Coccoliths of the coccolithophorid *Emiliania huxleyi* are calcified biomineral scales composed of calcium carbonate and coccolith polysaccharide (CPs). Coccolith production is regulated by inorganic phosphate (Pi) availability, but no information currently exists on how this process occurs. In this study CP was experimentally characterized by HPLC analysis as an acid polysaccharide of mannose, galacturonic acid, xylose and rhamnose. Both calcification (estimated from 45Ca uptake) and CP production (estimated from uronic acid quantification) were stimulated under Pi-deficient conditions but strongly suppressed under Pi-sufficient conditions. When cells were transferred from Pi-sufficient to Pi-deficient conditions the production of neutral polysaccharides (NP)—storage glucans—ceased rapidly after a temporary increase in the presence of Pi, and CP production started to increase after Pi was almost depleted. Under Pi-sufficient conditions NP production increased, concomitant with stimulation of cell growth. Calcification increased gradually, but photosynthetic 14CO2 fixation was reduced by almost 40% for 5 d of culture during Pi depletion. [14C]CP production was maintained at almost constant, high levels under Pi-deficient conditions but gradually decreased under Pi-sufficient conditions in conjunction with cell growth. In contrast, [14C]NP production increased about 3-fold under Pi-sufficient conditions for 3 d. The present study indicates that *E. huxleyi* switches the direction of carbon flow toward CP and NP production under Pi-deficient and Pi-sufficient conditions, respectively.

**Keywords:** Acid polysaccharide • Calcification • Carbon metabolism regulation • Coccolith polysaccharide • *Emiliania huxleyi* • Phosphate deficiency.

**Abbreviations:** ABEE, 4-aminobenzoic acid ethyl ester; AP, acid polysaccharide; BAS, bio-imaging analyzer system; CP, coccolith polysaccharide; IP, imaging plate; NP, neutral polysaccharide; TCA, trichloroacetic acid.
Gephyrocapsa oceanica (Ozaki et al. 2004). APs, including species-specific sugar compositions, are located in coccoliths and share common chemical features; they are likely to play an important role in the formation of highly elaborate species-specific coccoliths, but as yet there is no direct evidence that APs, and hence CPs, possess this function.

In addition, there are few studies reporting on the biosynthesis of AP. One study into the cytological localization of CP in E. huxleyi implied that it is produced in the Golgi body, transported to the coccolith-producing vesicle, and then deposited on the cell surface together with the coccolith (van Emburg et al. 1986). Such behavior was also observed in P. carterae (Marsh et al. 1992, Marsh 1996). However, the corresponding biosynthetic pathway and its metabolic regulation are unknown.

Environmental factors affecting coccolith formation, such as macronutrients (CO₂, P, and NO₃⁻), trace elements (Mg, Mn and Zn), temperature and salinity, have been studied in detail (Zondervan 2007). P limitation increases the number of coccoliths produced by E. huxleyi cells (Paasche 1998) and calcification is stimulated by low temperature signals (Sorrosa et al. 2005). P limitation is the primary signal triggering coccolith production, and low temperature is the secondary signal (Satoh et al. 2009). These results were based on the observation that coccolith production increased along with P deprivation, as indicated by the induction of alkaline phosphatase activity as a cellular response, and that the process proceeded even at the optimum temperature for growth (Satoh et al. 2009). However, how P deprivation regulates calcification is still unclear.

In the present study, we investigated the regulation of CP production by changing P availability in E. huxleyi cells using a radiotracer technique when giving NaH¹⁴CO₃ as a substrate under P₄-sufficient and P₄-deficient conditions. In addition, ⁴⁰Ca radionuclide was used to monitor CaCO₃ crystal formation and to compare with data on ¹⁴C]CP production in coccolith formation. We found that upon polysaccharide production carbon metabolism switches from storage polysaccharides, namely neutral polysaccharides (NPs), to CP production, and that CaCO₃ crystal formation and CP production are regulated simultaneously during coccolith formation when P₄ availability decreases. These results led to a better understanding of the regulation of coccolith production by E. huxleyi cells and the function of CP in vivo.

