JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 1186–1192 Vol. 179, No. 4
0021-9193/97/$04.00+0
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Utilization of Phosphocholine from Extracellular Complex Polysaccharide as a Source of Cytoplasmic Choline Derivatives in *Penicillium fellutanum*

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Received 5 August 1996/Accepted 28 November 1996

***Penicillium fellutanum* produces a phosphorylated, choline-containing extracellular polysaccharide, peptidophosphogalactomannan (pP$_x$GM) [where *x* is the number of phosphodiester residues]). The ^{13}C-methyl-labeled pP$_x$GM ([*methyl*-^{13}C]pP$_x$GM) was prepared from the cultures supplemented with L-[*methyl*-^{13}C]methionine and was used as a probe to monitor the fate of phosphocholine in this polymer. The addition of [*methyl*-^{13}C]pP$_x$GM to growing cultures in low-phosphate medium resulted in the disappearance within 5 days of [*methyl*-^{13}C]phosphocholine and *N,N′*-dimethylphosphoethanolamine from the added [*methyl*-^{13}C]pP$_x$GM. Two ^{13}C-methyl-enriched cytoplasmic solutes, choline-*O*-sulfate and glycine betaine, were found in mycelial extracts, suggesting that phosphocholine-containing extracellular pP$_x$GM of *P. fellutanum* is a precursor of intracellular choline-*O*-sulfate and glycine betaine. The mycelia cultured in low-phosphate (2 mM) medium contained glycine betaine and 1.5-fold more choline-*O*-sulfate than those grown in high-phosphate (20 mM) medium. The high levels of extracellular nonspecific phosphocholine:phosphocholine hydrolase and acid phosphomonoesterase observed in the low-phosphate culture medium are likely related to the release of phosphocholine from pP$_x$GM and hydrolysis of phosphocholine, respectively. These results suggest that extracellular pP$_x$GM of *P. fellutanum* provides phosphate needed as the environment becomes depleted of this nutrient. Choline, in excess of that needed immediately, is stored in the cytoplasm in forms that can be reutilized.**

Fungi produce complex phosphorylated polysaccharides and glycopeptides which are membrane bound, cell wall bound, or extracellular (1, 3, 13, 17, 30, 31). Although the structural chemistry of these polymers has been studied (2, 13), their role(s) in fungal physiology is mostly unknown. Some extracellular polysaccharides or glycopeptides of fungi may be reserve nutrient sources of carbon (6, 19, 34), and others function in animal and plant pathogenesis (8, 14).

Ubiquitous filamentous fungi, in the genera of *Penicillium* or *Aspergillus*, produce soluble extracellular polysaccharides and glycopeptides, called peptidophosphogalactomannans (pP$_x$GMs [where *x* is the number of phosphodiester residues]) (13, 20). *Penicillium fellutanum* (formerly *Penicillium charlesii* G. Smith NRRL 1887) produces two classes of complex exocellular polysaccharides, (i) soluble pP$_x$GM with a mass of about 70 kDa and (ii) membrane-bound lipo-pP$_{30}$GM (11, 24, 25). The structure of the major extracellular pP$_x$GM has been examined by wet chemistry and by nuclear magnetic resonance (NMR) spectroscopy (5, 10, 25, 35, 36). The polymer has oligomannopyranosyl residues and a mannan attached to a 3-kDa peptide through an O-glycosidic linkage; 8 to 10 galactofuran chains are attached to the mannan backbone. Approximately 10 phosphocholine and phosphoethanolamine residues are attached to the C-6 position of mannopyranosyl residues (35); *N*-peptidyl phosphoethanolamine phosphodiester residues are attached to the C-6 position of galactofuranosyl residues. The mannan backbone consists of 10 repeating units (Fig. 1).

Some microbial cell wall or extracellular polymers are known to contain phosphocholine phosphodiesters. Examples are teichoic and lipoteichoic acids of several viridans group streptococci and clostridia (18, 23, 33), pP$_x$GM of *P. fellutanum* (35), and the cyclic β-(1,6)-β-(1,3)-glucans of *Bradyrhizobium japonicum* (27). Although phosphocholine phosphodiester is a structural component of these biological polymers, its physiological role is largely unknown. Phosphocholine in wall and membrane teichoic acids is necessary for autolysin amidase activity required for cleavage of peptidoglycan muramoyl residues in *Streptococcus pneumoniae* (15, 22). The wall teichoic acid is involved in the maintenance of normal cell shape and physiology in *Streptococcus oralis* (16).

P. fellutanum cultured in a medium containing 2 mM phosphate produces extracellular nonspecific phosphocholine: phosphocholine hydrolase (PC:PCH) that catalyzes the release of phosphocholine from *p*-nitrophenyl phosphocholine or glycerol-3-phosphocholine (28). A crude enzyme preparation from the same culture filtrate catalyzed the removal of phosphocholine from pP$_x$GM (28).

During research to elucidate the physiological role(s) of the phosphodiester residues in extracellular pP$_x$GM, we found, using ^{13}C NMR analysis, that this fungus accumulates two major choline derivatives inside of the mycelia. In this paper, we report that phosphocholine in extracellular pP$_x$GM is a precursor of intracellular choline-*O*-sulfate (COS) and glycine betaine (GB).

MATERIALS AND METHODS

Chemicals and strain. L-[*methyl*-^{13}C]methionine was purchased from Sigma, St. Louis, Mo. Deuterium oxide (D_2O) was from Sigma. Sodium (trimethylsilane)-1-propanesulfonate (sodium TSP) was obtained from Wilmad Glass Co., Buena, N.J. COS was synthesized according to the method of Stevens and Vohra (32). All other chemicals were reagent grade and were obtained from commercial sources. *P. fellutanum* (previously *P. charlesii* G. Smith NRRL 1887) was a gift from Kenneth Raper, Department of Bacteriology, University of Wisconsin, Madison.

