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Choline Uptake in *Agrobacterium tumefaciens* by the High-Affinity ChoXWV Transporter▽

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Agrobacterium tumefaciens **is a facultative phytopathogen that causes crown gall disease. For successful plant transformation *A. tumefaciens* requires the membrane lipid phosphatidylcholine (PC), which is produced via the methylation and the PC synthase (Pcs) pathways. The latter route is dependent on choline. Although choline uptake has been demonstrated in *A. tumefaciens*, the responsible transporter(s) remained elusive. In this study, we identified the first choline transport system in *A. tumefaciens*. The ABC-type choline transporter is encoded by the chromosomally located *choXWV* operon (ChoX, binding protein; ChoW, permease; and ChoV, ATPase). The Cho system is not critical for growth and PC synthesis. However, [^{14}C]choline uptake is severely reduced in *A. tumefaciens choX* mutants. Recombinant ChoX is able to bind choline with high affinity (equilibrium dissociation constant [K_D] of ≈2 μM). Since other quaternary amines are bound by ChoX with much lower affinities (acetylcholine, K_D of ≈80 μM; betaine, K_D of ≈470 μM), the ChoXWV system functions as a high-affinity transporter with a preference for choline. Two tryptophan residues (W40 and W87) located in the predicted ligand-binding pocket are essential for choline binding. The structural model of ChoX built on *Sinorhizobium meliloti* ChoX resembles the typical structure of substrate binding proteins with a so-called "Venus flytrap mechanism" of substrate binding.**

Choline, a quaternary amine (trimethyl-β-hydroxy-ethylammonium), is widespread in nature. As a precursor of various metabolites, it fulfills numerous biological functions in eukaryotes and prokaryotes (4, 31). In many *Rhizobiaceae*, choline can serve as a sole carbon/nitrogen source (5, 46) and is a direct precursor of glycine betaine, one of the most potent osmoprotectants (23, 33). Since choline uptake from exogenous sources is energetically more favorable than its *de novo* synthesis, bacteria have evolved different uptake mechanisms. Most often, protein-dependent ATP-binding cassette (ABC) transporters (8, 13, 50) are used, but uptake via secondary transporters has also been described (7, 24).

Two choline uptake systems are known in *Escherichia coli*: the ABC transporter ProU and the proton motive force-driven, high-affinity uptake system BetT. At low external concentrations, choline is mainly taken up by BetT, whereas at higher concentrations choline is also transported by ProU (24, 30, 50). In *Bacillus subtilis*, two closely related high-affinity ABC transport systems (OpuB and OpuC) for choline uptake were identified. Expression of the *opuB* and *opuC* operons is regulated in response to increasing osmolality (19). OpuC transports various osmoprotectants, whereas OpuB is highly specific for choline (19). The structural reasons for this discrimination have recently been revealed (37).

Several choline uptake activities have been examined in the plant symbiont *Sinorhizobium meliloti*. One constitutive system exhibits low affinity. The other two systems have high affinity and are either inducible by choline or constitutively expressed (38). Recently, two choline ABC-type transporters have been identified and characterized in *S. meliloti*. The Prb system (PrbABCD) belongs to the oligopeptide subfamily and mediates the uptake of proline betaine as well as glycine betaine and choline (2) while the Cho system (ChoXWV) is specific for choline (13). The Cho system has been extensively characterized, and crystal structures of the substrate binding protein ChoX are now available (35, 36).

Apart from its function as nutrient or osmoprotectant, choline is also a direct precursor of phosphatidylcholine (PC), a typical eukaryotic membrane component present in only about 10% of all bacterial species, in particular, in bacteria interacting with eukaryotes (1, 48). Recent studies revealed that PC plays a fundamental role in pathogenic and symbiotic microbe-plant interactions (1, 11, 12, 34, 53). Like many members of the *Rhizobiaceae* family, *Agrobacterium tumefaciens* contains PC as a major membrane component (45, 53). An *A. tumefaciens* mutant lacking PC shows a dramatic virulence defect due to the lack of the type IV secretion system, essential for virulence. In addition, PC is critical for motility, biofilm formation, and stress resistance in *A. tumefaciens* (20, 53).

