre-fixed in 2% (v/v) glutaraldehyde at pH 7 and post-fixed in OsO_4 (Millonig, 1962). They were then dehydrated in ethanol followed by propylene oxide, embedded in Epon (Luft, 1961) and sectioned with an LKB Ultratome. The sections were stained with uranyl acetate (Stempak & Ward, 1964) for 5 min and with lead citrate (Venable & Coggeshall, 1965) for 2 min, unless stated otherwise. The silver methenamine method for localizing polysaccharide material in ultrathin sections was performed essentially as described by Walker (1969): the sections were placed on grids and stained with silver hexamethylenetetramine solution at 60 °C for 15 to 60 min and subsequently treated with 2% (w/v) sodium thiosulphate for 5 min. Some sections were post-stained with uranyl acetate and lead citrate as described above.

RESULTS

Electron microscopic studies

The morphological changes in *Cytophaga* sp. during transition from Mg^{2+} -excess to Mg^{2+} -limited growth conditions were studied in two batch cultures, differing only in Mg^{2+} concentration. At different times, as indicated by the arrows in Fig. 1, culture samples were centrifuged, fixed and examined in the electron microscope.

Bacteria in exponential growth (point 2*a*, Fig. 1) varied in length between 1.9 and 3.6 μm and had a diameter of about 0.6 to 0.7 μm (Fig. 2*a*). The outer triple-layered membrane of the wall had a regular waviness which could not be seen on the inner parts. After exponential growth had ended (point 2*b*, Fig. 1), no morphological changes were seen if Mg^{2+} was still available in the medium (Fig. 2*b*). In the stationary phase (point 2*c*, Fig. 1), the bacteria appeared similar, but none were longer than 1.5 μm (Fig. 2*c*).

In the medium with a low concentration of Mg^{2+} , the bacteria had the exponential phase appearance (Fig. 2*a*) as long as free Mg^{2+} was present. Some 2 to 3 h after all the Mg^{2+} had been consumed (point 3*a*, Fig. 1), the wall membrane region appeared both thicker and more wavy (Fig. 3*a*). The number of ribosomes was reduced. After depletion of Mg^{2+} for 18 h (point 3*b*, Fig. 1), the heterogeneity of the cells was further enhanced (Fig. 3*b*). Some ribosomes could still be observed. On further incubation of the bacteria in the Mg^{2+} -free medium, some thread-like forms appeared (Fig. 3*c*). In all samples, except those from old Mg^{2+} -depleted cultures, the bacteria seemed to divide after distinct septum formation.

Thread-like slime material extended radially from the outer surface of the bacteria (Figs 2 and 3). Silver methenamine-stained sections of *Cytophaga* sp. showed silver granules round the bacteria in a pattern similar to the thread-like structures (Fig. 4), suggesting that the slime excretion consisted, at least partly, of polysaccharide material. More slime seemed to be produced under Mg^{2+} -limited conditions, i.e. more silver granules were seen between the bacteria (Fig. 4*a*) compared with cultures grown with excess Mg^{2+} (Fig. 4*b*) (see, also, chemical analysis). Since thin sections of silver methenamine-stained bacteria had a rather pale appearance, attempts were made to post-stain lightly silver-stained sections with uranyl acetate and lead citrate to enhance the basic bacterial structures (Fig. 4*c*). In such micrographs the silver grains could be seen along the thread-like slime.

Gross chemical analysis

The bacterial content of Mg^{2+} , protein, carbohydrate and RNA was followed during growth of *Cytophaga* sp. in medium with a limited amount of Mg^{2+} (Fig. 5). The amounts of these components remained relatively constant until the medium was free of Mg^{2+} . There

Fig. 1. Growth of *Cytophaga* sp. NCMB 1314 in basal medium with limited (●) or excess (○) MgSO_4 . Numbered arrows show the times at which samples were taken for electron microscopy and refer to Figs 2 and 3. The vertical line shows the time of onset of Mg^{2+} limitation for bacteria grown in Mg^{2+} -limited medium.

was then a transition phase of about 3 to 4 h, during which the dry weight approximately doubled, and so the intracellular content of Mg^{2+} approximately halved, the protein content in the cells increased by 60% and the carbohydrate content by 100%, while the RNA content decreased to 70% of the earlier value (all values calculated on a dry weight basis).