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[-M-α1,2-M-α1,6 P-choline
M-α1,2-M-α1,2-M-α1,2-M-α1,6
(5-O-β-Gf)$_{2\text{-}30}$ M-α1,2-M-]$_{10}$ -peptide

FIG. 1. Repeating unit of the mannan backbone. Mannopyranosyl (M) and galactofuranosyl (G_f) residues are indicated. cP-choline, phosphocholine.

Media and growth conditions. Conidiospores for liquid shake culture were harvested from cultures grown on the Czapek Dox plates (7) for 12 days at room temperature under constant light. Conidiospore suspensions were prepared in sterile water containing 0.9% (wt/vol) NaCl and 0.125% (wt/vol) Tween 20. For the production of pP_xGM, *P. fellutanum* was grown in either the standard growth (SG) medium containing 20 mM phosphate or low-phosphate standard growth (LPSG) medium containing 2 mM phosphate (28). Medium compositions are described elsewhere (29). *P. fellutanum* was grown in 200 ml of medium with an inoculum of $\sim 10^6$ conidiospores in a 500-ml notched widemouthed Erlenmeyer flask at 22°C under constant light and under constant shaking at 40 rpm on a New Brunswick model G-10 shaker.

Enzyme assays. Activities of nonspecific PC:PCH and acid phosphomonoesterase (AP) were determined daily during the incubation of cultures. Enzyme activities were assayed, following 20 min of incubation at room temperature, in a reaction mixture of 500 μl of 10 mM sodium citrate buffer (pH 3.0 for AP or pH 5.0 for PC:PCH), 2.0 μmol of *p*-nitrophenyl phosphocholine for PC:PCH or *p*-nitrophenyl phosphate for AP, and 100 μl of culture filtrate with deionized water to a total volume of 850 μl. Reactions were stopped by addition of 1 ml of 0.2 N NaOH. *p*-Nitrophenol released was determined at 410 nm in a Hewlett-Packard model 8450A diode array spectrophotometer.

Isolation and fractionation of extracellular soluble pP_xGMs. Soluble pP_xGMs were obtained from 2 liters of day-8 cultures in SG or LPSG medium. Procedures for the isolation and fractionation of extracellular soluble pP_xGMs followed established methods (10). Crude preparations of pP_xGMs were fractionated on DEAE-cellulose preequilibrated with 50 mM $K_2B_4O_7$. Fractions were assayed for total carbohydrates at 490 nm (9). Carbohydrate-containing fractions were pooled, dialyzed against deionized water, and freeze dried. Extracellular pP_xGMs used in these experiments were eluted with 0.01 N HCl–0.06 M LiCl from a DEAE-cellulose (DE-52) column. This fraction of pP_xGM contains more phosphocholine phosphodiester than occurs in other fractions of pP_xGM (29).

Preparation of [*methyl*-^{13}C]pP_xGM by metabolic labeling. *P. fellutanum* was cultured for 8 days in 4 liters of SG medium enriched with L-[*methyl*-^{13}C]methionine (50 mg/200 ml of medium). ^{13}C-methyl-labeled pP_xGM was isolated from the culture filtrate as described above. Since extracellular pP_xGM obtained from high-phosphate (SG) medium contains more phosphocholine residues than those obtained from low-phosphate (LPSG) medium (29), the SG medium was used to obtain [*methyl*-^{13}C]pP_xGM. Thirty hours after inoculation, 50 mg of L-[*methyl*-^{13}C]methionine dissolved in deionized water was filter sterilized (filter pore size, 0.22 μm; Millipore) and added to each culture flask. The [*methyl*-^{13}C]pP_xGM obtained was analyzed by proton-decoupled ^{13}C NMR spectroscopy.

Release of phosphocholine from extracellular pP_xGM. The ^{13}C-methyl-labeled pP_xGM obtained was used to determine the release of phosphocholine from extracellular pP_xGM. Purified [*methyl*-^{13}C]pP_xGM (200 mg) was dissolved in 4 ml of deionized water and added 72 h after inoculation, by filtration through a 0.22-μm-pore-size membrane filter (Millipore), to 200-ml cultures in LPSG medium. This time was selected to include the interval (3 to 8 days) of maximum PC:PCH activity (Fig. 2). The same quantity of unlabeled pP_xGM was used as a control. At day 8, the cultures were harvested and the added [*methyl*-^{13}C]pP_xGM was recovered from the culture filtrates through a modified isolation procedure. Compositional changes in the recovered [*methyl*-^{13}C]pP_xGM were analyzed by proton-decoupled ^{13}C NMR spectroscopy.

Ten milliliters of 1 M sodium borate, pH 7.0, was added to 200 ml of culture filtrate from each culture flask. The negatively charged [*methyl*-^{13}C]pP_xGM bound to DE-52 matrix preequilibrated with 50 mM $K_2B_4O_7$. The column was washed with 50 mM $K_2B_4O_7$ to remove residual glucose in the culture filtrate; [*methyl*-^{13}C]pP_xGM was then eluted with 0.01 N HCl–0.4 M LiCl. The carbohydrate-containing fractions were dialyzed against deionized water and then freeze-dried. The residue was dissolved in 4 ml of deionized water and analyzed for any compositional changes by ^{13}C NMR spectroscopy. The intracellular solute pools of mycelia obtained from the same cultures were examined for the existence of any intracellular ^{13}C-methyl-labeled substance(s) (see below).

Preparation of mycelial extracts. Intracellular solute pools of mycelia were analyzed by proton-decoupled ^{13}C NMR spectroscopy after extraction with 80% ethanol. Culture conditions are described above. Fifty milligrams of L-[*methyl*-^{13}C]methionine was added to each 200-ml culture at 30 h, which is the approximate time of germination. According to the activity profile of PC:PCH in culture filtrates collected daily (Fig. 2), unless otherwise stated all the mycelial extracts for subsequent experiments were obtained from the cultures grown for 8 days. It was noted that PC:PCH activity gradually decreased after day 6. Mycelia were obtained by filtration in vacuo, washed with water, and then treated with 80% ethanol for 24 h at room temperature and centrifuged at 8,000 × *g* for 30 min at 4°C. Insoluble debris were removed by vacuum filtration through M-type sintered glass filters. The filtrates were evaporated in vacuo to near dryness at 40°C with enough time to completely remove any residual ethanol. The dried materials were dissolved in 4 ml of deionized water, and only water-soluble substances were analyzed by ^{13}C NMR spectroscopy.