Two pathways are known to produce PC in *A. tumefaciens*: the *S*-adenosyl-L-methionine-consuming methylation pathway and the energetically more favorable choline-dependent PC synthase (Pcs) pathway. In the latter, PC is formed directly by Pcs-catalyzed condensation of choline with CDP-diacylglycerol (CDP-DAG). Since there is no clear evidence that *A. tumefaciens* can synthesize choline *de novo*, this pathway might rely on exogenously provided choline. In early studies radioactively labeled choline uptake from the medium and incorporation into phospholipids have been demonstrated (45). However, a corresponding choline transporter(s) was not revealed. Recently, we analyzed whether the predicted ABC transporter (Atu1792, Atu1791, Atu1790, and Atu1789) encoded immediately downstream of the *pcs* gene is responsible for choline

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) or description[a]	Reference or source
E. coli strains		
DH5α	Cloning host	16
BL21(DE3)	Expression host	49
A. tumefaciens strains		
C58	Wild type	C. Baron, Montreal, Canada
C58 *choX*::Gm>	*choX*::Gm> insertion mutant of C58	This study
C58 *choX*::Gm<	*choX*::Gm< insertion mutant of C58	This study
C58 Δ*pmtA*	*pmtA* deletion mutant of C58	53
C58 Δ*pmtA choX*::Gm>	*pmtA choX*::Gm> double mutant of C58	This study
C58 Δ*pmtA choX*::Gm<	*pmtA choX*::Gm< double mutant of C58	This study
C58 Δ*pcs*	*pcs* deletion mutant of C58	53
C58 Δ*pcs choX*::Gm<	*pcs choX*::Gm< double mutant of C58	This study
Plasmids		
pBluescriptKS(+)	*E. coli* cloning vector, *lacZ*, Amp^r	Stratagene, Santa Clara, CA
pAC01	Transcriptional *lacZ* fusion vector containing promoterless *lacZ* gene; Tc^r Ap^r	26
pET24b(+)	Expression vector, T7 promoter; 3′ His codon, Km^r	Novagen, Darmstadt, Germany
pVSBADNco	*A. tumefaciens* expression vector; Sp^r Sm^r	55
pK19*mobscacB*	Suicide plasmid; Km^r	43
pYP5	pBSL15 derivative carrying Gm>	18
pBO893	pVSBADNco carrying *choX*	This study
pBO2002	pET24b carrying *choX*	This study
pBO2017	pAC01 carrying a *choX-lacZ* fusion	This study
pBO890	pAC01 carrying a *choW-lacZ* fusion	This study
pBO891	pAC01 carrying an *atu2278-lacZ* fusion	This study
pBO2004	pBluescript-*choX*::Gm>	This study
pBO2005	pBluescript-*choX*::Gm<	This study
pBO2006	pK19*mobsacB-choX*::Gm>	This study
pBO2007	pK19*mobsacB-choX*::Gm<	This study

[a] Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

uptake in *A. tumefaciens* (21). Our data suggested that either this predicted transporter is not responsible for choline uptake or that additional choline uptake systems exist in *A. tumefaciens*. Six putative ABC-type transporters for quaternary amines are predicted in *A. tumefaciens* (40, 54). One of these putative transporters is highly similar to the well-characterized ChoXWV system in *S. meliloti*. Atu2281 is similar to ChoX (85% similarity), the periplasmic binding protein of the Cho transport system. Genes for a putative permease (Atu2280) and an ATPase (Atu2279) are encoded downstream of *atu2281*. Based on the functional analysis reported in this study, these genes were designated *choXWV*.

MATERIALS AND METHODS

Chemicals. Choline dihydrogen citrate, acetylcholine chloride, and betaine were purchased from Sigma-Aldrich. Radioactively labeled [*methyl*-^{14}C]choline chloride (0.1 mCi/ml and 55 mCi/mmol) was obtained from Hartmann Analytic. HAWP 02500 filters (0.45-μm pore size) for [^{14}C]choline-binding assays were purchased from Millipore GmbH. The HPTLC silica gel 60 plates were from Merck, and Molybdenum Blue Spray reagent was purchased from Sigma-Aldrich. All other reagents were of the highest standard commercially available.