**Results**

**Identification of CP**

Polysaccharides were extracted with 5% trichloroacetic acid (TCA) from whole E. huxleyi, including coccoliths on the cell surface, to enable their characterization. The extracts were subjected to anion exchange chromatography after dialysis to obtain AP and NP. Following SDS–PAGE, AP was visualized as a strong band as a result of staining with both Stains-all and Alcian blue. This band was very similar to that of the polysaccharide extracted from isolated coccoliths of the E. huxleyi strain used in this study (Fig. 1A). There were also some minor positive bands in addition to the major one, which we consider to be derivatives of CP. Although the SDS–PAGE profiles of the AP from P₄-deficient and P₄-sufficient cells looked almost identical, the AP of P₄-deficient cells varied slightly from that of P₄-sufficient cells in mobility and also in terms of staining less intensely with Alcian blue, showing that the molecules contained more uronic acid residues. In addition, quantification of the polysaccharide content using a phenol–H₂SO₄ assay, followed by analysis of uronic acid content using a carbazol–H₂SO₄ assay, revealed that 4 µg of AP from P₄-deficient and P₄-sufficient cells contained 0.83 and 0.68 µg of uronic acid, respectively. CP isolated from the coccoliths of P₄-deficient cells was shown to contain more uronic acid (0.98 µg per 4 µg of total sugar components). Composition analysis of the AP by HPLC showed that it was composed of at least four sugars (mannose, xylose, rhamnose and galacturonic acid), which were previously reported as constitutive sugars of CP by Fichtinger-Scheperman et al. (1981; Fig. 1B). These data clearly show that the AP from the E. huxleyi strain used in the present study was the same as the CP reported previously (Westbroek et al. 1973, de Jong et al. 1976). However, the NP was composed solely of glucose (Fig. 1B) and no positive bands were observed following SDS–PAGE and staining of gels with Stains-all or Alcian blue.

**Change in CP content and calcification in whole cells during growth under various Pi concentrations**

The growth of E. huxleyi cells was strongly suppressed following the limitation of P (Fig. 2A). In basal medium containing 28.7 µM P, the linear growth of cells was maintained for 4 d; however, the growth slowed after about 5 d as a result of P depletion (Figs. 2A, 3A). These data show that the concentration of P, as opposed to other nutrients was a limiting factor for Emiliania growth in culture. In contrast to algal growth, ⁴⁰Ca uptake by whole cells (a measure of calcification; Satoh et al. 2009) and uronic acid content detected using the carbazole–H₂SO₄ assay (a measure of the amount of CP) gradually increased under P₄-deficient conditions, whereas neither phenomenon was observed under P₄-sufficient conditions (Fig. 2B, C). A complete absence or reduction in calcified cells was consistently observed under microscopic examination (data not shown). It is possible that the amount of uronic acid determined might have been underestimated because CP contains many neutral sugar residues, which the carbazole–H₂SO₄ assay is also able to detect as a result of non-specific reactions (Bitter and Muir 1962). However, the data show that the rate of calcification, namely ⁴⁰CaCO₃ production, far exceeds that of CP production.
The data calculated per unit of cells show a clearer difference between both Pi conditions (Fig. 2E, F). These data imply that Pi limitation is a trigger for both calcification and CP production.

When CP and NP were determined separately after purification, we found that the amount of CP increased slowly for 3 d and rapidly thereafter (Fig. 3). In contrast, NP content increased immediately after the transfer from Pi-sufficient to Pi-deficient conditions and then stabilized at day 3. Although the reason for this increase is not known, it is clear that the increase in NP content occurred slightly before Pi depletion, and was followed by a significant increase in CP production (Fig. 3B). Both NP and CP content per cell remained very low in the presence of Pi (final concentration 28.7 µM; Fig. 3C), but this seems to have been caused by the marked increase in cell number due to cell division (Fig. 3A, B).