^{13}C NMR spectroscopy of mycelial extracts and pP_xGMs. Natural-abundance proton-decoupled ^{13}C NMR spectra were recorded at 75.45 MHz using an NT-300 spectrometer with a 7-T Oxford magnet operating in the pulsed Fourier transform mode. Unless otherwise stated, all samples were examined at 22°C, with broad-band decoupling of protons, with full nuclear Overhauser enhancement, and without sample spinning. Four milliliters of each mycelial extract was analyzed by proton-decoupled ^{13}C NMR spectroscopy with addition of 0.5 ml of D_2O containing sodium TSP as an internal reference. Frequencies and acquisition parameters are described in the legends of each figure.

Synthesis of COS. COS was synthesized from 4 g of choline chloride (Sigma) in 10 ml of concentrated H_2SO_4 according to the methods of Stevens and Vohra (32). One hundred seventy-one milligrams of dried, white crystals was obtained. The purity and identity of synthesized COS were confirmed by ^{13}C NMR, ^{1}H NMR spectroscopy, and by Dragendorff-positive reaction (4) on the silica gel thin-layer chromatography plate (not shown).

Identification of COS and GB. Three authentic choline derivatives (COS, GB, and choline chloride) were dissolved directly in mycelial extracts, and their chemical shifts and signal intensities were compared with those of signals in mycelial extracts. Preliminary experiments showed that this procedure, rather than dissolving authentic chemicals in deionized water, was necessary to obtain valid chemical shift values; this created the same environment for the added authentic chemicals as that of biological intracellular choline derivatives. Thus, to the mycelial extract obtained from the 8-day-grown mycelia in LPSG medium and transferred to a 12-mm NMR tube, authentic solid GB, COS, and choline chloride (30 mM each) were added sequentially. ^{13}C NMR spectra were obtained after each addition and were analyzed for the influence of the added authentic substance on the signal intensities.

Quantification of intracellular COS and GB. Intracellular COS and GB were quantified by integrating their individual methyl signals from the ^{13}C NMR spectrum; sodium TSP was used as the reference. Integration was performed with Felix for Windows 1.02 software (Molecular Simulations Inc.). The mycelial dry weight was determined by taking the mean value of triplicate samples. Mycelia obtained from each 200-ml culture grown for 8 days were filtered in vacuo through an M-type sintered glass filter with the preweighed, demoisturized Whatman no. 4 filter paper. Mycelia on filter papers were then separately placed in each beaker and heated for 24 h at 110°C.

RESULTS

Production of extracellular phosphoesterases in low-phosphate (LPSG) and high-phosphate (SG) medium. A nonspecific PC:PCH and an AP were produced at low level in high-phosphate (SG) medium (20 mM NaH_2PO_4) (Fig. 2A). However, in 2 mM phosphate medium (LPSG), much greater activities of AP and PC:PCH were observed (Fig. 2B). This suggests that the high level of PC:PCH activity in low-phosphate culture medium could be involved in recruiting phosphorus by releasing phosphocholine from extracellular pP_xGM. The production of PC:PCH occurred for a longer period of time than that of AP in LPSG medium.

Preparation of [*methyl*-^{13}C]pP_xGM by metabolic labeling. The ^{13}C NMR spectrum of Fig. 3A represents normal pP_xGM

FIG. 2. Production of phosphoesterases in high-phosphate (SG) medium (A) and low-phosphate (LPSG) medium (B). Two to three milliliters of culture filtrates was passed through Whatman no. 4 filter paper and immediately assayed for two enzyme activities, a nonspecific PC:PCH (□) and an AP (○).