Bacterial strains, plasmids, and growth conditions. All strains, plasmids, and oligonucleotides used in this work are listed in Tables 1 and 2. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) or M9 medium (42) supplemented with ampicillin (Ap; 100 μg/ml), kanamycin (Km; 50 μg/ml), tetracycline (Tc; 10 μg/ml), and streptomycin or spectinomycin (Sm/Sp; 50 μg/ml) if appropriate. *E. coli* DH5α was used as host for all cloning procedures. *E. coli* BL21(DE3) served as host for overproduction of the ChoX wild type and mutated variants from the corresponding pET24b-based expression plasmids. *A. tumefaciens* strain C58 (wild type) and derivatives (*choX* mutants) were routinely grown at 30°C in yeast extract broth (YEB) complex, AB minimal medium (pH 5.5; 1% [wt/vol] glucose) (44) or M9 minimal medium supplemented with 1.5 μg/ml Tc, 10 μg/ml gentamicin (Gm), and 100 μg/ml Sm and/or 300 μg/ml Sp, if necessary. For β-galactosidase assays choline was added to the AB medium at a final concentration of 0.1 mM.

Plasmid and mutant construction. Recombinant DNA work was carried out according to standard protocols (42). For overproduction of ChoX in *E. coli*, the *A. tumefaciens* C58 *choX* gene was PCR amplified with chromosomal DNA as a template and appropriate primers (Table 2). The PCR product was digested with NdeI and XhoI and cloned into pET24b(+) treated with the same enzymes, resulting in hybrid plasmid pBO2002 coding for a C-terminally His-tagged ChoX protein.

For overproduction of ChoX in *A. tumefaciens*, the coding region, including its own ribosome binding site, was amplified via PCR. The PCR product was digested with EcoRI and SalI and cloned into the corresponding site of the arabinose-inducible vector pVSBADNco (55), resulting in plasmid pBO893.

To generate ChoX with the mutations W41A, W88A, W283A, and W294A and the double mutation W41A W88A, site-directed mutagenesis was conducted using a QuikChange mutagenesis kit (Stratagene) following the supplier's protocol. The vector pBO2002 (pET24b containing wild-type *choX*) was subjected to site-directed mutagenesis. Mutated *choX* variants were verified by sequencing.

To construct transcriptional fusions to the *lacZ* gene, PCR-generated fragments of the promoter regions of *choX*, *choW*, and *atu2278* (14, 54) were digested with KpnI and XhoI and ligated into pAC01 treated with the same enzymes.

To construct *choX* insertion mutants, a 1,155-bp *A. tumefaciens* DNA fragment was PCR amplified using the oligonucleotide pair choX_Kpn_fw and choX_Eco_rv (Table 2). The PCR product was cloned into the Kpn- and EcoRV-restricted pBluescript KS(+) vector. Subsequently, a SphI fragment carrying the Gm resistance cassette from plasmid pYP5 was inserted into the single SphI site within *choX*, leading to the hybrid plasmids pBO2004 and pBO2005. The 1,703-bp HindIII-*choX*::Gm-EcoRI fragment from pBO2004 and pBO2005 was then excised with EcoRI and HindIII and cloned into the suicide vector pK19*mobsacB* (43), resulting in the plasmids pBO2006 and pBO2007. The plasmids were transferred into wild-type *A. tumefaciens* C58 or *pmtA* or *pcs* mutants via electroporation. Single-crossover integration mutants were selected on LB plates containing kanamycin. Single colonies were grown overnight in liquid LB

TABLE 2. Oligonucleotides used in this study

Oligonucleotide function and name	Sequence (5′→3′)[a]
Transcriptional *lacZ* fusions	
choX-Kpn_fw	CCCCGGTACCGTCTGCGGACGCTTCGC
choX-Xho_rv	CCCCCTCGAGTGTTTGCTCCCTGTTTTTTC
choW-up-Kpn-fw	CCCCGGTACCCGAATGTCGGCGCGTTTCTTAAG
choW-up-Xho-rv	CCCCCTCGAGGGCCTTCGATGCGAGAAAAATTC
nolR_up_Kpn_fw	CCCCGGTACCGGTAATAGATTGAAGCGCCA
nolR_up_Xho_rv	CCCCCTCGAGACGCTCAAGTCTCCTGATGAC
Construction of pVSBAD-*choX* expression vector	
ChoX_Eco_fw	CCCGAATTCTTGATGGCCGGAAGATATTGATA
ChoX_Sal-rv	CCCGTCGACCTAAAGGCCGAGAGCCTTT
Construction of pET24b-choX expression vector	
choX_Nde_fw	GGAATTCCATATGTTTGCAAATAGAAGTCGC
choX_Sal-Xho_rv	CCGGTCGACTCACTCGAGAAGGCCGAGAGCC
QuikChange mutagenesis of *choX*	
ChoX-W41A-fw	TGATGTCGGCGCGACCGATATCACCGCC
ChoX-W41A-rv	GGCGGTGATATCGGTCGCGCCGACATCA
ChoX-W88A-fw	CCTCGGCAACGCGATGCCGACAATGGAAGG
ChoX-W88A-rv	CCTTCCATTGTCGGCATCGCGTTGCCGAGG
ChoX-W283A-fw	CCGCAACCGCGGCGCTGAAGGCCAATCC
ChoX-W283A-rv	GGATTGGCCTTCAGCGCCGCGGTTGCGG
ChoX-W294A-fw	GATCGAGCCCGCGTTCGCCAACGTTAAGACAA
ChoX-W294A-rv	TTGTCTTAACGTTGGCGAACGCGGGCTCGATC
Construction of *A. tumefaciens choX* mutants	
choX_Kpn_fw	GCAGGGTACCAGGCGGCGATTTTCACG
choX_Eco_rv	CCGGAATTCGTATTTCCCACTGCATCCG

