Purification, Characterization, and Identification of a Sphingomyelin Synthase from *Pseudomonas aeruginosa*

PlcH IS A MULTIFUNCTIONAL ENZYME

Chiara Luberto§, Martin J. Stonehouse§, Elizabeth A. Collins‡, Norma Marchesini‡, Samer El-Bawab‡, Adriana I. Vasil¶, Michael L. Vasil¶, and Yusuf A. Hannun‡

*From the ‡Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425 and the §Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262*

Sphingomyelin synthase is the enzyme that synthesizes sphingomyelin (SM) in mammalian cells by transferring a phosphorylcholine moiety from phosphatidylcholine to ceramide. Despite its importance, the gene and/or the protein responsible for this activity has not yet been identified. Here we report the purification, identification, and biochemical characterization of an enzyme that synthesizes SM in *Pseudomonas aeruginosa*. SM synthase-like activity was found secreted in the culture medium of *P. aeruginosa*, strains PA01 and PAK, whereas it could not be detected in cultures of *Escherichia coli*. From the medium of PAK cultures, SM synthase was purified through sequential chromatographic columns. After separation on polyacrylamide-SDS gels and visualization by silver staining, the purified enzyme showed two bands, one of 75 kDa and one of 35–35 kDa. Interestingly, the highly purified SM synthase preparation also showed neutral sphingomyelinase activity. We therefore investigated whether the protein we purified as SM synthase could actually be the previously identified PlcH, a 78-kDa phospholipase C known to hydrolyze phosphatidylcholine and SM in *P. aeruginosa*. First, the purified SM synthase preparation contained a 78-kDa protein that reacted with monoclonal antibodies raised against purified PlcH. Second, purified PlcH showed SM synthase activity. Third, using different knockout mutant strains for the *PlcH* operon, *PlcH* was found to be necessary for SM synthase activity in *P. aeruginosa*. Interestingly, SM synthase activity was specific to the *Pseudomonas* PlcH as other bacterial phospholipases did not display SM synthase activity. Biochemical studies on the *Pseudomonas* SM synthase confirmed that it is a transferase, similar to the mammalian enzyme, that specifically recognizes the choline head-group and the primary hydroxyl on ceramide. This SM synthase did not have reverse transferase activity. In conclusion, the *Pseudomonas* PlcH also exerts SM synthase activity; therefore, for the first time, we have identified a structural gene for a SM synthase.

Sphingomyelin (SM)

1 The abbreviations used are: SM, sphingomyelin; PlcH, hemolytic phospholipase C; N-SMase, neutral sphingomyelinase; SMase, sphingomyelinase; PC-PLC, phosphatidylcholine-specific phospholipase C; p-NPPC, p-nitrophenolphosphoholme; DAG, diacylglycerol; deH₂O₂, double distilled H₂O; MES, 4-morpholineethanesulfonic acid; CDase, ceramidase; NBD, (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)).
Identification of SM Synthase in P. aeruginosa

Blue Sepharose—Fractions with peak activity from the octyl-Sepharose column were pooled, adjusted to pH 6.0, and loaded (0.3 mg of proteins/ml of column) at 0.5 ml/min on a 2-ml Blue Sepharose high performance column equilibrated with buffer C (20 mM MES, pH 6.0, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.005% Triton X-100). A step elution with 7.5 column volumes of 12% buffer D (buffer C + 1.5 mM NaCl) was followed by an increasing linear gradient of NaCl (from 12 to 75% buffer D) and a column wash with 75% buffer for 2.1 column volumes each. One-ml fractions were collected, and the SM synthase activity (50 µl) and protein concentration were determined. The fractions with peak activity were then pooled, desalted, and buffer-exchanged with buffer E (20 mM Tris, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.005% Triton X-100) on PD-10 desalting columns.

Mono P—The desalted, buffer-exchanged fractions were applied to a 1-ml Mono P column equilibrated with Buffer E. The column was washed at 0.5 ml/min with 7 column volumes of Buffer E, and then eluted by decreasing the pH with Buffer F (1:8 dilution of Polybuffer 74, pH 4.0). Fractions of 0.5 ml were collected and neutralized with concentrated Tris buffer, pH 7.4—9.0. SM synthase activity (50 µl) was determined.