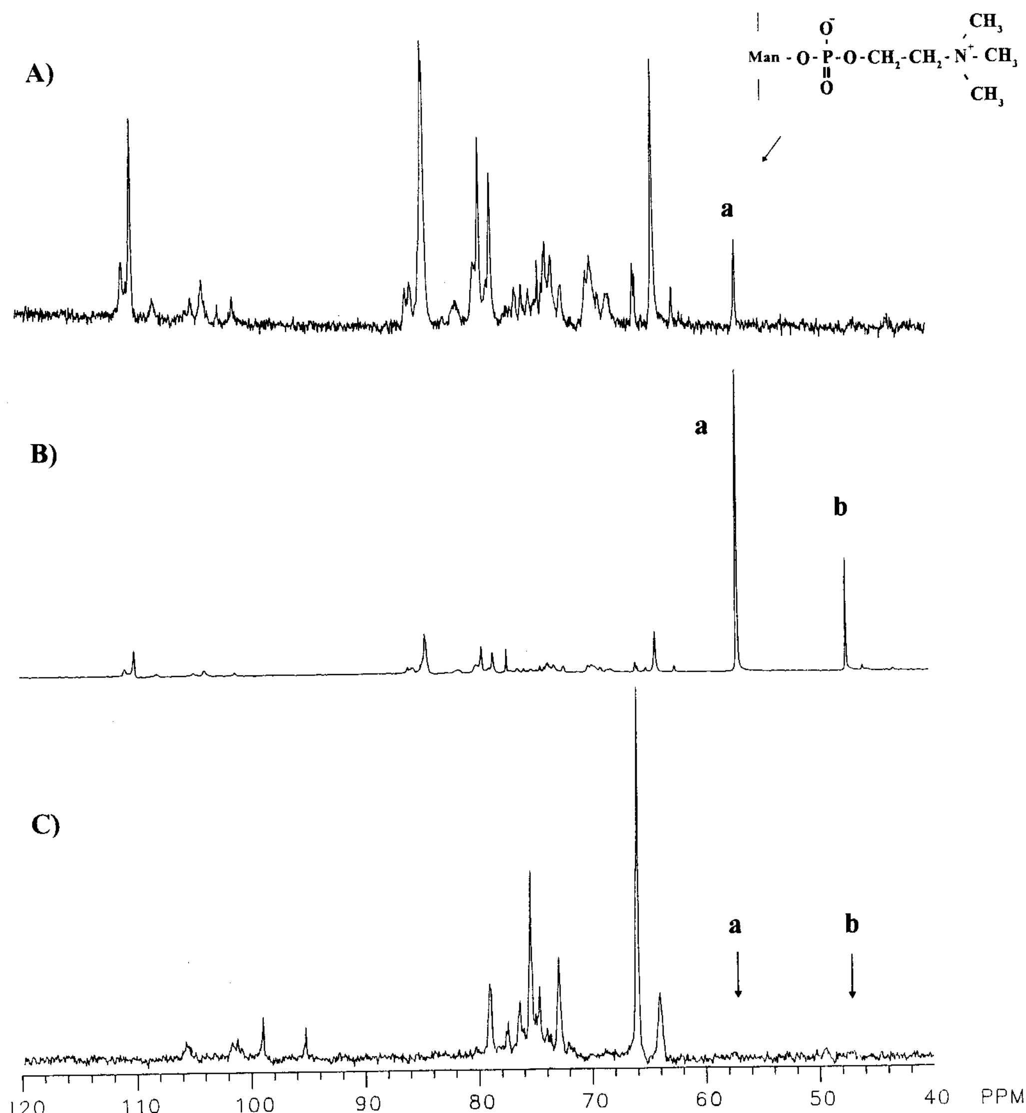

FIG. 3. Proton-decoupled ^{13}C NMR spectra of extracellular pP$_x$GMs isolated from the culture filtrates. (A) Day-8 culture in 20 mM phosphate (SG) medium. (B) Day-8 culture from the same medium enriched with L-[*methyl*-^{13}C]methionine (50 mg/200 ml of medium) 30 h after inoculation. (C) The [*methyl*-^{13}C]pP$_x$GM was added to low-phosphate (LPSG) medium (200 mg/200 ml of medium) on day 3, reisolated from the culture filtrate 5 days later, and then subjected to ^{13}C NMR spectroscopy. Spectra were recorded, with 13,476, 18,557, and 13,649 acquisitions for panels A to C, respectively, and with 90° radio frequency pulses of 26 μs applied at 4-s intervals. The signals at 56.75 ppm (designated "a") and 47.68 ppm (designated "b") represent the methyl carbons of phosphocholine and *N,N'*-dimethylphosphoethanolamine, respectively, attached to C-6 of mannopyranosyl residues in pP$_x$GM. Signals were previously assigned by Unkefer et al. (35, 36).

obtained from SG medium; that of Fig. 3B represents the [*methyl*-^{13}C]pP$_x$GM from the same medium but enriched with L-[*methyl*-^{13}C]methionine. The chemical shifts of each ^{13}C signal in the spectra of pP$_x$GM have been established (35, 36). In Fig. 3A, the signal at 56.83 ppm (designated "a") represents methyl carbons of phosphocholine attached to C-6 of mannopyranosyl residues. The methyl carbon signal at 47.24 ppm (designated "b") was tentatively identified as that of *N,N'*-dimethylphosphoethanolamine (36) attached to C-6 of other mannopyranosyl residues in pP$_x$GM. In Fig. 3B, the signal intensities of methyl groups of phosphocholine and *N,N'*-dimethylphosphoethanolamine increased about 25-fold compared with those in Fig. 3A, indicating that the methyl carbons of both choline and dimethyl ethanolamine of extracellular pP$_x$GM were metabolically enriched with ^{13}C. The purified [*methyl*-^{13}C]pP$_x$GM was then used as a probe for the initial survey of ^{13}C-methyl-labeled substances in intracellular solute pools by ^{13}C NMR spectrometry.

Release of phosphocholine from extracellular pP$_x$GM. The fate of phosphocholine in extracellular pP$_x$GM was determined during the growth of *P. fellutanum* in low-phosphate medium. Purified [*methyl*-^{13}C]pP$_x$GM was added to the low-phosphate (LPSG) cultures and recovered from the culture filtrates 5 days later and then analyzed by ^{13}C NMR for any compositional changes. The two signals, representing ^{13}C-labeled phosphocholine (56.83 ppm) and *N,N'*-dimethylphosphoethanolamine (47.24 ppm), completely disappeared from the added [*methyl*-^{13}C]pP$_x$GM (Fig. 3C). This interpretation of the data was confirmed by ^{31}P NMR spectroscopy, which showed that the phosphocholine signal resonating at 0.22 ppm significantly decreased (data not shown).

