Cloning of a Cholesterol-α-glucosyltransferase from Helicobacter pylori‡1,2

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O-Glycans of the human gastric mucosa show antimicrobial activity against the pathogenic bacterium Helicobacter pylori by inhibiting the bacterial cholesterol-α-glucosyltransferase (Kawakubo, M., Ito, Y., Okimura, Y., Kobayashi, M., Sakura, K., Kasama, S., Fukuda, M. N., Fukuda, M., Katsuyama, T., and Nakayama, J. (2004) Science 305, 1003–1006). This enzyme catalyzes the first step in the biosynthesis of four unusual glycolipids: cholesteryl-α-glucoside, cholesteryl-6′-O-acetyl-α-glucoside, cholesteryl-6′-O-phosphatidyl-α-glucoside, and cholesteryl-6′-O-lysophosphatidyl-α-glucoside. Here we report the identification, cloning, and functional characterization of the cholesterol-α-glucosyltransferase from H. pylori. The hypothetical protein HP0421 from H. pylori belongs to the glycosyltransferase family 4 and shows similarities to some bacterial dicylglycerol-α-glucosyltransferases. Deletion of the HP0421 gene in H. pylori resulted in the loss of cholesteryl-α-glucoside and all of its three derivatives. Heterologous expression of HP0421 in the yeast Pichia pastoris led to the biosynthesis of ergosteryl-α-glucoside as demonstrated by purification of the lipid and subsequent structural analysis by nuclear magnetic resonance spectroscopy and mass spectrometry. In vitro enzyme assays were performed with cell-free homogenates obtained from cells of H. pylori or from transgenic Escherichia coli, which express HP0421. These assays revealed that the enzyme represents a membrane-bound, UDP-glucose-dependent cholesterol-α-glucosyltransferase.

Steryl glycosides are membrane lipids that are synthesized by all plants, most fungi, slime molds (Physarum polycephalum and Dictyostelium discoideum) and some animals (1–3). Recently, Kunimoto et al. (4, 5) found cholesteryl glucoside in cultured human fibroblasts and in several rat tissues. Even some pathogenic bacteria, which do not produce cholesterol, but take it up from their hosts, are able to glycosylate cholesterol by innate glycosyltransferases (6–12). Some of these sterol glycosyltransferases have been identified and cloned from plants and fungi (1, 3), but representatives from animals or from bacteria have not been characterized. The plant, fungal, and mammalian sterol glucosides (1, 3, 4) as well as the bacterial lipids from Mycoplasma (6) and Borrelia (10, 12) carry the sugar β-glucosidic-linked to the sterol. In contrast, up to now sterol α-glycosides have not been detected only in the bacteria Acholeplasma axanthum (8) and Helicobacter pylori (9, 14). In the human gastric pathogen H. pylori, cholesteryl-α-D-glucopyranoside (αCG) is accompanied by two derivatives, which carry either an acyl moiety or a phosphatidyl moiety at C6 of the glucose: cholesteryl-6′-O-tetradecanoyl-α-D-glucopyranoside (αCAG) and cholesteryl-6′-O-phosphatidyl-α-D-glucopyranoside (αCPG) (9) (Fig. 1).

So far only a few functions have been ascribed to sterol glycosides. The yeast Pichia pastoris requires a functional sterol glucosyltransferase to prevent degradation of peroxisomes (15, 16). A mutant of the plant pathogenic fungus Colletotrichum gloeosporioides defective in the sterol glucosyltransferase shows reduced pathogenicity on avocado plants (17). Sitosterol glucoside in cotton was shown to act as a suppressor for the biosynthesis of sitosteryl celloxodrin to be used for the initial step of cellulose synthesis (18).

The cholesteryl-α-glucosides of H. pylori support the pathogenicity of this organism, because inhibition of the cholesterol glucosyltransferase by O-glycans of the human gastric mucosa suppresses growth of the bacterium (19). Interestingly, morphological changes of the bacterium or changes in colony variants are accompanied by alterations in the total amount of sterol glucosides and the relative proportions of αCG, αCAG, and αCPG (20, 21).