During the exponential growth phase little or no carbohydrate and RNA were found in the supernatant. The extracellular carbohydrate content started to increase rapidly during the transition phase. However, RNA, indicating lysis, did not appear in the culture supernatant as a direct response to Mg^{2+} -limited growth, and not until the end of the transition phase and in the stationary phase were readily measurable amounts detected.

Results comparable with those in batch cultures were obtained when Mg^{2+} -limited and non- Mg^{2+} -limited (unknown limitation) continuous cultures were run at different dilution rates. Dry weight and intracellular Mg^{2+} , protein and carbohydrate contents of the bacteria depended on the Mg^{2+} content of the growth medium, while the RNA content appeared to be a function only of growth rate (Fig. 6). Extracellular carbohydrates were also found in continuous cultures, especially at low dilution rates. Greater amounts were found during Mg^{2+} -limited growth than during growth at the same dilution rate in a non- Mg^{2+} -limited culture.

Adenine nucleotide pool

Adenine nucleotide contents (calculated on a dry weight basis) in an exponentially growing culture were approximately constant. When the culture became Mg^{2+} -limited, the total adenylate pool rapidly decreased and then remained constant at the new level (Fig. 7). The decrease was almost solely due to an approximately 40% decrease in ATP content, while only a slight decrease in ADP content and a slight increase in AMP content were noted. Before Mg^{2+} limitation, the ATP, ADP and AMP contents were about 66, 18 and 16%, respectively, of the total adenine nucleotide content. After Mg^{2+} limitation, the proportions were changed to 52, 11 and 37%. Analyses for extracellular adenine nucleotides generally showed values <1% of those found in the whole culture. Similar results were obtained for continuous cultures run at different dilution rates.

DISCUSSION

No dramatic morphological changes could explain the sudden increase in extracellular S-factor activity shortly after the onset of Mg^{2+} starvation. The electron micrographs, however, show a more complex and wavy wall membrane region in Mg^{2+} -starved bacteria

Fig. 2. Sections of *Cytophaga* sp. NCMB 1314, taken 5, 7 and 24 h (*a*, *b* and *c*, respectively) after inoculation into basal medium with excess Mg^{2+} (points 2*a*, 2*b* and 2*c* in Fig. 1, respectively). Bar markers represent $0.1\ \mu m$.

Fig. 3. (a, b) Sections of *Cytophaga* sp. NCMB 1314 grown in basal medium with limited amounts of Mg^{2+} , taken 2.5 and 18.5 h (a and b, respectively) after the onset of Mg^{2+} limitation (points 3a and 3b in Fig. 1, respectively). Bar markers represent 0.1 μm .

(c) Section of a thread-shaped *Cytophaga* sp. taken 48 h after inoculation into a Mg^{2+} -limited medium. Bar marker represents 0.5 μm .

Fig. 4. (a, b) Thin sections of *Cytophaga* sp. NCMB 1314 grown in Mg^{2+} -limited (a) or Mg^{2+} -excess (b) medium, stained by the silver methenamine method. Apart from non-specific large silver grains, silver deposits indicating polysaccharide-containing structures can be seen on the wall, sometimes in a double layer, and in a thread-like pattern outside the organisms. Bar markers represent $0.5 \mu m$.

(c) Thin section of *Cytophaga* sp. grown in Mg^{2+} -limited medium, stained with silver methenamine and post-stained with uranyl acetate and lead citrate. Silver deposits can be seen on the inner dense layer of the wall and on the fibril-like threads outside the wall. Bar marker represents $0.1 \mu m$.

Fig. 5

Fig. 5. Changes in gross chemical content during batch cultivation of *Cytophaga* sp. NCMB 1314 in Mg^{2+} -limited medium: (a) bacterial dry weight (○) and extracellular S-factor activity (□); (b) intracellular Mg^{2+} (○) and protein (□) contents; (c) intracellular (○) and extracellular (□) carbohydrate contents; (d) intracellular (○) and extracellular (□) RNA contents. The vertical line shows the time of onset of Mg^{2+} limitation.