It was also observed that signals at ~110 and 84 ppm, which

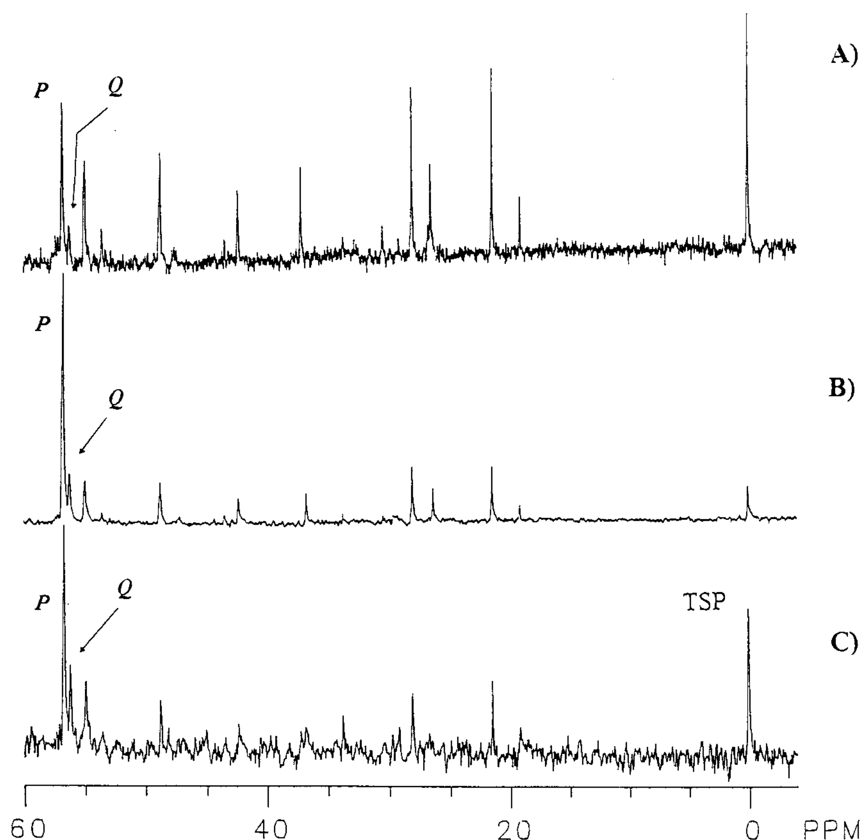

FIG. 4. Proton-decoupled ^{13}C NMR spectra of mycelial extracts. Mycelial extracts were obtained from 200 ml of low-phosphate LPSG medium (A) and of the same medium supplemented with either 200 mg of [*methyl*-^{13}C]pP_xGM (B) or 200 mg of unlabeled pP_xGM (C). All cultures were harvested 8 days after inoculation, and the extract in the spectrum in panel B was surveyed for the presence of any ^{13}C-enriched substances in the intracellular solute pools. Either ^{13}C-labeled or unlabeled pP_xGM was added 72 h after inoculation. To 4 ml of each extract, 0.5 ml of D_2O containing 2% sodium TSP was added. Sodium TSP was used as an internal reference for the chemical shifts and also as a reference for integrating the peak intensity of each signal. Integration was performed with Felix for Windows 1.02 software (Molecular Simulations Inc.), and relative values were calculated by setting the intensity of the sodium TSP signal to 1.0. The integrated values were 8.64, 1.42, and 1.00 (B) and 2.00, 0.63, and 1.00 (C) for *P*, *Q*, and sodium TSP, respectively. Spectra were recorded with 1,000 acquisitions, and 90° radio frequency pulses of 35 μs were applied at 4-s intervals for all three spectra. *P*, 56.77 ppm; *Q*, 56.23 ppm.

represent galactofuranosyl residues (see reference 36 for signal assignment), were no longer present in the polymer (Fig. 3C).

Evidence for the cytosolic localization of methyl carbons of phosphocholine from extracellular pP_xGM. Intracellular solute pools of mycelia obtained from cultures initially containing 2 mM phosphate and either unsupplemented (Fig. 4A), supplemented with [*methyl*-^{13}C]pP_xGM (Fig. 4B) or supplemented with unlabeled pP_xGM (Fig. 4C) were surveyed for the presence of any ^{13}C-labeled substance(s) (Fig. 4B). Each extract contained the same concentration of sodium TSP (0.22%) as a reference for integration of peak intensities of individual signals. Integration was performed with Felix for Windows 1.02 Software (Molecular Simulations Inc.). The spectral window of 0 to 60 ppm covers the chemical shift range of methyl carbons. Signals that resonated at 56.77 and 56.23 ppm (designated *P* and *Q*, respectively) in each spectrum are at positions in the region expected for N^+-trimethylated carbons.

With the sodium TSP signal as a reference, comparison of relative intensities of signals *P* and *Q* in Fig. 4B with those in Fig. 4A and C showed that the relative intensities of signals *P* and *Q* were about 4.3-fold and 2.3-fold higher than those in the extract from the culture supplemented with unlabeled pP_xGM (Fig. 4C). These data show that signals *P* (56.77 ppm) and *Q* (56.23 ppm) in Fig. 4B are the ^{13}C-enriched signals and that they originated from the extracellular ^{13}C-methyl-labeled phosphocholine of the [*methyl*-^{13}C]pP_xGM added to the culture. This suggests that at least the methyl groups of phosphocholine from extracellular pP_xGM were relocalized as a component of intracellular *methyl*-carbon-containing solutes. Addition of unlabeled pP_xGM to the culture (Fig. 4C) resulted in a small increase in signals *P* and *Q*, compared with those in Fig. 4A.

Identification of two intracellular choline derivatives of *P. fellutanum* as COS and GB. Identification of the two methyl-containing substances that accumulated inside of the mycelia was performed by proton-decoupled ^{13}C NMR spectroscopy. Dissolving authentic commercial GB (30 mM) directly into a mycelial extract resulted in a significant increase in signals at 56.23, 69.04, and 172.04 ppm (designated *Q*, *Q*1, and *Q*2, respectively, in Fig. 5A and *G*1, *G*2, and *G*3, respectively, in Fig. 5B). These results demonstrated that the mycelial extract contained GB derived from extracellular pP_xGM. Subsequently, solid authentic COS (30 mM) was dissolved in the mycelial extract containing added authentic GB. This resulted in an increase of the three signals resonating at 56.77, 64.90, and 67.68 ppm (designated *P*, *P*1, and *P*2, respectively, in Fig. 5A and *COS*1, *COS*2, and *COS*3, respectively, in Fig. 5C), indicating that the 56.77-ppm substance in the original extract was COS. Finally, authentic choline chloride (30 mM) was dis-