Mono S—The fractions with peak activity from the Mono P column were combined and adjusted to 50 mM sodium acetate, pH 4.0, then loaded onto a 1 ml Mono S column previously equilibrated with Buffer G (50 mM sodium acetate, pH 4.0, 0.2 mM phenylmethylsulfonyl fluoride, 0.005% Triton X-100). A column wash at 0.5 ml/min with 5 column volumes of Buffer G preceded the elution step with Buffer H (Buffer G + 1.5 mM NaCl) consisting of a linear gradient (0—0.75 mM NaCl) of 10 column volumes and 20 column volumes of 1.5 mM NaCl. One-ml fractions were collected in tubes containing 0.1 ml of 1.5 ml Tris, pH 9.0. SM synthase assay was performed on 100 µl fraction, and active fractions were concentrated on YM-10 Microcon centrifugal filters, separated on an 8% Tris-glycine gel, and visualized by silver staining.

SM Synthase Assay—The SM synthase assay was performed using aliquots (50–100 µl depending on the column) from the fractions of interest. The substrate was prepared as a 2× mixture of 40 µM NBD-C6-ceramide and 240 µM PC suspended in 100 mM Tris, pH 7.4, 50 mM KCl, 1 mM EDTA by sonication and vortexing until clear. The substrate was diluted 1:1 with the protein, and the incubation was carried out for 1 h in the dark at 37 °C. The reaction was stopped on ice by addition of a volume of chloroform:methanol (1:1, v/v) 3 times the assay volume. After vortexing, the phases were clarified by centrifugation at 2500 × g for 5 min. The lower phase was transferred to new tubes, dried down, and lipids were resuspended with 30 µl of chloroform:methanol (2:1, v/v). NBD-C6-sphingomyelin was separated from the substrate by thin layer chromatography in chloroform, methanol, and 15 mM CaCl2 (60:35:8). Fluorescence was measured by using a Storm 860 Imaging Analysis System from Amersham Biosciences. Data were analyzed using ImageQuant software from Amersham Biosciences.

Characterization Experiments—Pierce Biochel was purified as previously described (26) and diluted to 0.1 µg/ml in 50 mM Tris, pH 7.4 for the SM synthase assay. The reaction mixture contained 16.7 µg/ml of purified protein, 75 mM Tris, pH 7.4, 100 µM NBD-C6-ceramide, and 120 µM PC in a total volume of 100 µl and was carried out for 15 and 30 min at 30 °C. Formation of NBD-C6-SM was determined as previously indicated. In some experiments, radioactive PC was used instead of fluorescent C6-ceramide as the labeled substrate. Briefly, a 2× substrate mixture containing 200 µM C6-ceramide and 240 µM [14C]-choline)PC (specific activity of ~45 nCi/nmol) was resuspended in 0.1 mM Tris, pH 7.4. The substrate was diluted 1:1 with the protein (200 µl final reaction volume) and incubated for 30 min at 30 °C in the dark. The reaction was stopped with 1.5 ml of chloroform:methanol (2:1, v/v) and protein and vortexing performed. The butyl-Sepharose column was then washed with 5 column volumes of double distilled water (ddH2O) at a rate of 2 ml/min, and fractions of 5 ml were collected. Water wash fractions were also tested for SM synthase activity and protein concentration, and fractions with peak activity were combined with fractions with peak activity from the elution of the Butyl-Sepharose.

Octyl-Sepharose—Pooled fractions with peak activity from the butyl-Sepharose column were adjusted to 1 mM ammonium sulfate and loaded on a 1-ml octyl-Sepharose column (0.7 mg of proteins/ml of column) previously equilibrated with buffer B (buffer A + 1 mM ammonium sulfate) at a flow rate of 0.5 ml/min. The bound proteins were washed with 5 column volumes of buffer B at 1 ml/min flow rate. Proteins were eluted by decreasing the ammonium sulfate concentration from 1 M to zero applying a 6-column volume gradient of buffer A. Fractions of 3 ml were collected, and SM synthase activity and protein concentration, and fractions with peak activity were combined with fractions with peak activity from the elution of the Butyl-Sepharose.