These data suggest that a better understanding of cholesteryl glucoside biosynthesis in H. pylori would be useful for further studies on host-pathogen interactions. Therefore, the aim of

The abbreviations used are: αCG, cholesteryl-α-D-glucopyranoside; αCAG, cholesteryl-6′-O-tetradecanoyl-α-D-glucopyranoside; αCAG, cholesteryl-6′-O-phosphatidyl-α-D-glucopyranoside; αEG, ergosta-5,7,22-trienyl-3-α-D-glucopyranoside (ergosteryl-α-glucoside); ESI FT-MS, electrospray ionization Fourier-transform mass spectrometry; GlC/Cer, glycosylceramide; HP-TLC, high performance thin-layer chromatography; MS, mass spectrometry; ORF, open reading frame; TLC, thin-layer chromatography; peracetylatedepidiodystosteryl-α-glucoside, 3α-O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)oxy)-5α,8α-epideoxy-5α-ergosta-6,22-dien.
our study was to identify the gene encoding the cholesterol-α-glucosyltransferase in the genome of the bacterium. A single candidate gene was selected and its function in lipid biosynthesis determined by the generation of a knock-out mutant of *H. pylori*. The activity of the corresponding enzyme was characterized by its heterologous expression in *Escherichia coli* and the yeast *P. pastoris* with subsequent lipid analyses and *in vitro* enzyme assays.

**MATERIALS AND METHODS**

**Bacterial and Yeast Strains, Growth, and Recombinant DNA Techniques—** *E. coli* strains XL1-Blue (MRF') (Stratagene) and C41(DE3) (22) were routinely grown aerobically at 37 °C in Luria-Bertani medium (23). Ampicillin (100 mg liter⁻¹) or kanamycin (45 mg liter⁻¹) were included for growth of plasmid-bearing cells. The yeast strain used in this study was *P. pastoris* JC 308Δgcs/Δgt51B1 (24), grown at 30 °C in YPD medium (10 g liter⁻¹ yeast extract, 20 g liter⁻¹ peptone, 20 g liter⁻¹ glucose). For gene expression driven by the AOX1 promoter, 0.5% methanol was added to minimal medium (13.4 g liter⁻¹ Yeast Nitrogen Base). *H. pylori* strain P12 was grown on agar plates containing 10% horse serum in a microaerophilic atmosphere (generated by Campy-Gen, Oxoid, Basingstoke, UK) at 37 °C for 48 h. Bacteria were harvested, suspended to an optical density at 550 nm (A550) of 0.1 in brain heart infusion broth containing 10% heat inactivated fetal calf serum, and grown for 18 h under microaerophilic conditions. *H. pylori* was harvested, washed twice in ice-cold phosphate-buffered saline, and the bacterial pellet was used for further lipid extraction or *in vitro* assays. The vectors pBluescript (Stratagene), pET24d(+) (Novagen), and pPIC3.5 (Invitrogen) were used for cloning. Standard methods were followed for DNA isolation, restriction endonuclease analysis, and ligation (23).

**Deletion of HP0421 in H. pylori—** For the construction of the P12Δ0421 strain two 0.5-kb DNA fragments upstream and downstream of the HP0421 open reading frame (ORF) (GenID: 900074) were amplified by polymerase chain reaction and cloned into pBluescript, separated by a chloramphenicol resistance cassette. The plasmid was introduced into *H. pylori* strain P12 by natural transformation. Transformation and homologous recombination was performed by harvesting bacteria from serum plates and suspending to an A550 of 0.1 in brain heart infusion broth containing 10% fetal calf serum. DNA was added (1 µg), and incubation was extended for 5 h under microaerophilic conditions before the suspension was plated on selective serum plates. Correct allelic exchange of the HP0421 gene with the resistance gene was verified by polymerase chain reaction.