Change in calcification and CP production under various Pi conditions

After pre-culturing E. huxleyi cells for 4 d to deplete Pi in the medium, the culture suspension was divided in two. One half was maintained continuously in Pi-deficient medium, and the other was transferred to Pi-sufficient medium to which Pi was added periodically to avoid Pi limitation (Fig. 4). The algal cells grew well in the Pi-sufficient medium for 3 d and growth then plateaued, whereas no growth occurred in the Pi-deficient medium (Fig. 4A). Cells harvested at 2, 3 and 5 d were individually transferred to a glass reaction vessel and incubated separately with either 2 mM NaH14CO3 or 10 mM 45CaCl2 for 8 h. After terminating the reaction by harvesting, we extracted CP and subjected it to SDS–PAGE as described in Materials and Methods. Radioactive bands of [14C]CP were then visualized on the imaging plate (IP; Fig. 4B). The bio-imaging analyzer system (BAS) image analysis of SDS–PAGE profiles of 14C-labeled CP showed only one major radioactive band (Fig. 4B), although other minor bands stained with Alcian blue were also observed (Fig. 1A). The radioactivity was determined as the IP response with a unit of photostimulated luminescence by bio-imaging analysis (Fig. 4C). The accumulation of [14C]CP within both cells and the coccoliths increased during 2–8 h of incubation. [14C]CP synthesis by Pi-deficient cells remained at high levels during 5 d of cultivation, whereas that by Pi-sufficient cells decreased markedly over this time period. 45Ca uptake by Pi-deficient cells increased significantly with cultivation time, whereas that of Pi-sufficient cells remained at low levels (Fig. 4C). These results suggest that 45CaCO3 crystal formation increases if accompanied by CP production under Pi-deficient conditions, although the extent of calcification exceeds that of CP production. The rate of total 14C uptake by cells was 40–100 times higher than that of 45Ca uptake, even though the NaH14CO3 concentration (2 mM) was five times lower than that of 45CaCl2 (10 mM; Fig. 4C). Therefore, the amount of 14C incorporation into CaCO3, namely Ca14CO3 production, was not as significant as the amount of 14C fixed by photosynthesis. Thus, the amount of 14C inorganic carbon in the medium decreased to about half of the initial concentration at 8 h because of utilization by cells, whereas most added 45Ca remained in the medium (Fig. 4C). In Pi-deficient cultures the total 14C uptake per cell gradually decreased, whereas that in Pi-sufficient medium remained unchanged, although 14C uptake per cell in Pi-deficient conditions was slightly greater than that under Pi-sufficient conditions (Fig. 4C). These results suggest that the regulation of carbon metabolism by Pi availability does not apply to the...
photosynthetic CO₂ fixation stage. It is possible that other regulatory mechanisms exist for the control of CP production by Pi. CP can be considered to be associated with calcification, but more detailed information on the function of CP itself and the relationship between amounts of CP and CaCO₃ produced is required.

**Regulation of NP and CP production by Pi availability**

To compare NP and CP synthesis, we pre-cultured cells in Pi-deficient medium for 2 d. The culture was then divided in two and incubated in Pi-sufficient or Pi-deficient medium for a further 3 d (Fig. 5A). ¹⁴C uptake during 8 h of photosynthesis was approximately 25% higher in Pi-deficient cells than in Pi-sufficient cells (Fig. 5B). The accumulation of [¹⁴C]NP increased 3-fold in Pi-sufficient cells, whereas no change was observed in Pi-deficient cells. In contrast, [¹⁴C]CP production increased in both Pi-deficient and Pi-sufficient cells, although the extent of the increase was small in the latter compared with the former (Fig. 5C). These results suggest that the synthesis of storage polysaccharides such as NP is strongly stimulated by Pi.