Butyl-Sepharose—The filtered solution was adjusted to 1 mM ammonium sulfate and applied to a 20-ml HiTrap butyl-Sepharose column (1.2 mg of proteins/ml of column) previously equilibrated with buffer B (buffer A + 1 mM ammonium sulfate) at a flow rate of 0.5 ml/min. The bound proteins were washed with 5 column volumes of buffer B at 1 ml/min flow rate. Proteins were eluted by decreasing the ammonium sulfate concentration from 1 M to zero applying a 6-column volume linear gradient of buffer A. Fractions of 3 ml were collected, and SM synthase activity and protein concentration, and fractions with peak activity were combined with fractions with peak activity from the elution of the Butyl-Sepharose.
After vortexing and centrifugation at 1500 × g for 5 min, 400 µl from the upper phase were counted in 4 ml of scintillation fluid.

**PC-PLC Activity**—Purified PlcHR, (40–140 pg/µl) was incubated with 20 µM p-nitrophenylphosphorylcholine (p-NPPC) in 100 mM Tris, pH 7.4, and 25% glycerol, in a total volume of 200 µl. Incubation was carried out at 37 °C for 1 h in a 96-well plate, and production of the chromogenic product p-nitrophenol was measured at 405 nm (26).

**BCA Protein Determination**—Protein concentration was determined according to the instructions from the manufacturer (Pierce).

**Western Blotting**—The Western blot analysis for PlcHR, was performed as previously described (26). Proteins were separated on a 7% PAGE-SDS gel and transferred onto nitrocellulose at 80 V for 1 h. The membranes were blocked with 5% milk in phosphate-buffered saline containing 0.05% Tween, and blotted with antibodies (H952, 1:250 dilution) in the blocking solution. After extensive washing, the membranes were blocked with secondary anti-mouse horseradish peroxidase-conjugated antibodies (1:4000 dilution) in blocking medium (Amersham Biosciences).

**RESULTS**

**Purification of SM Synthase from P. aeruginosa**—Given that *P. aeruginosa* produces and secretes enzymes with sphingolipid-metabolizing activities such as neutral sphingomyelinase and neutral/alkaline ceramidase, we wondered whether this bacterium also produced SM synthase activity. Indeed we found that two different strains of *P. aeruginosa*, PA01 and PAK (but not other bacteria such as *Escherichia coli*) produced and secreted in the medium an enzymatic activity able to synthesize SM using phosphatidylcholine and ceramide as substrates (data not shown). The activity found in the medium from PAK cultures was greater than that measured from PA01; therefore, the protocol was optimized for the purification of the secreted SM synthase activity from *Pseudomonas* using the PAK strain.

SM synthase present in the medium of an overnight culture was concentrated by incubation with 60% ammonium sulfate, and then the precipitated proteins were resuspended in 20 mM MES, pH 6.5. The suspension was filtered, adjusted to a final concentration of 1 mM ammonium sulfate, and subjected to hydrophobic interaction chromatography using a butyl-Sepharose column previously equilibrated with the same buffer containing 1 mM ammonium sulfate (buffer B) (Fig. 1A). In these conditions, most of the activity was found to bind to the matrix whereas the bulk of the proteins (~84% of the total proteins), represented mainly by small size proteins and/or peptides, were found in the flow-through. Elution of the activity was observed in two distinct peaks upon decrease of the ammonium sulfate concentration, a first peak at ~0.43 mM ammonium sulfate and a second peak at ~100 mM ammonium sulfate. Moreover, when the column was regenerated with a wash of double distilled water, ~30% of the initial SM synthase activity and 10% of proteins were eluted from the column. The fractions containing the two peaks of activity were combined with the activity recovered in the water wash, and the buffer and salt concentration were adjusted to 20 mM MES, pH 6.5, and 1 mM ammonium sulfate. The total volume was applied to an octyl-Sepharose column (Fig. 1B) previously equilibrated with the same buffer. The SM synthase activity was found to bind to the column, as did most of the other proteins. Elution of SM synthase activity contained only two bands, one at ~75 kDa and one at ~30 kDa, and both correlated with the activity (data not shown).