**Fractionation of H. pylori Homogenate—** To isolate cytosolic and membrane fractions of *H. pylori*, bacteria were resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM MgCl₂ including a mixture of protease inhibitors (Complete¹¹, Roche) and disrupted by 4 passages through a French press at 15,000 lb/in². Removal of intact bacteria by two centrifugations at 4,000 g and 4 °C for 10 min resulted in a cell-free homogenate. This homogenate was subjected to centrifugation at 164,000 × g at 4 °C for 1 h. The supernatant represented the cytosolic fraction, whereas the pellet representing the membrane fraction was washed and resuspended in buffer (see above).

**Cloning of the Cholesterol-α-gluco-}

![FIGURE 1. Structures of cholesteryl-α-glucoside and its derivatives found in *H. pylori*. R1 and R2 represent the alkyl chains of fatty acyl moieties (14:0, 18:1, and 19:0 cyclopropane fatty acid).](image-url)
specific oligonucleotide primer pairs HP0421_F (5′-GTACGGAGTTCCACCATGGTTATTGTTTAGCGTGGATA-3′) and HP0421_R (5′-AGCTGGATCCGGCGCCGCTGATAAGGTTTTAAAAGATGGGGG-3′). Herculese-enhanced DNA polymerase (Stratagene) was used for the amplification of the 1169-bp product containing the entire HP0421 ORF sequence. This amplicon was digested with Ncol/BamHI and inserted into pET24d(Ncol/BamHI) leading to pET24d-HP0421, which was used for transformation of E. coli C41(DE3) cells. The amplicon was also digested with BamHI/NotI to be inserted into pPIC3.5(BamHI/NotI) resulting in pPIC3.5-HP0421 which was also digested with BamHI/NotI to be inserted into pET24d(NcoI/BamHI) leading to pET24d-HP0421, which was used for transformation of P. pastoris C41(DE3) (22) was used as expression host for the plasmid pET24d-HP0421. After transformation, 50-ml cultures of E. coli were grown at 37 °C to an A600 of 0.8–1.2. Induction was performed by adding 0.4 mM isopropyl β-D-thiogalactoside and further incubation for 3 h at 30 °C. A P. pastoris strain deficient in glycolipid biosynthesis was used to express HP0421: JC 308 Δgcs:Sh ble, Δugt51B1::URA3, ade1, arg4, his4 (24). In a first step, this strain was transformed with the empty vectors pBLADE and pBLARG (25) to increase its rate of growth. Subsequently, the resulting strain, DKO (JC 308 Δgcs:Sh ble, Δugt51B1::URA3, his4) was used for transformation with pPIC3.5-HP0421 leading to DKO-Hp. Transformed cells were grown at 30 °C in 50 ml of YPD medium to an A600 between 1 and 2. Expression was driven by the strong AOX1 promoter and induced by transferring the cells to minimal medium with 0.5% methanol as sole carbon source followed by additional incubation for 20 h.

In Vitro Sterol Glucosyltransferase Assay—E. coli cells were harvested by centrifugation (4 °C, 10 min, 3200 × g), resuspended in 2–3 ml buffer (50 mM Tris-HCl, pH 7.5, 5 mM 1,4-di-thiothreitol), and cooled in an ice bath. Disruption of E. coli cells was performed by ultrasonication (probe tip, 10 times for 10 s). Cell debris were removed by centrifugation (4 °C, 10 min, 3200 × g), and the supernatant fractions representing the cell-free homogenates were used for in vitro assays. The assay for the cholesterol-α-glucosyltransferase activity was performed similar to the assay used for the eukaryotic sterol-β-glucosyltransferases (3, 26). Shortly, the assay mixture contained in a total volume of 100 μl: 5–30 μl (40–240 μg of protein) of H. pylori or E. coli cell homogenate, either 5 μl of a solution of 4 mM cholesterol in ethanol (200 μM final concentration) or 4 μl of 270,000 dpm [14C]-cholesterol in ethanol (final concentration 44 μM, specific activity 2.1 GBq/mmol) and either 100,000 dpm of UDP-[U-14C]glucose (final concentration 1.5 μM, specific activity 12.2 GBq/mmol) or 10 μl of 5 mM unlabeled UDP-glucose (final concentration 0.5 mM). After incubating for 30 min at 30 °C the reaction was terminated by the addition of 0.7 ml of 0.45% NaCl solution and ether 2 ml of ethyl acetate or 3 ml of chloroform/methanol 2:1. After vortexing and phase separation by centrifugation, the radioactivity in the organic phase was determined by scintillation counting or the extracted lipids were separated by thin-layer chromatography (TLC) or high performance TLC (HPTLC). The radioactivity on the silica gel plate was detected by radioscanning with a BAS-1000 Bio Imaging Analyser (Raytest, Straubenhardt, Germany).