In the ¹⁴C labeling experiment with Pi-sufficient and Pi-deficient cells, almost half of the ¹⁴C radioactivity (48 and 50%, respectively) was incorporated into the lipid and low molecular metabolite fraction, which probably included predominantly storage lipids such as alkenones, a few membrane lipids and low molecular metabolites (data not shown). Approximately 28 and 16%, respectively, of the total ¹⁴C taken up by cells was incorporated into proteins (20 and 26%, respectively) and low molecular polymers filtered through dialysis membrane (4.5 and 6.2%, respectively; data not shown). These results suggest that NP and CP production are closely regulated by Pi availability at the cellular level.
The presence of CP in coccoliths was first reported by Westbroek et al. (1973) and the complicated structure of the molecule was determined by Fichtinger-Schepman et al. (1981). However, the detailed physiological functions of CP and the metabolic process and regulation of CP production have yet to be elucidated. In this study, we found that the regulation of CP production is positively associated with calcification. Although P deficiency is known to trigger the calcification process in *E. huxleyi* cells (Paasche 1998, Satoh et al. 2009), here we present evidence that both CP production and CaCO₃ precipitation are regulated simultaneously by the change in P availability. Through a partial analysis of sugar components, we first confirmed that the AP extracted with 5% TCA from the *E. huxleyi* strain used in this study is the same as the polysaccharide isolated from the coccoliths (Fig. 1) and corresponds to the CP reported previously (Fichtinger-Schepman et al. 1981; Fig. 1). We then determined that both CP production (monitored by ¹⁴C labeling) and calcification (monitored by ⁴⁵Ca uptake) are stimulated under P₃-deficient conditions, as compared with P₃-sufficient conditions (Fig. 4B, C).

As [¹⁴C]CP production was maintained at almost constant, high levels for 5 d under P₃-deficient conditions (Fig. 4C), CP production could proceed almost linearly, as shown in Fig. 2. Evidence exists that calcification is strongly stimulated after 3 d (Satoh et al. 2009). This is compatible with the increase in the ratio of calcification to uronic acid content (Fig. 2D), and suggests that CaCO₃ production occurs at a higher rate than CP production, which proceeds in a constant manner. In addition, the synthesis of CP may occur continuously in cells because the amount of [¹⁴C]CP in both the cells and coccoliths increased continuously for 8 h (Fig. 4). Thus, a substantial amount of CP—about a half of that synthesized—is likely to remain in the cell without being excreted with the coccoliths. However, the fate of the remainder of the CP with regard to degradation, metabolism to other molecules or reuse for coccolith production is unknown. According to cytological observations, polysaccharides in the coccolith-producing compartment that have not yet been experimentally identified as CP disappeared after the completion, but before the excretion of coccoliths (van der Wal et al. 1983). In *P. carterae*, which contains three kinds of AP (PS-1, PS-2 and PS-3), about 25% of PS-3 remained in cells even after 6h of [¹⁴C]pulse–chase labeling, whereas most PS-1 and PS-2 was excreted with coccoliths in 2 h (Marsh 1996). Only PS-3 contains mannose, xylose and sulfate groups as components and is reported to be the same as the CP in *E. huxleyi* (Marsh et al. 2002). It should be noted that PS-3 was reported to be the main molecule controlling the specific morphology of coccoliths in a study using a natural variant that had lost the ability to produce PS-3 (Marsh et al. 2002).

Immunological quantification of CP showed that 0.44 µg (10⁶ cells)⁻¹ and 0.56 µg (10⁶ cells)⁻¹ of CP was excreted to the medium under nutrient-sufficient and -deficient conditions, respectively, for 6d in a batch culture of *E. huxleyi* (Nanninga et al. 1996). These amounts are comparable with those of CP measured in this study, although the experimental conditions were different (Fig. 3). The determination of CP released into the medium is required to enable further understanding of CP metabolism.