**Purified SM Synthase Also Shows N-SMase Activity**—*P. aeruginosa* PA01 is known to express and secrete a N-SMase activity (encoded by the *plcH* gene) for which the theoretical isoelectric point and molecular mass are ~5.0 and 78 kDa, respectively (20), similar to what was observed with the purified SM synthase. Because SM synthase activity was observed in both PAK and PA01 strains, and the gene encoding for N-SMase activity in PAK is identical to the *plcH* gene in PA01, we wondered whether the purified preparation of SM synthase from PAK also contained N-SMase activity. To this end, N-SMase activity was measured in the fractions collected from the Mono P chromatofocusing column (Fig. 2A). As shown in the figure, the enzyme activity was detected in the fractions but this activity mimicked the profile of the SM synthase activity (Fig. 1D). In contrast, no significant activity was detected (data not shown). N-SMase activity was then measured in fractions collected from the last column of the purification protocol, the cation exchange chromatography (Fig. 2B). Also in this case, N-SMase activity paralleled exactly SM synthase activity.

To confirm the existence of a N-SMase in the highly purified SM synthase preparation, a Western blot analysis was performed on the fractions with peak activity for SM synthase from a Blue Sepharose column using monoclonal antibodies raised against purified PlcH from *P. aeruginosa*, strain PA01 (26) (Fig. 3). As shown in the figure, fractions from the elution

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*M. L. Vasil, unpublished observations.
step with peak SM synthase activity (fractions 42–46, Fig. 3A) tested positive for the presence of a 78-kDa protein, which reacted with the specific antibodies against PlcH and that run similarly to the purified PlcH from PA01 (Fig. 3B). These results show that the preparation of SM synthase purified from PAK indeed contained also a protein homologous to the PlcH isolated and purified from strain PA01. Therefore, these results raised the question of whether the SM synthase from PAK is identical to this N-SMase.

PlecHR₂ Is Sufficient and Necessary for SM Synthase Activity—Given the high homology between N-SMase from PAK and PlcH from PA01, we utilized purified PlcH from PA01 to determine whether this enzyme also had SM synthase activity in vitro (Fig. 4). The protein responsible for the N-SMase activity in PA01 (PlcH) is secreted as a complex with two accessory proteins (PlecR₁ and PlecR₂), believed to regulate PlcH secretion (26, 27). In the purification process, however, only PlecR₂ maintains its association with PlcH, forming the PlecHR₂ complex. Therefore, purified PlecHR₂ was incubated with vesicles containing phosphatidylcholine and NBD-C₆-ceramide, and the
formation of NBD-C₆-sphingomyelin was monitored as described under “Experimental Procedures.” As shown in Fig. 4, purified PlcHR₂ was able to synthesize SM in a protein-dependent (Fig. 4A) and time-dependent (Fig. 4B) manner. Therefore, these results clearly show that PlcHR₂ is sufficient for SM synthase activity.

Next, we asked whether PlcH was necessary for SM synthase activity in *P. aeruginosa*, PA01. To this end, SM synthase activity was measured in different cultures of mutant strains lacking the genes responsible for the synthesis and secretion of PlcH. Because the plcR₁₂ genes are located 3’ to plcH and are expressed from in-phase overlapping genes, the single deletion of plcH causes a block of transcription of the plcR₁₂, thus delivering in fact a plcH⁻⁻/plcR⁻⁻ double knock-out. In the following experiments, strains were used that were lacking in either plcR₁₂ (H₉₀₀₄R) or plcH and plcR₁₂ (H₉₀₀₄HR). As a control we employed a strain that was deficient in the neutral/alkaline ceramidase (H₉₀₀₄CDase) gene, which is adjacent to the PlcHR operon (Fig. 5). As expected, the ΔR strain secreted significantly less N-SMase activity in the media compared with the parental strain (PA01) (left top panel) retaining the activity in the cells (left bottom panel). The lack of the PlcHR operon (ΔHR) caused an almost total disappearance of N-SMase activity in both medium and cells. On the other hand, absence of the CDase gene did not affect significantly N-SMase activity in either medium or cells, demonstrating that the observed effects on N-SMase activity are specific to the PlcHR₁₂ genes. When SM synthase activity was measured in these same cultures, an almost identical profile was observed; the ΔR strain retained the activity in the cells, and lack of the PlcHR operon caused an almost total disappearance of SM synthase in both cells and medium. Moreover, no significant difference between the parental strain and the ΔCDase in both cells and medium was observed when measuring the SM synthase activity, thus confirming also in this case the specificity of the results (Fig. 5). These results therefore support the necessity of the expression of the PlcH for SM synthase activity in *P. aeruginosa*, PA01.