Lipid Extraction and Analysis—P. pastoris or H. pylori cells were harvested by centrifugation (4 °C, 10 min, 3200 × g) and the sedimented cells were boiled for 10 min in a water bath. Lipid extraction was performed with chloroform/methanol 1:2 (v/v) and chloroform/methanol 2:1 (v/v). The lipid extract was washed by Folch partitioning (27) (chloroform/methanol/ 0.45% NaCl solution, 2:1:0.75) and the organic phase was evaporated. The residue was redissolved in pyridine (because of the low solubility of steryl glucosides in chloroform/methanol mixtures) and subjected to analytical and preparative TLC. For NMR spectroscopy and mass spectrometry (MS), the purified glycolipids were acetylated (with acetic anhydride in pyridine, 1:1) overnight at room temperature and subjected to repurification by preparative TLC in diethyl ether.

Electrospray Ionization Fourier Transform Mass Spectrometry—High resolution electrospray ionization Fourier-transform mass spectrometric (ESI FT-MS) analyses of acetylated glycolipids were performed in the positive and/or negative ion mode using an APEX II-Instrument (Bruker Daltonics, Billerica, MA) equipped with an actively shielded 7 Tesla magnet and an Apollo II ESI source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. For the positive ion mode samples (~10 ng μl−1) were dissolved in a 50:50:0.03 (v/v/v) mixture of 2-propyl alcohol, water, and triethylamine (pH 8.5). The samples were sprayed at a flow rate of 2 μl min−1. Capillary entrance voltage was set to 3.8 kV, and drying gas temperature to 150 °C. Because the spectra comprise the molecular species in different charge states, the spectra were charge-deconvoluted, using the XMASS-6.1 software, and mass numbers given refer to monoisotopic masses of the neutral molecules.

Proton (1H) Nuclear Magnetic Resonance (NMR) Spectroscopy—1H NMR spectra were recorded at 360 MHz (Bruker Avance DPX 360) according to Hözl et al. (28).

RESULTS

A Single Candidate Gene in the Genome of H. pylori Was Identified as the Putative Cholesterol-α-glucosyltransferase Gene—To discover putative cholesterol-α-glucosyltransferase genes in H. pylori, a BLAST data base search (29) with the amino acid sequence of the sterol β-glucosyltransferase from Arabidopsis thaliana (1) was performed. No sequence of significant similarity was found in the genome of H. pylori. This result was not surprising considering that glycosyltransferases operate with two different catalytic mechanisms and are classified according to the stereoenzymes between the reaction substrates and products as either retaining or inverting enzymes (30). While the plant β-glucosyltransferase belongs to the inverting enzymes, the α-glucosyltransferase from H. pylori probably works by the retaining mechanism.

As a result, candidate cholesterol-α-glucosyltransferase genes from H. pylori had to be identified by a different approach. Cholesterol biosynthesis is a typical eukaryotic fea-
ture and this lipid is available for bacteria only from eukaryotic sources. Therefore, we speculated that the cholesterol-glucosyltransferase from Helicobacter pylori may have developed from an ancient bacterial -glycosyltransferase of originally different acceptor specificity. This hypothesis is supported by a similar situation in another group of enzymes, the acyl-CoA-dependent acyltransferases. Whereas an acyl-CoA-dependent cholesterol acyltransferase had been cloned and characterized in 1993 (31), all efforts to clone a diacylglycerol acyltransferase failed for many years. This important enzyme was finally identified, since a protein with amino acid sequence similarities to the cholesterol acyltransferase turned out to transfer the acyl group not to cholesterol, but to diacylglycerol (32). Thus, cholesterol and diacylglycerol acyltransferases have a common ancestor.