NP containing only glucose, which was isolated by anion exchange chromatography, was also discovered in the present study. This appears to correspond to β-glucan, which was identified as an aqueous acid-soluble sugar and reported to be a cytosolic storage polysaccharide in *E. huxleyi*.
However, the amount of polysaccharide determined as NP in this study (Figs. 3, 5) was smaller than that of β-glucan, which was reported to be equivalent to 16% (w/w) of the dry cell weight (Vårum et al. 1986), because the small glucan molecule was fractioned as a low molecular weight polymer following dialysis treatment. Vårum et al. (1986) reported that the average degree of polymerization of β-glucan was 106, corresponding to the average molecular weight of approximately 17,000 Da in E. huxleyi. The small glucan molecule equated to 4.5–6.2% of the total 14C incorporation, irrespective of P$_i$ availability. These compounds may include intermediates of the NP metabolic pathway. Thus, not all β-glucan was determined to be NP in the present study.

NP production seems to be stimulated under P$_i$-sufficient conditions (Figs. 3, 5). The consumption or metabolism of NP may also be stimulated in P$_i$-sufficient cells because the cells actively divide for growth, as shown in Fig. 3A. NP

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**Fig. 4** Change in cell growth, calcification and CP production during the culture of *Emiliania huxleyi* cells under P$_i$-sufficient and P$_i$-deficient conditions. (A) Change in cell number. Cells were harvested by centrifugation from a batch culture maintained under aeration for 3 d and then transferred to fresh medium, with or without 28.7 µM P$_i$, at day 0 for a standing culture. To maintain a sufficient P$_i$ concentration, we added 28.7 µM K$_2$HPO$_4$ every day as indicated by white arrows. At 2, 3 and 5 d (black arrowheads on top), part of the culture was subjected to 14C and 45Ca labeling using NaH14CO$_3$ (final concentration, 2 mM; specific activity, 9.25 MBq mmol$^{-1}$) and 45CaCl$_2$ (final concentration, 9.5 mM; specific activity, 0.39 MBq mmol$^{-1}$) as substrates. (B) Bio-imaging analysis of radioactive bands of [14C]CP isolated from whole cells including coccoliths (a), and naked cells from which coccoliths were removed artificially (b), on SDS–PAGE. (C) Time course of 45Ca and 14C uptake by cells and [14C]CP produced in the cellular and coccolith fractions during 8 h radiolabeling. CP, coccolith polysaccharide; PSL, photostimulated luminescence.
production was stimulated about 3-fold under P<sub>i</sub>-sufficient conditions (Fig. 5), suggesting that the cells actively synthesize storage polysaccharides, resulting in an increase in growth. In contrast, NP production remained constantly low for 3 d of cultivation under P<sub>i</sub>-deficient conditions, resulting in a strong suppression of cell growth (Fig. 5).

In general, two types of response to P<sub>i</sub> deficiency are observed in higher plants and algae: a primary response related to the efficient acquisition of P<sub>i</sub> (e.g. induction of phosphatase and P<sub>i</sub> transporters) and subsequent metabolic changes (e.g. accumulation of storage polysaccharides; Nilsson et al. 2007). With regard to the production of storage polysaccharides, several nucleotide diphosphate sugar pyrophosphorylases, which provide substrates for polysaccharide synthesis as key enzymes, are stimulated by P<sub>i</sub> deficiency (Collén et al. 2004, Nilsson et al. 2007). In *E. huxleyi*, the induction of alkaline phosphatase activity occurred before calcification under conditions of P<sub>i</sub> deficiency (Satoh et al. 2009), suggesting that calcification requires a trigger after receiving a P<sub>i</sub>-deficient signal. In contrast, CP production does not seem to be directly regulated by P<sub>i</sub> itself because CP production in 2-day-old cells not fully acclimated to P<sub>i</sub>-sufficient conditions showed no substantial difference, irrespective of P<sub>i</sub> condition, and required 5 d to acclimatize to complete suppression of [14C]CP production (Fig. 4C, a). Moreover, another coccolithophorid, *Pleurochrysis*, continuously produces coccoliths independently of P<sub>i</sub> availability, even as it synthesizes the CP-like AP, PS-3, and accumulates β-glucan as a storage polysaccharide (van der Wal et al. 1987, Hirokawa et al. 2008).