Free PlcR₂ Enhances SM Synthase Activity of Free PlcH—As mentioned, PlcR₁₂ chaperones PlcH out of the cells where their interaction is maintained. More recently it has also been shown that the interaction of free PlcR₂ with free PlcH increased the phospholipase activity of PlcH (26). Therefore we investigated the effect of free PlcR₂ on SM synthase activity of free PlcH (Fig. 6). As shown in the figure, addition of increasing amount of free PlcR₂ to a constant amount of free PlcH increased the phospholipase activity of PlcH (26). Therefore we investigated the effect of free PlcR₂ on SM synthase activity of free PlcH (Fig. 6). As shown in the figure, addition of increasing amount of free PlcR₂ to a constant amount of free PlcH significantly augmented PlcH ability to synthesize NBD-C₆-sphingomyelin. No significant SM synthase activity was detected from free PlcR₂ alone (data not shown). These results suggest that in the case of SM synthase, as for PC-PLC, PlcH may represent the catalytic subunit of the PlcHR₂ complex whereas PlcR₂ could represent a possible regulatory subunit.

Characterization of pH Dependence and Effects of Cations on SM Synthase Activity of PlcHR₂—To characterize and further optimize the conditions for the SM synthase activity of PlcHR₂,
the pH optimum of the reaction was determined using purified PlcHR₂ (Fig. 7A). As shown in the figure, purified PlcHR₂ showed significant activity over a wide range of pH (from 7 to 9.5) with peak activity observed between 7.5 and 8.5. At pH lower than 6.0 and higher than 9.5, activity was almost undetectable.

Next, the effects of cations were studied. It has been described that some divalent cations significantly affect PC-PLC activity of PlcHR₂ (26); therefore, the effects of these cations on SM synthase activity were investigated (Fig. 7B). As shown in the figure, SM synthase activity was effectively inhibited by zinc and to a lesser extent by nickel, thus demonstrating effects similar to those observed on PC-PLC activity. Manganese significantly inhibited SM synthase activity but at higher concentrations than zinc and nickel, also reproducing the effects observed with PC-PLC activity. Calcium, magnesium, and low concentrations of manganese slightly but reproducibly activated SM synthase, whereas they did not significantly affect PC-PLC, suggesting a possible difference in the regulation of these activities.

SM Synthase from P. aeruginosa Is a Transferase—PlcH has been previously described as a phospholipase C that hydrolyzes both PC and SM, releasing phosphorylcholine (20). In mammalian cells, SM synthesis occurs upon transfer of a phosphorylcholine (choline-P) moiety onto the primary hydroxyl of ceramide through a transferase-like reaction (2). Therefore, it became important to determine the biochemical nature of the bacterial SM synthase activity. A screen with different donors of choline-P was set up, and the possibility of each compound to be used as substrate for the synthesis of SM was tested (Fig. 8A). As shown in the figure, free phosphorylcholine (Ch-P) was not a substrate for SM synthesis (as compared with PC). Moreover, neither CDP-choline (CDP-Ch) nor p-NPPC (often used as substrate for the phospholipase C activity in vitro) was used as substrate for SM synthesis. On the other hand, PC, platelet activating factor, and their respective lyso-forms were efficiently recognized as substrates. SM itself and its lyso-counterpart were also used as donors of the phosphorylcholine group but with significantly less efficiency. These results suggest that the bacterial SM synthase is a transferase that requires at least a fatty acyl chain on the choline-P donor molecule to be recognized as a substrate. Next, the possibility that the bacterial SM synthase would use phospholipids other than PC as substrates was investigated. As shown in Fig. 8B, none of the phospholipids tested (phosphatidylethanolamine, phosphatidylinositol, phosphatidylinerine, phosphatidic acid, phosphatidylglycerol) other than PC induced formation of SM. These results suggest that SM synthase specifically recognizes the choline head group on its substrate.