By extrapolating this cholesterol/diacylglycerol correlation to glycosyltransferases, we concluded that the cholesterol-glucosyltransferase candidate HP0421 from H. pylori with bacterial diacylglycerol-α-glucosyltransferase activity might have developed from a bacterial diacylglycerol-α-glucosyltransferase. Indeed, such diacylglycerol-α-glucosyl and galactosyltransferases have been identified in Acholeplasma laidlawii, Streptococcus pneumoniae, Deinococcus radiodurans, and Thermotoga maritima (33, 34). A BLAST search with the sequence of the diacylglycerol-α-glucosyltransferase from Helicobacter pylori (accession number: NP_0421) showed significant homology with the sequences of these bacterial enzymes.
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Deletion of the HP0421 gene in \(H.\) pylori led to the loss of cholesteryl-\(\alpha\)-glucosides and its three derivatives. A, cell-free homogenates from \(H.\) pylori strain P12 (WT) and from the corresponding HP0421 knock-out mutant (KO) were used in \textit{in vitro} determination of cholesterol glucosyltransfer activity by incubation with UDP-glucose and cholesterol. Either UDP-glucose ([\(^{14}\)C]Glc) or cholesterol ([\(^{14}\)C]Chol) were radiolabeled. The lipophilic ferase activity by incubation with UDP-glucose and cholesterol. Either UDP-

The deletion of HP0421, was the most promising candidate to be tested for (42% similarity, 19% identity). This hypothetical protein, glucose ([\(^{14}\)C]Glc) or cholesterol ([\(^{14}\)C]Chol) were radiolabeled. The lipophilic ferase activity by incubation with UDP-glucose and cholesterol. Either UDP-

FIGURE 3. Deletion of the HP0421 gene in \(H.\) pylori led to the loss of cholesteryl-\(\alpha\)-glucosides and its three derivatives. A, cell-free homogenates from \(H.\) pylori strain P12 (WT) and from the corresponding HP0421 knock-out mutant (KO) were used in \textit{in vitro} determination of cholesterol glucosyltransfer activity by incubation with UDP-glucose and cholesterol. Either UDP-glucose ([\(^{14}\)C]Glc) or cholesterol ([\(^{14}\)C]Chol) were radiolabeled. The lipophilic reaction products were separated by TLC in chloroform/methanol 85:15 and detected by radioscanning. B, lipid extracts from the two strains were separated by TLC in chloroform/methanol/water 70:30:5. After drying of the plate, a second development was performed in diethylether. Lipids were visualized by spraying with \(\alpha\)-naphthol/sulfuric acid and subsequent heating to 160 °C. Glycolipids were identified (after purification) by mass spectrometry (\(\alpha\)CG, \(\alpha\)CAG, \(\alpha\)CPG, lyso-\(\alpha\)-CPG) and NMR spectroscopy (\(\alpha\)CG, \(\alpha\)CAG, \(\alpha\)CPG), and phospholipids were identified by co-chromatography with authentic standards. Chol, cholesterol; CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

the other hand, the compound with the lowest \(R_f\) value had not been described previously. Therefore, it was isolated, and its structure elucidated by MS. The mass spectrum (supplemental Fig. S1) comprised two abundant and one minor molecular species the masses of which were in agreement with the mass of peracetylated cholesteryl-lyso phosphatidyl-hexoside which carried either a \(C_{18:1}\), a cyclopropane \(C_{19:0}\) or a \(C_{14:0}\) fatty acid (supplemental Fig. S1). From these data we conclude that the glycolipid of \(H.\) pylori with the smallest \(R_f\) value represents cholesteryl-\(\alpha\)-lyso phosphatidyl-\(\alpha\)-glycopranoside derived from \(\alpha\)-CPG by the loss of one fatty acid residue. The deletion of HP0421 in \(H.\) pylori and lipid analysis of this mutant demonstrate that this gene is essential for the biosynthesis of \(\alpha\)CG and its three derivatives in \(H.\) pylori.