A model of the regulatory profile of CP and NP production by P<sub>i</sub> availability in *E. huxleyi* is shown in Fig. 6. CP production is stimulated under P<sub>i</sub>-deficient conditions and suppressed under P<sub>i</sub>-sufficient conditions. In contrast, NP production is activated under P<sub>i</sub>-sufficient conditions. The present study suggests that the adverse regulation of the production of CP and NP is due to the polysaccharides’ different localization and function. Understanding the metabolic relationship between CP and coccolith production, as regulated by P<sub>i</sub> availability, requires further detailed study of the metabolic pathways for and gene regulation of CP and NP. In addition, a comparative study of CP production in coccolithophorids that possess inducible and constitutive processes for coccolith production (e.g. *E. huxleyi* and *P. haptonomoides*, respectively) will be very useful for understanding cell for 8 h. (C) Change in the amount of cellular [14C]CP and [14C]NP. The polysaccharides were isolated from naked cells from which coccoliths on the cell surface were removed by EDTA treatment, as described in Materials and Methods. CP, coccolith polysaccharide; NP, neutral polysaccharide.
the physiological and molecular regulation of coccolith production.

Materials and Methods

Organism details and culture conditions

The strain of coccolithophorid *E. huxleyi* (Lohman) Hay & Mohler (Haptophyceae; NIES 837) used in the present study was collected by Dr. I. Inouye of the University of Tsukuba in the South Pacific Ocean in 1990. Cells were grown in natural seawater for stock culture and in artificial seawater (Marine Art SF; produced by Tomita Seiyaku Co., Ltd., Tokushima, formerly distributed by Senju Pharmaceutical Co., Osaka, Japan, and recently by Osaka Yakken Co. Ltd., Osaka, Japan) enriched with Erd-Schreiber’s medium containing 10 nM sodium selenite instead of soil extracts (Danbara and Shiraiwa 1999). Cells were maintained under constant illumination at 100 µmol m⁻² s⁻¹ and 20°C (standard condition). Erd-Schreiber’s medium contains 28.7 µM K₂HPO₄, but Marine Art SF does not. To produce the phosphate-deficient condition, K₂HPO₄ was removed from the medium.

Extraction of polysaccharides and subsequent fractionation of CP and NP

Polysaccharides were extracted from *E. huxleyi* cells using TCA according to de Jong et al. (1979). Briefly, cells were harvested by centrifugation and stored at −80°C prior to ultrasonic lysis at 4°C using 5% (w/v) TCA. The homogenates were centrifuged at 15,000×g for 1 h, and the supernatant was either analyzed for uronic acid content or used for CP or NP purification. For the purification of CP, the supernatant was dialyzed against dialysis membrane (molecular weight cut-off 12–14 kDa; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the solvent changed to 20 mM Tris–HCl (pH 8.0). The sample was then subjected to anion exchange liquid chromatography using a HiTrap DEAE FF (GE Healthcare UK Ltd., Buckinghamshire, UK). The fractions eluted with the same buffer containing 0.5 M NaCl were collected as the AP, whereas the fractions that flowed through the column were collected as the NP. Each polysaccharide fraction was dialyzed against deionized water and either stored or lyophilized for further analysis.

Assays

Uronic acid content was estimated using a carbazole–H₂SO₄ assay (Bitter and Muir 1962). For calibration, 0–90 µg ml⁻¹ glucuronic acid (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was used as the standard. The amount of polysaccharides was estimated using a phenol–H₂SO₄ assay (Hodge and Hofreiter 1962). For calibration, 0–90 µg ml⁻¹ glucose was used as the standard. Inorganic phosphate concentration in the medium was determined using the molybdenum blue method (Murphy and Riley 1962). To obtain a cell-free medium, cells were removed by centrifugation at 15,000×g for 2 min, twice.