On the other hand, we then determined the specificity of the phosphorylcholine-acceptor molecule (i.e., ceramide). First, the stereospecificity of the transferase reaction when ceramide was used as substrate was evaluated by directly measuring the conversion of 14C-fatty acid-labeled C₆-ceramide stereoisomers to their respective short chain sphingomyelins. As shown in Fig. 9A, the enzyme best operated when the erythro-C₆-ceramide isomers were used as substrates, whereas the three isomers showed lower activity. Importantly, these results suggest that the transferase reaction is stereoselective. Then the ability of C₆-dihydroceramide to be recognized as substrate was evaluated as compared with the ceramide isomers (Fig. 9A), and it was found to be significantly lower than the erythro isomers and similar to the three isomers. This observation might implicate the relevance of the presence of the 4–5 double bond on the ceramide molecule to be efficiently recognized as a substrate. Next, the specificity of the reaction was further tested using short chain diacylglycerol, a molecule biochemically similar to short chain ceramide, and in particular having an accessible hydroxy-group in the sn-3 position. As shown in Fig. 9A, it is apparent that the bacterial enzyme does not recognize diacylglycerol as acceptor molecule for the transferase reaction. This result would suggest that the transferase reaction discriminates among acceptor hydroxyls. To further verify this hypothesis, other alcohols were tested as substrates for the bacterial enzyme (Fig. 9B) in competition with NBD-C₆-ceramide (30 μM; Kᵡ = 20 μM). In this experiment, 1-erythro- and 1-threo-C₆-ceramides were used as controls. As expected from the results in Fig. 9A, 1-erythro-C₆-ceramide, being a good substrate for the transferase activity, efficiently competed with the NBD-C₆-ceramide for the formation of NBD-C₆-SM (~80 and 90% inhibi-
bition at 60 and 90 \( \mu \)M, respectively) whereas L-threo-C\( _6 \)-ceramide was not as effective (20 and 50% inhibition at 60 and 90 \( \mu \)M, respectively). Importantly, none of the alcohols used in the assay, namely ethyl, lauryl, or stearyl alcohol, were able to compete with the NBD-C\( _6 \)-ceramide. Thus, they were neither inhibitors nor substrates because, according to this approach, a substrate would act as a substrate competitor of NBD-C\( _6 \)-ceramide. These results therefore strengthen the hypothesis that the transferase reaction discriminates among acceptor hydroxyl groups.

Altogether, these results show that the transferase reaction is selective in the choice of the phosphorylcholine acceptor molecule at different levels; the reaction is stereoselective, it favors the presence of the 4–5 double bond on the ceramide, and it exchanges only with the ceramide primary hydroxyl and not other hydrophobic alcohols.

**SM Synthase from P. aeruginosa Has No Reverse Activity**—It has been suggested that the enzyme responsible for SM synthesis in Madin-Darby canine kidney cells also has reverse activity (28). Therefore, we investigated whether this was also the case for the bacterial SM synthase. By using radiolabeled sphingomyelin in the choline moiety and diacylglycerol as substrates, formation of radioactive phosphatidylcholine was monitored. To determine the production of phosphatidylcholine, the lipids were extracted with 1.5 ml of chloroform:methanol (2:1, v/v) and 200 \( \mu \)l of dd\( \text{H}_2\text{O} \), and then processed with two alternative protocols. First, the lower phase was base-hydrolyzed (causing liberation of radioactive choline-P from the newly synthesized PC), and, after a second lipid extraction, aliquots of the aqueous phase were counted (Table I). Alternatively, the whole lower phase from the first extraction was dried down and lipids separated by thin layer chromatography (TLC) (Fig. 10A). As shown in the table and in the figure, no formation of radioactive PC and therefore transferase activity was detected by either protocol, even though hydrolysis of SM occurred (Table I).

The optimum pH for SM synthesis by PlcHR\(_2\) is between 7.5 and 8.5 (Fig. 7A); however, the reverse reaction may demonstrate a different pH optimum (a condition seen with neutral ceramidase; Ref. 29). Therefore, PC synthesis was examined at different pH by TLC analysis (Fig. 10B). Also in this case, no
SM Synthase Activity Is Specific to the Phospholipase C of P. aeruginosa—Other bacterial phospholipases C have been identified, such as sphingomyelinase and PC-specific phospholipase C from B. cereus (15, 30) or S. aureus (16). These enzymes show similar substrate preferences (PC or SM) to the P. aeruginosa PlcH and, therefore, they were evaluated for SM synthase activity. Purified sphingomyelinase (bSMase) or PC-PLC (bPC-PLC) from B. cereus, which gave comparable NSMase (cpm) or PC-PLC activity to the PlcHR2 from Pseudomonas were tested for SM synthase activity. As shown in Table II, neither bSMase nor bPC-PLC showed detectable SM synthase activity. Additionally, several other dilutions of the two enzymes were tested for SM synthase and found to have no activity under any condition (data not shown). It has been shown that magnesium activates bSMase and upon magnesium activation bSMase would also act as a PC-PLC. Therefore, the effects of magnesium on possible SM synthase activity of bSMase were examined (Table II). Whereas PC-PLC activity could be clearly measured under these conditions (Table II), there was no detectable SM synthase activity. These results clearly show that the ability to synthesize SM is specific to the Pseudomonas PlcH.