Heterologous Expression of HP0421 in \(P.\) pastoris Resulted in the Biosynthesis of Ergosteryl-\(\alpha\)-glucoside. Lipid extracts from different \(P.\) pastoris strains were separated by TLC in chloroform/methanol 85:15. The wild-type strain (WT) contained ergosteryl-\(\beta\)-glucoside (\(\beta\)EG) and glucosylceramide (GlcCer). A double knock-out mutant (DKO) deficient both in sterol glucosyltransferase and ceramide glucosyltransferase activities lacked these glycolipids (24). The DKO mutant expressing HP0421 from \(H.\) pylori (\(H.\) pylori (DKO Hp)) synthesized the novel glycolipid ergosteryl-\(\alpha\)-glucoside (\(\alpha\)EG). In contrast, the DKO mutant expressing the ORF TM0744 from \(T.\) maritima (DKO Tm) contained diacylglycerol-\(\alpha\)-glucoside (\(\alpha\)DG) (34).

FIGURE 4. Expression of HP0421 in \(P.\) pastoris resulted in the biosynthesis of ergosteryl-\(\alpha\)-glucoside. Lipid extracts from different \(P.\) pastoris strains were separated by TLC in chloroform/methanol 85:15. The wild-type strain (WT) contained ergosteryl-\(\beta\)-glucoside (\(\beta\)EG) and glucosylceramide (GlcCer). A double knock-out mutant (DKO) deficient both in sterol glucosyltransferase and ceramide glucosyltransferase activities lacked these glycolipids (24). The DKO mutant expressing HP0421 from \(H.\) pylori (\(H.\) pylori (DKO Hp)) synthesized the novel glycolipid ergosteryl-\(\alpha\)-glucoside (\(\alpha\)EG). In contrast, the DKO mutant expressing the ORF TM0744 from \(T.\) maritima (DKO Tm) contained diacylglycerol-\(\alpha\)-glucoside (\(\alpha\)DG) (34).

Deletion of the HP0421 Gene in \(H.\) pylori Led to the Loss of Cholesteryl-\(\alpha\)-glucoside and Its Three Derivatives—A HP0421 knock-out strain of \(H.\) pylori P12 was generated by insertion of a chloramphenicol resistance cassette. The deletion of HP0421 in \(H.\) pylori resulted in the loss of cholesterol-\(\alpha\)-glucosyltransferase activity determined by \textit{in vitro} enzyme assays with homogenates of \(H.\) pylori cells and radiolabeled substrates (Fig. 3A). In addition, TLC of lipid extracts of the cells revealed that 4 glycolipids present in wild-type extracts were absent in the extracts of the knock-out mutants (Fig. 3B). Three of them, \(\alpha\)CG, \(\alpha\)CAG, and \(\alpha\)CPG have been identified before (9). Analysis of the purified and peracetylated lipids by MS and NMR-spectroscopy confirmed their structures (data not shown). On the other hand, the compound with the lowest \(R_f\) value had not been described previously. Therefore, it was isolated, and its structure elucidated by MS. The mass spectrum (supplemental Fig. S1) comprised two abundant and one minor molecular species the masses of which were in agreement with the mass of peracetylated cholesteryl-lyso phosphatidyl-hexoside which carried either a \(C_{18:1}\), a cyclopropane \(C_{19:0}\) or a \(C_{14:0}\) fatty acid (supplemental Fig. S1). From these data we conclude that the glycolipid of \(H.\) pylori with the smallest \(R_f\) value represents cholesteryl-\(\alpha\)-lyso phosphatidyl-\(\alpha\)-glycopranoside derived from \(\alpha\)-CPG by the loss of one fatty acid residue. The deletion of HP0421 in \(H.\) pylori and lipid analysis of this mutant demonstrate that this gene is essential for the biosynthesis of \(\alpha\)CG and its three derivatives in \(H.\) pylori.
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in the strain expressing HP0421 (Fig. 4). This glycolipid was isolated, peracetylated and appeared as two partially separated compounds after purification by TLC, which were subjected to MS and NMR spectroscopy.