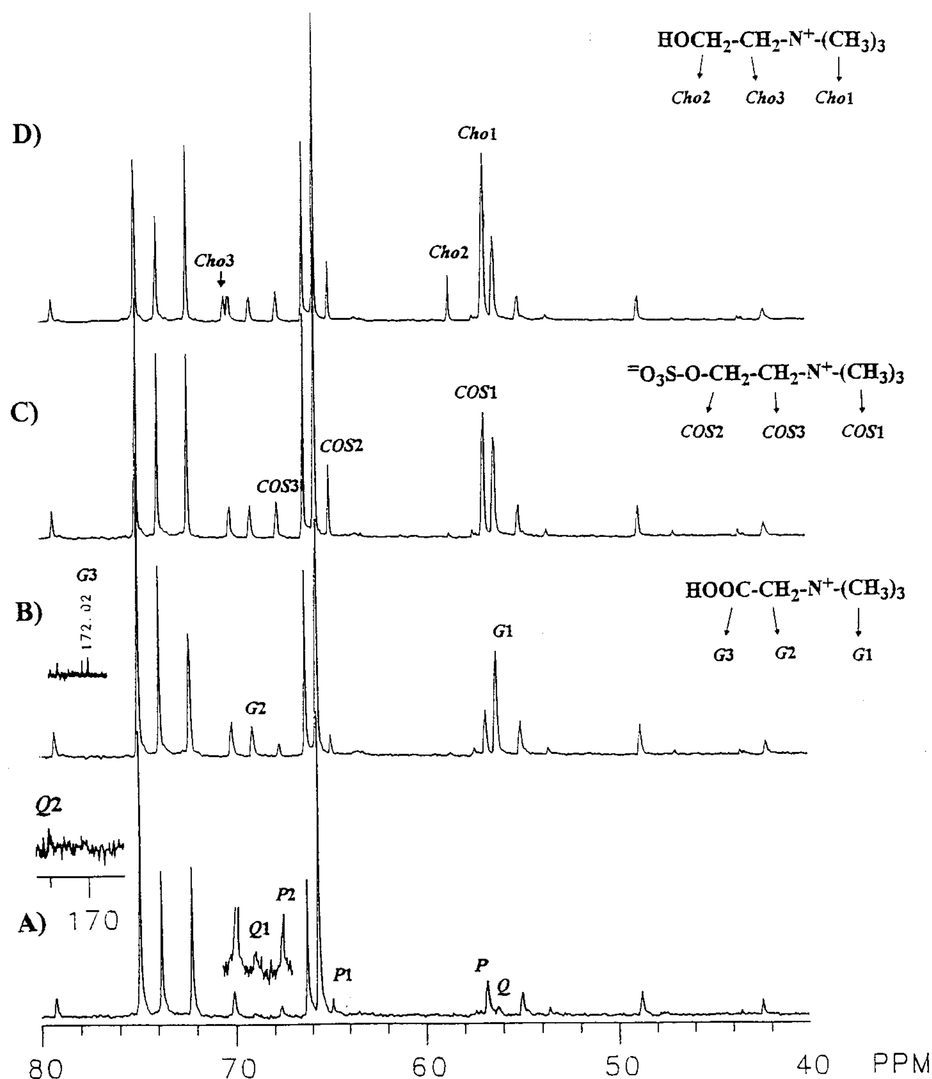

FIG. 5. Identification of two intracellular choline derivatives as COS and GB. Spectra A through D (proceeding from the bottom to the top) represent mycelial extract (A), mycelial extract plus added authentic solid GB chloride (B), mycelial extract plus added GB and COS (C), and mycelial extract plus added GB, COS, and choline chloride (D). The final concentrations of each authentic chemical was 30 mM. Spectra were recorded after 2,000 acquisitions, with a pulse width of 35 μs (90°) and an acquisition time of 204.80 ms. *P*, 56.77 ppm; *Q*, 56.23 ppm; *G*1, *G*2, and *G*3, N^+-$(CH_3)_3$, CH_2-N^+, and COOH of GB, respectively; *COS*1, *COS*2, and *COS*3, N^+-$(CH_3)_3$, CH_2-N^+, and CH_2O-SO_3 of COS, respectively; *Cho*1, *Cho*2, and *Cho*3, N^+-$(CH_3)_3$, CH_2-N^+-, and CH_2OH of choline chloride, respectively. The insets were plotted with twofold enlargement. The chemical shift of the signal in the inset of the spectrum in panel A is 172.00 ppm.

solved in the mycelial extract containing authentic GB and COS. This resulted in, as shown in Fig. 5D, further increase of the 56.77-ppm signal and appearance of two new signals resonating at 58.59 (designated *Cho*2) and 70.22 ppm (*Cho*3). These two signals (*Cho*2 and *Cho*3 in Fig. 5D) are missing in Fig. 5A, indicating that there was no detectable choline in the original mycelial extract.

Intracellular COS and GB were partially fractionated through a Bio-Rad AG-50w (H^+) column (not shown). The substance responsible for the 56.77 ppm signal did not bind to the column preequilibrated with 0.1 N HCl, whereas that responsible for the 56.23 ppm signal bound at this pH. The two fractions were separately spotted on a thin-layer chromatography plate, and both showed positive reactivity to sprayed Dragendorff reagent (4), indicating that these fractions contained quaternary amine molecules (not shown).

These results, taken together, showed that phosphocholine (and possibly *N,N'*-dimethylphosphoethanolamine) attached to extracellular pP_xGM accumulates primarily as COS and GB inside of the mycelia of *P. fellutanum* cultured for 8 days in low-phosphate medium.

Effect of the concentrations of phosphate in the culture medium on the accumulation of intracellular COS and GB. Mycelial extracts obtained from the cultures after 8 days of growth in the LPSG medium containing 2 mM phosphate or in the SG medium containing 20 mM phosphate were analyzed by proton-decoupled ^{13}C NMR spectroscopy. The reference relating the values obtained upon integration was 0.22% sodium TSP added to each sample.

Mycelia obtained from LPSG medium contained 9.3 and 1.5 mg of COS and GB, respectively, per g (dry weight) of mycelia (Table 1). In contrast, no GB was detected in mycelia from high-phosphate medium and only 6.4 mg of COS per g (dry weight) of mycelia was found. This result could be correlated to the high-level production of extracellular phosphodiesterase, PC:PCH, and AP in low-phosphate culture medium.