SM Synthase Activity of P. aeruginosa Is Inhibited by D609—Mammalian SM synthase has been previously shown to be inhibited by an inhibitor of the bacterial PC-PLC, D609 (10, 12). Therefore, the effect of D609 on the Pseudomonas SM synthase was also tested. As shown in Fig. 11, preincubation of the enzyme with different concentrations of D609 significantly inhibited the reaction in a dose dependent manner. The extent of inhibition is comparable with that observed with mammalian SM synthase and PC-PLC.3 These results provide an additional level of similarity between mammalian and bacterial SM synthase.

**DISCUSSION**

In this study we have purified and identified a protein able to synthesize sphingomyelin from the culture supernatant of P. aeruginosa. The synthesis of sphingomyelin by this bacterial SM synthase occurs through the transfer of the phosphorylcholine moiety from phosphatidylcholine to ceramide. The bacterial enzyme therefore acts as a transferase similar to the mammalian counterpart, and selectively recognizes as substrate glycerophospholipids carrying a choline head-group and at least one fatty acyl chain (1, 2). Moreover, the bacterial transferase reaction is stereoselective as it favors erythro ceramides versus threo ceramides as substrates, and it specifically recognizes the primary hydroxyl on ceramide as other alcohols are not used as substrates. As with the mammalian reaction, synthesis of SM by the bacterial enzyme proceeds best at neutral to alkaline pH and does not appear to require a particular cation, although activity of mammalian SM synthase has been shown at times to be favored by manganese, calcium and magnesium (31). Similarly to the mammalian SM synthase, the bacterial enzyme is inhibited by D609.

On the other hand, the bacterial SM synthase is a soluble protein, whereas the mammalian enzyme has been described as a tightly membrane-bound enzyme mainly residing in the Golgi apparatus (32–37), but also present in the plasma membrane (28, 31, 32, 38–41) and in the nucleus (42, 43). Interestingly, however, during the purification protocol, the bacterial

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3 C. Luberto, E. Collins, and Y. A. Hannun, unpublished observations.
SM synthase was found to bind strongly to butyl- and octyl-Sephrose matrices, highlighting an important hydrophobic component of this soluble protein. This aspect is not surprising given the lipid-metabolizing activity of the enzyme, and thus the necessity for a lipid/protein interaction exemplified by the requirement of at least one fatty acyl chain in the donor molecule for SM synthesis.

The difference in the solubility between sphingolipid-metabolizing enzymes from *Pseudomonas* and the mammalian counterparts is not unique to the SM synthase. In fact, a similar situation is observed in the case of both neutral/alkaline ceramidase and neutral SMase. Both are secreted proteins from *P. aeruginosa* (23) and *P. fluorescens* (21), respectively, whereas their mammalian counterparts, the mitochondrial/neutral ceramidase and the nSMase2, are associated with membranes. Despite this difference, a significant homology between the amino acidic sequences can be observed. For instance, the *Pseudomonas* ceramidase and the mammalian mitochondrial ceramidase share 35% identity and 53% similarity. Importantly, a similar correspondence could support the possibility of finding a mammalian homologue to the bacterial SM synthase.