The masses of these two compounds were in agreement with the masses of peracetylated ergosteryl monohexoside and epidioxyergosteryl-monohexoside (supplemental Fig. S2). Subsequent NMR analyses revealed further structural details. The chemical shift of the anomeric proton in the sugar residue of each compound (H-1, 5.260, and 5.211 ppm, respectively) in combination with a small coupling constant ($J_{1,2} = 3.5$ Hz) showed an α-anomeric proton for the pyranoside (Table 1). In addition, high coupling constants of the ring protons ($J_{3,4} = J_{3,5} = 9.7$ Hz) revealed gluco-configuration. Comparison of the NMR data of signals derived from the sterol residue in both compounds showed a diagnostic downfield shift in the epidioxyergosteryl-α-glucopyranoside for the two H-6 and H-7 protons (Table 1). This assignment was in excellent agreement with $^{13}$C NMR data (not shown) and $^1$H NMR resonances published for the 5α,8α-epidioxyergosta-6,22-dienyl-β-D-glucopyranoside (39). From these data we conclude that expression of HP0421 in P. pastoris results in the biosynthesis of ergosta-5,7,22-trienyl-3-α-D-glucopyranoside (ergosteryl-α-glucoside, α-EG) and 5α,8α-epidioxyergosta-6,22-dienyl-3-α-D-glucopyranoside (epidioxyergosteryl-α-glucoside).

A parallel expression of a bacterial GT4 member from T. maritima in P. pastoris led to the biosynthesis of diacylglycerol-α-glucoside as described before (34). These data demonstrate that HP0421 from H. pylori is a sterol-α-glucosyltransferase.

**HP0421 Is a Membrane-bound UDP-glucose-dependent Cholesterol-α-glucosyltransferase**—Further functional characterization of the cholesterol-α-glucosyltransferase from H. pylori was performed by *in vitro* determination of enzyme activity. *E. coli* cells expressing HP0421 were disrupted by ultrasonication and the sterol glycosyltransferase activity in cell-free homogenates was measured using radiolabeled substrates. After HPTLC of the lipophilic reaction products, radio scanning revealed that cholesteryl-α-glucosides were synthesized, which differ from the well known steryl-β-glucosides by a smaller $R_f$ value (Fig. 5). The clear resolution of the two anomic steryl glucosides was achieved by HPTLC. Equivalent separations were observed before with diacylglycerol glucosides (34).

In the sterol glycosyltransferase assay, the recombinant HP0421 exhibited a pH optimum of 8.5, and the apparent $K_m$ for UDP-Glc was 25 µM. Table 2 shows the substrate specificities of the recombinant enzyme HP0421 for glucosyl acceptor substrates. Despite the fact that the assays were performed under linearized conditions, these data should not be considered as a quantitative determination of substrate affinities. Because the lipophilic substrates in the assay were not uniformly dissolved, but were present as both monomers and components of lipid vesicles, the exact determination of their concentrations in the assay was not possible. Therefore, these results indicate which substrates are accepted or discriminated by the cholesterol-α-glucosyltransferase...
TABLE 2

| Acceptor | Steryl glucoside synthesis |
|----------|----------------------------|
| None     | 0                          |
| Cholesterol | 100                       |
| Ergosterol | 10                        |
| β-Sitosterol | 7                         |
| Stigmasterol | 5                       |
| Campesterol | 12                       |
| Ceramide‡ | 0                         |
| 1,2-Dioleoyl glycerol | 0                 |

‡ Sphing-4-ene with amide-linked stearic acid.

cosyltransferase. With UDP-[14C]glucose as the donor, the glycosyltransferase used various sterols as sugar acceptors, but neither ceramide nor diacylglycerol were glucosylated. Under linearized assay conditions the enzyme showed a distinct preference for cholesterol. Whereas all other sterols led to at most 13% of the incorporation into steryl glucosides compared with cholesterol. Despite this preference for cholesterol, the enzyme is able to synthesize substantial amounts of ergosteryl-α-glucoside in transgenic *P. pastoris* expressing HP0421 (Fig. 4).