TABLE 1. Effect of phosphate concentration in culture medium on accumulation of intracellular choline derivatives

Medium (concn. of P_i [mM])[a]	Growth[b]	Choline derivative (mg/g [%])[c]	
		COS	GB
LPSG (2)	3.46 ± 0.35	9.3 (0.93)	1.5 (0.15)
SG (20)	4.38 ± 0.21	6.4 (0.64)	ND[d]

[a] Cultures were grown for 8 days, and the water-soluble materials of mycelial extracts were analyzed by ^{13}C NMR spectroscopy.

[b] Means of triplicates ± standard deviations, in grams (dry weight) per 200 ml of culture medium.

[c] The amounts of COS and GB (in milligrams per gram [dry weight]) were determined by integrating individual signals on ^{13}C NMR spectra, using Felix for Windows 1.02 software (Molecular Simulations Inc.), with sodium TSP (0.22%) used as reference. Numbers in parentheses represent COS and GB as percentages of mycelial dry weight.

[d] ND, no detectable signal.

DISCUSSION

P. fellutanum produces extracellular phosphocholine-containing polysaccharide (pP_xGM) (13, 35). The number of phosphodiester residues varies depending upon culture conditions such as C source or phosphate starvation (26, 28, 29, 34). Although it was suggested (12) that pP_xGM had a role in survival of *Penicillium* when some nutrients became depleted, the selective removal of a component of pP_xGM and uptake of that component from the medium have not been demonstrated previously.

During this study to determine possible role(s) of phosphocholine attached to the C-6 position of mannopyranosyl residues of extracellular pP_xGM, we found that, under low-phosphate conditions, phosphocholine disappeared from pP_xGM (Fig. 3). The methyl groups of COS and GB, enriched with ^{13}C derived from the [*methyl*-^{13}C]pP_xGM, accumulated as soluble constituents in mycelia (Fig. 4). Choline did not accumulate significantly as a soluble intermediate in the mycelia. COS is known as a storage form of choline and sulfate in filamentous fungi (21). Our results suggest that extracellular pP_xGM is a reserve source of choline in *P. fellutanum*. However, these data do not exclude another role(s) for phosphocholine and phosphoethanolamine diester residues of pP_xGM in fungal physiology. No significant enrichment of methyl carbons of either COS or GB occurred in a similar experiment performed in a medium containing 20 mM phosphate instead of 2 mM phosphate (data not shown). These data, coupled with the severalfold increase in AP and nonspecific PC:PCH activities in cultures initially containing (i) 2 mM phosphate, compared with those containing (ii) 20 mM phosphate, are consistent with the following: *P. fellutanum* has a mechanism for recycling phosphate and for conserving excess choline not immediately required for synthesis of new membranes. COS is a primary storage form of choline; however, GB could be a secondary storage form. To our knowledge, this is the first time that an extracellular choline-containing complex polysaccharide has been demonstrated as a precursor of intracellular COS, GB, or other choline derivative.

The overall process of reutilization of specific nutrients from complex polysaccharides is likely associated with derepression of extracellular enzyme synthesis and secretion of nonspecific PC:PCH and an AP to accommodate phosphate depletion from the culture medium. Coupling the release of choline with that of phosphate from a storage polymer, pP_xGM, is consistent with synthesis of membranes which are major sinks for both phosphate and choline derivatives. Possibly, small quantities of other important metabolites are also covalently attached to pP_xGM and can be released when culture medium becomes depleted of this component. This type of recycling of nutrients back into the organism provides a means of conserving important nutrients or metabolites until they are needed and also a means of selectively releasing them. This may be a general mechanism for providing for continued survival and growth in a nutritionally unbalanced environment.

ACKNOWLEDGMENTS

This work was supported by the Florida Agricultural Experiment Station (Journal Series No. R-05277). This research was supported by a Research Project Enhancement Award from the Office of the Dean for Research, Institute of Food and Agricultural Sciences, University of Florida, Gainesville.

We thank J. F. Preston and K. T. Shanmugam for helpful comments.