Electrophoresis of polysaccharides

To characterize the molecular properties, namely electrostatic mobility and specific staining, of the polysaccharides, we subjected isolated CP, AP and NP to SDS–PAGE. The amount of polysaccharides applied to each lane was 4 µg, corresponding to the glucose used as a standard in the phenol–H₂SO₄ assay. Notably, APs from Pᵢ-sufficient and Pᵢ-deficient cells were estimated to contain 0.68 and 0.83 µg uronic acid, respectively, corresponding to glucuronic acid used as a standard in the carbazole–H₂SO₄ assay. In other experiments, CP isolated from only the coccoliths was used as a standard and it was found that 4 µg of CP contained 0.98 µg of uronic acid (see above). After electrophoresis, the gels were stained with Stains-all or Alcian blue. Alcian blue staining was carried out with 7.5% (v/v) acetic acid. No positive bands were detected as a result of Coomassie brilliant blue staining in any of the samples. For standard CP, coccoliths were isolated from cells using the method of de Jong et al. (1976), except that 80% (w/v) sucrose was used instead of 50% Ludox in the coccolith purification process.

Analysis of polysaccharide composition

CP and NP isolated from Pᵢ-deficient cells were hydrolyzed with 4 M trifluoroacetic acid for 3 h at 100°C. Hydrolyzed samples were modified with 4-aminobenzoic acid ethyl ester (ABEE) according to the manufacturer’s instructions (Seikagaku Co., Tokyo, Japan). The ABEE-modified monosaccharides were analyzed by HPLC using Honenpak C-18 (Seikagaku Co.). Each monosaccharide was identified with the standard sugars, individually prepared and subjected to HPLC in the same way.
**45Ca uptake**

The amount of coccolith production was measured based on Satoh et al. (2009) by determining the incorporation of 45Ca into the cells, based on evidence that >95% of 45Ca was taken up by whole cells, located in the coccoliths. An adequate amount of 45CaCl2 (Perkin-Elmer, Inc., Waltham, MA, USA) was added to the culture (see respective figures and legends). At intervals, 0.2–1 ml of culture was harvested, and the cells were separated from the medium by centrifugation at 15,000×g for 2 min after the addition of 0.01% (w/v) Tween-20. The pellet was washed twice with fresh medium, and radioactivity in the pellet (cells) and supernatant (medium) was determined using a liquid scintillation counter (LS 5000TD; Beckman Coulter, Inc., Fullerton, CA, USA) with a scintillation cocktail (Hionic-Fluor; Perkin-Elmer).

**Determination of 14C incorporated into CP and NP**

The initial rate of CP production was estimated using a 14C labeling technique. An adequate amount of NaH14CO3 (GE Healthcare) was added to the pre-culture (see respective figures and legends). At intervals, an aliquot of cell suspension was harvested for further analysis. After the addition of 0.01% (w/v) Tween-20 to the suspension for smooth precipitation, the cells were obtained by centrifugation at 15,000×g for 2 min. The supernatant was treated with 1 M NaOH to avoid volatilization of CO2 and the pellet was washed twice with fresh medium. Radioactivity in the pellet (cells) and supernatant (medium) was determined using a liquid scintillation counter.

For analysis of 14C-labeled CP, a 5% TCA extract was prepared from whole cells, as described above. To determine CP located inside the cells, we treated part of the sample with a solution containing 0.1 M EDTA and 60 mM Tris–HCl (pH 8.0) to dissolve the extracellular coccoliths attached to the cell surface prior to 58.0) to dissolve the extracellular coccoliths attached to the solution containing 0.1 M EDTA and 60 mM Tris–HCl (pH 8.0) to obtain low molecular weight polymers fraction 2 in the dialysate. The CP and NP fractions were separated using anion exchange chromatography as described above. The radioactivity in each fraction was determined using a liquid scintillation counter.

**References**


Regulation of coccolith polysaccharide production


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