The bacterial SM synthase was found to be the previously isolated PlcH, a phospholipase C known to hydrolyze phosphatidylcholine and sphingomyelin, and responsible for the hemolytic activity of *P. aeruginosa*. The results from this work, by using a combination of biochemical and genetic approaches, demonstrate that PlcH is sufficient and necessary for SM synthase activity. First, the purified protein from PAK strain showed neutral SM hydrolytic activity and similar molecular weight and isoelectric point to the PlcH from PA01 strain.
Second, the purified SM synthase from PAK specifically reacted with monoclonal antibodies raised against purified PlcH from PA01. Third, purified PlcH from PA01 was able to synthesize SM. Fourth, SM synthase activity is abolished when the PlcH gene in P. aeruginosa PA01 is deleted. Interestingly, SM synthase activity is associated only with PlcH and not PlcN, a second phospholipase C from P. aeruginosa. Deletion mutants for PlcN did not display any significant defect in SM synthesis (data not shown). Although PlcH and PlcN share 40% identity in their amino acidic sequences, they show different substrate preference. PlcH recognizes PC and SM whereas PlcN hydrolyzes PC and phosphatidylserine (PS) (20). This observation brings up the possibility that the ability of PlcH to recognize SM might be a requirement for SM synthase activity.

The ability of PlcH to hydrolyze PC (PC-PLC) and at the same time to synthesize SM (SM synthase) raises the interesting question on how these reactions might be regulated. Interestingly, several ceramidases (yeast, mammalian, and bacterial) have been reported to have dual activity, hydrolytic (ceramidase) and synthetic (ceramide synthase) (23, 29, 44, 45). Biochemical characterization has shown that regulation of these reactions might involve different mechanisms such as different pH optima, product inhibition (sphingosine on ceramidase), and differential effects of lipid cofactors (cardiolipin and phosphatidyl-

![Figure 10](Image)

**Fig. 10.** PlcHR does not show SM synthase reverse activity. PlcHR (16.7 pg/μl) was incubated in the presence of vesicles loaded with 120 μM [14C-choline]SM (specific activity ~3 × 10^4 dpm/nmol) plus or minus 100 μM DAG in 100 mM Tris, pH 7.4 (A and B), MES, pH 6.5, or sodium acetate (Na Acetate), pH 5.5 (B). After a 30-min incubation at 30 °C, the 200-μl reaction mixtures were neutralized and then extracted by addition of 1.5 ml of chloroform:methanol (2:1, v/v) and 200 μl of ddH2O. After clarification of the phases by centrifugation, the organic phases were dried down and analyzed by TLC using a mixture of chloroform, methanol, and 15 mM CaCl2 (60:35:8). The plates were then subjected to autoradiography.

![Figure 11](Image)

**Fig. 11.** D609 inhibits Pseudomonas SM synthase activity. After a 15-min preincubation at 30 °C with different concentrations of D609 (Biomol), PlcHR (16.7 pg/μl) was incubated in the presence of vesicles loaded with 120 μM PC and 100 μM [14C-hexanoyl]De-C6-ceramide for additional 30 min. The reaction was terminated by addition of three volumes of a mixture of chloroform:methanol (1:1), and the organic phase was dried and lipids separated by TLC using a mixture of chloroform, methanol, and 15 mM CaCl2 (60:35:8). The plate was analyzed by autoradiography, and the radioactivity associated with the short chain sphingomyelin was determined. The preincubation with D609 was found to be important to obtain maximal inhibition of SM synthase activity.
Identification of SM Synthase in P. aeruginosa

glycerol promote ceramide activity and inhibit the ceramide synthase). In the case of PlcH, hydrolytic and synthetic reactions occur at similar pH optima excluding a differential regulation at different pH. Interestingly, it has been previously reported that PlcH and its chaperone PlcR bind to calcium and that the latter predicted to contain four EF-hands (26). Therefore, one possible regulatory mechanism might occur through the interaction of specific cations, in particular calcium. Although no effect of calcium has been reported on PC-PLC and SM synthase reactions share a common hydrolytic site for PC.

Importantly, bacterial SM synthase activity is specific to P. aeruginosa as it has been found neither in other bacteria such as E. coli nor in other purified phospholipases C with similar substrate preference to PlcHR. This observation is not surprising, given the lack of sequence homology between, for instance, PC-PLC and N-SMase from B. cereus and S. aureus and PlcH, which appears to belong to a distinct family of enzymes. Also this observation would lead to the prediction that the mammalian phospholipases homologue to these bacterial enzymes (such as nSMase1) should not exhibit SM synthase activity.

In conclusion, this study identifies the first structural gene for a SM synthase, providing the first molecular tool for the study of this class of enzymes. Importantly, it may potentially lead to the identification of the elusive mammalian SM synthase.

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