To assess the intracellular localization of the cholesterol-α-glucosyltransferase, a cell-free homogenate from *H. pylori* cells was centrifuged for 1 h at 164,000 × g, and the cholesterol glucosyltransferase activity was determined in the supernatant (cytosolic fraction) and in the resuspended pellet (membrane fraction). The activity of the cholesterol glucosyltransferase was present in the homogenate of *H. pylori* cells and in the membrane fraction, but was barely detectable in the cytosolic fraction (Fig. 5). In summary, these *in vitro* data demonstrate that HP0421 from *H. pylori* represents a membrane-bound UDP-glucose-dependent cholesterol-α-glucosyltransferase.

**DISCUSSION**

To identify the enzyme, which is responsible for the biosynthesis of cholesteryl-α-glucoside, in *H. pylori*, we have employed a strategy based upon both insertional inactivation in *H. pylori* and heterologous expression in *E. coli* and *P. pastoris*. This has led to the characterization of HP0421, a glycosyltransferase of the GT4 family (35), the members of which display a retaining chemistry. The complete loss of cholesteryl-seryltransferase of the GT4 family (35), the members of which have developed from such an enzyme after a new substrate, cholesterol, was available for the pathogenic bacterium. Like other bacteria, *H. pylori* is not able to synthesize cholesterol, but it is able to acquire the lipid from epithelial cells of its host organisms.5

Because the bacterial cholesterol glucosyltransferase has been proposed to be accessible by extracellular α,1,4-GlcNAccapped O-glycans (19), we assessed the intracellular localization of the enzyme by differential centrifugation. The activity of the cholesterol glucosyltransferase was found in total *H. pylori* lysates and in the membrane fraction, but was barely detectable in the cytosolic fraction. These data are in agreement with the assumption that an enzyme using a membrane lipid, cholesterol, as substrate would be membrane-bound. On the other hand, it is doubtful whether HP0421 contains a transmembrane domain, because the results of various prediction methods at the ExPASy Molecular Biology server are contradictory (data not shown). Berg et al. (33) found similar inconsistencies with the DAG-α-glucosyltransferase from *A. laidlawii*. Fold predictions indicated several amphipathic α-helices, which may attach the enzyme to negatively charged domains of the bacterial membrane (33). Regardless of the mechanism of membrane attachment/integration, it is not yet clear whether the enzyme is present in the plasma membrane or the outer membrane or both. Because of its UDP-glucose-dependence it seems likely that the enzyme localizes to the cytoplasmic face of the plasma membrane. Regarding the inhibitory effect of α,1,4-GlcNAccapped O-glycans on the cholesterol-α-glucosyltransferase, we suggest either a signaling effect by O-glycans, or the internalization of O-glycans by the bacterium to permit access to the enzyme.

The biosynthesis of cholesteryl-α-glucoside is of particular importance for *H. pylori*, since it allows the pathogen to evade the immune response of the mammalian host, and *H. pylori* mutants lacking cholesteryl glucosides show impaired infection in a murine model. Therefore, the identification of the cholesterol-α-glucosyltransferase provides the basis for further studies on a new aspect of the interaction between the pathogenic bacterium and its hosts. The availability of the DNA sequence of HP0421 allows the manipulation of cholesteryl glucoside synthesis in *H. pylori*. On the other hand, we demonstrated the formation of recombinant HP0421 protein in two different expression hosts. These expression systems will be used for the purification of the recombinant protein, which could be subjected to a high throughput screen for the identification of specific inhibitors of cholesterol-α-glucosyltransferase activity (40).

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