Fig. 6

Fig. 6. Changes in gross chemical content during continuous cultivation of *Cytophaga* sp. NCMB 1314 at different dilution rates in Mg^{2+} -limited (○, □) or non- Mg^{2+} -limited (●, ■) (unknown limitation) medium: (a) bacterial dry weight (○, ●) and S-factor activity (□, ■); (b) intracellular Mg^{2+} (○, ●) and protein (□, ■) contents; (c) intracellular (○, ●) and extracellular (□, ■) carbohydrate contents; (d) intracellular (○, ●) and extracellular (□, ■) RNA contents.

Fig. 7. Adenine nucleotide content of *Cytophaga* sp. NCMB 1314 during batch cultivation in Mg^{2+} -limited medium; $\circ$, AMP+ADP+ATP; ∇ , ATP; $\square$, ADP; $\triangle$, AMP. The vertical line shows the time of onset of Mg^{2+} limitation. The accompanying changes in dry weight (---) and S-factor activity (-.-.-) are also indicated.

than in cells grown normally, and this difference appeared within 3 h after the onset of starvation. The increased waviness could be a morphological artefact arising during the preparation for electron microscopy when the bacteria are exposed to chemical and physical stress. However, as the same preparation technique was used throughout, these observed differences must reflect some change in the wall structure or composition. Mg^{2+} deficiency in Gram-negative bacteria, induced either by growth in a Mg^{2+} -free medium or by addition of EDTA, is known to cause permeability changes (Brock, 1962) and weakened bacterial cell walls (Asbell & Eagon, 1966). The more wavy wall during Mg^{2+} starvation might then result from shrinkage during the dehydration process when the wall is not stabilized to the same extent as in the presence of Mg^{2+} ions. In agreement with the results of other investigations (e.g. Webb, 1970), a diminished ribosome content as a result of Mg^{2+} starvation was clearly seen.

No difference was observed in the division patterns of *Cytophaga* sp. NCMB 1314 when grown under conditions of Mg^{2+} excess or limitation. All dividing cells formed a cross-wall prior to division. In apparent disagreement is the observation (Mitchell *et al.*, 1969) that *Cytophaga* sp. NCMB 1314 forms constrictions after extended periods (3 to 10 d) of cultivation. However, in the present study of *Cytophaga* sp. NCMB 1314, the cultivation time was normally limited to 24 h. In old Mg^{2+} -limited cultures in which thread-like forms began to appear, a division pattern characterized by a constriction of the wall without septum formation could be seen.

The silver methenamine method (Walker, 1969) was used mainly to detect whether the bacteria had a capsule or produced polysaccharide-containing slime, which is one of the properties said to characterize most species of the genus *Cytophaga* (Christensen, 1977), but it also gave an indication of whether different amounts of polysaccharide were produced when the bacteria were starved of Mg^{2+} . The results suggest that more carbohydrate-containing slime is produced during Mg^{2+} -limited growth. This observation is supported by the results from the chemical analyses of supernatants from both batch and continuous cultures which showed an increasing carbohydrate content after the bacteria became Mg^{2+} -limited.

In batch cultures the amounts of cell-bound protein, carbohydrate, RNA and ATP (calculated on a dry weight basis) during bacterial growth changed after Mg^{2+} depletion in the medium.

The changes in gross chemical composition were further analysed in Mg^{2+} -limited and non- Mg^{2+} -limited continuous cultures run at different dilution rates. A good correlation between results found in batch and continuous cultures was obtained when the measured parameters were compared at the same specific growth rate. The continuous culture experiments indicated that Mg^{2+} content, bacterial dry weight, S-factor production and extra-cellular carbohydrate, as well as intracellular protein and carbohydrate contents, are all dependent both on the Mg^{2+} content of the medium and on the growth rate, while the change in RNA content is independent of Mg^{2+} content and reflects only the bacterial growth rate. A similar series of experiments measuring protein, carbohydrate and RNA content in *Klebsiella* (*Aerobacter*) *aerogenes* and *Bacillus subtilis* (Tempest *et al.*, 1965, 1967) showed the same tendency, but the percentage differences obtained were smaller than those reported here. A change to Mg^{2+} -limited growth results in morphological and chemical changes in *Cytophaga* sp. reflecting at least a quantitative difference in metabolism in this system. In a batch culture all these changes are most rapid during the transient phase when the bacteria change from one metabolic state to another. It is also during this phase that the sharp increase in S-factor production is observed, although the functional relationship remains unclear.

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