REFERENCES

1. **Ballou, C. E.** 1976. Structure and biosynthesis of the mannan component of the yeast cell envelope. Adv. Microb. Physiol. **14:**93–158.
2. **Barreto-Bergter, E., and P. A. J. Gorin.** 1983. Structural chemistry of polysaccharides from fungi and lichens. Adv. Carbohydr. Chem. Biochem. **41:** 67–103.
3. **Berkeley, R. C. W., G. W. Gooday, and D. C. Ellwood (ed.).** 1979. Special Publications of the Society for General Microbiology. Microbial polysaccharides and polysaccharases. Academic Press, London, United Kingdom.
4. **Blunden, G., and S. M. Gordon.** 1986. Betaines and their sulphonio analogues in marine algae. Prog. Physiol. Res. **4:**39–80.
5. **Bonetti, S. J., J. E. Gander, and B. A. Tuekam.** 1993. The *N*-peptidyl ethanolamine phosphodiesters: phosphate bridging structures in the galactan chains of *Penicillium* glycopeptides. FASEB J. **7:**A1260.
6. **Bull, A. T.** 1972. Environmental factors influencing the synthesis and excretion of exocellular macromolecules. J. Appl. Chem. Biotechnol. **22:**261–292.
7. **Clutterbuck, P. W., A. E. Oxford, H. Raistrick, and G. Smith.** 1932. Studies in the biochemistry of micro-organisms. XXIV. The metabolic products of the *Penicillium brevi-compactum* series. Biochem. J. **28:**94–110.
8. **Dow, J. M., and J. A. Callow.** 1979. Leakage of electrolytes from isolated leaf mesophyll cells of tomato induced by glycopeptides from culture filtrates of *Fulvia fulva* (Cooke) Cifferi (syn. *Cladosporium fulvum*). Physiol. Plant Pathol. **15:**27–34.
9. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
10. **Gander, J. E., N. H. Jentoft, L. R. Drewes, and P. D. Rick.** 1974. The 5-O-β-D-galactofuranosyl-containing exocellular glycopeptide of *Penicillium charlesii*. Characterization of the phosphogalactomannan. J. Biol. Chem. **249:**2063–2072.
11. **Gander, J. E., J. C. Beachy, C. J. Unkefer, and S. J. Tonn.** 1980. Toward understanding the structure, biosynthesis, and function of a membrane-bound fungal glycopeptide. Structural studies. Am. Chem. Soc. Symp. Ser. **126:**49–79.
12. **Gander, J. E., and C. J. Laybourn.** 1981. Galactofuranosyl-containing lipoglycopeptide in *Penicillium*. Recent Adv. Phytochem. **15:**59–91.
13. **Gander, J. E.** 1974. Fungal cell wall glycoproteins and peptidopolysaccharides. Annu. Rev. Microbiol. **49:**103–119.
14. **Halverson, L. J., and G. Stacey.** 1986. Signal exchange in plant-microbe interactions. Microbiol. Rev. **50:**193–225.
15. **Holtje, J.-V., and A. Tomasz.** 1975. Specific recognition of choline residues in the cell wall teichoic acid by the *N*-acetylmuramyl-L-alanine amidase of pneumococcus. J. Biol. Chem. **250:**6072–6076.
16. **Horne, D. S., and A. Tomasz.** 1993. Possible role of a choline-containing teichoic acid in the maintenance of normal cell shape and physiology in *Streptococcus oralis*. J. Bacteriol. **175:**1717–1722.
17. **Jeanes, A., J. E. Pittsley, P. R. Watson, and R. J. Dimler.** 1961. Characterization and properties of the phosphomannan from *Hansenula holstii* NRRL Y-2448. Arch. Biochem. Biophys. **92:**343–350.
18. **Kilpper-Bälz, R., P. Wenzig, and K. H. Schleifer.** 1985. Molecular relationships and classification of some viridans streptococci as *Streptococcus oralis* and emended description of *Streptococcus oralis* (Bridge and Sneath 1982). Int. J. Syst. Bacteriol. **35:**482–488.
19. **Kohama, T., M. Fujimoto, A. Kuninaka, and H. Yoshino.** 1974. Structure of malonogalactan, an acidic polysaccharide of *Penicillium citrinum*. Agric. Biol. Chem. (Japan) **38:**127–134.
20. **Leal, J. A., C. Guerrero, B. Gomez-Miranda, A. Prieto, and M. Bernabe.** 1992. Chemical and structural similarities in wall polysaccharides of some *Penicillium*, *Eupenicillium* and *Aspergillus* species. Microbiol. Lett. **90:**165–168.

21. **Markham, P., G. D. Robson, B. W. Bainbridge, and A. P. J. Trinci.** 1993. Choline: its role in the growth of filamentous fungi and the regulation of mycelial morphology. FEMS Microbiol. Rev. **104:**287–300.
22. **Mosser, J. L., and A. Tomasz.** 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. **245:**287–298.
23. **Podvin, L., G. Reysset, J. Hubert, and M. Sebald.** 1988. Presence of choline in teichoic acid of *Clostridium acetobutylicum* NI-4 and choline inhibition of autolytic function. J. Gen. Microbiol. **134:**1603–1609.
24. **Preston, J. F., E. Lapis, and J. E. Gander.** 1969. Isolation and partial characterization of the exocellular polysaccharides of *Penicillium charlesii*. II. The occurrence of phosphate groups in high molecular weight polysaccharides. Arch. Biochem. Biophys. **134:**324–334.
25. **Rick, P. D., L. R. Drewes, and J. E. Gander.** 1974. The 5-O-β-D-galactofuranosyl-containing exocellular glycopeptide of *Penicillium charlesii*. Occurrence of ethanolamine and partial characterization of the phosphogalactomannan. J. Biol. Chem. **249:**2073–2078.
26. **Rietschel-Berst, M., N. H. Jentoft, P. D. Rick, C. Pletcher, F. Fang, and J. E. Gander.** 1977. Extracellular exo-β-D-galactofuranosidase from *Penicillium charlesii*. Isolation, purification and properties. J. Biol. Chem. **252:**3219–3226.
27. **Rolin, D. B., P. E. Pfeffer, S. F. Osman, B. S. Szwergold, F. Kappler, and A. J. Benesi.** 1992. Structural studies of a phosphocholine substituted β-(1,3):(1,6) macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110. Biochim. Biophys. Acta **1116:**215–225.
28. **Salt, S. D., and J. E. Gander.** 1988. Peptidophosphogalactomannans from *Penicillium charlesii*. Effects of culture pH and phosphate limitation. Exp. Mycol. **12:**243–257.
29. **Salt, S. D., and J. E. Gander.** 1985. Variations in phosphoryl substituents in extracellular peptidophosphogalactomannans from *Penicillium charlesii* G. Smith. Exp. Mycol. **9:**9–19.
30. **Sandford, P. A., and K. Matsuda (ed.).** 1980. American Chemical Society Symposium Series, vol. 126. Fungal polysaccharides. American Chemical Society, Washington, D.C.
31. **Slodki, M. E.** 1962. Phosphate linkages in phosphomannans from yeast. Biochim. Biophys. Acta **57:**525–533.
32. **Stevens, C. M., and P. Vohra.** 1955. Occurrence of choline sulfate in *Penicillium chrysogenum*. J. Am. Chem. Soc. **77:**4935–4936.
33. **Tomasz, A.** 1967. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in pneumococcus. Science **157:**694–697.
34. **Tuekam, B. A., and J. E. Gander.** 1993. Isolation, purification and characterization of *Penicillium* exo-β-D-galactofuranosidase. FASEB J. **7:**A1254.
35. **Unkefer, C. J., C. L. Jackson, and J. E. Gander.** 1982. The 5-O-β-D-galactofuranosyl-containing exocellular glycopeptide from *Penicillium charlesii*. Identification of phosphocholine attached to C_6 mannopyranosyl residues of the mannan region. J. Biol. Chem. **257:**2491–2497.
36. **Unkefer, C. J., and J. E. Gander.** 1979. The 5-O-β-D-galactofuranosyl-containing glycopeptide from *Penicillium charlesii*. Carbon-13 nuclear magnetic resonance studies. J. Biol. Chem. **254:**12131–12135.