[a] Restriction sites in the oligonucleotides are underlined.

medium without antibiotics and plated on LB medium containing 10% (wt/vol) sucrose to select for plasmid excision by double crossover. Sucrose- and gentamicin-resistant and kanamycin-sensitive clones were analyzed by colony PCR and Southern blot analysis (42).

β-Galactosidase assays. The β-galactosidase activity of *A. tumefaciens* cells grown in liquid AB minimal medium in the presence or absence of 1 mM choline was measured according to standard protocols (32). Cells from the exponential growth phase were taken for the β-galactosidase assays. Under these conditions, the addition of 1 mM choline did not influence growth of the tested strains. The plasmid pAC01 containing the promoterless *lacZ* gene was used as a negative control.

Choline transport assays. For initial [^{14}C]choline uptake analyses, overnight cultures of *A. tumefaciens* wild-type and *choX* mutant strains grown in M9 minimal medium with 0.1 mM choline were washed and adjusted to an optical density at 600 nm (OD_{600}) of 1.0 with M9 medium. Reactions were initiated by addition of [*methyl*-^{14}C]choline chloride (55 mCi/mmol) to a final concentration of 0.1 mM, and samples were incubated at 30°C. Fifty-microliter aliquots were taken at different time points (5 to 60 min), and reactions were terminated by rapid filtration of samples on HAWP 02500 filters. For the complementation assays, the strains were grown in M9 minimal medium to an OD_{600} of 0.5 to 0.8. Subsequently, the OD_{600}s of the cells were adjusted with M9 medium to 0.5. In the case of the *choX* mutants carrying the pVSBAD derivatives, cells were induced at an OD_{600} of 0.3 with 0.2% arabinose. Reactions were initiated by addition of [*methyl*-^{14}C]choline chloride (55 mCi/mmol) to a final concentration of 5 μM. Samples (1 ml) were incubated for 10 min at 30°C. Reactions were terminated by rapid filtration as mentioned above. Filters were washed four times with 1 ml of M9 medium, and the amount of radioactively labeled choline in the cells was quantified by liquid scintillation spectrometry (Beckman Counter LS-6000 TA).

Lipid analysis by TLC. The lipid composition of *A. tumefaciens* strains was determined via thin-layer chromatography (TLC). Cells were cultivated in M9 medium in the absence or presence of 1 mM choline for 24 h. Two milliliters of the cultures with identical optical densities was harvested by centrifugation, washed with 500 μl of water, and resuspended in 100 μl of water. The lipids were extracted according to the method of Bligh and Dyer (3), separated by one-dimensional TLC using high-performance TLC (HPTLC) silica gel 60 plates (Merck, Darmstadt, Germany), and stained with Molybdenum Blue Spray reagent (Sigma-Aldrich). As running solvent, *n*-propanol-propionate-chloroform-water (3:2:2:1) was used.

Overproduction and purification of ChoX and mutated variants. *E. coli* BL21(DE3) cells carrying the pBO2002 plasmid (pET24b_*choX*) or derivatives were grown in 1 liter of M9 minimal medium with glucose as the carbon source (42) and kanamycin (50 μg/ml) at 37°C. Protein production was induced at an OD_{580} of 0.5 by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM before the culture was incubated for 24 h at 20°C and harvested by centrifugation (6,000 × *g* for 10 min at 4°C). ChoX contains an N-terminal signal sequence for targeting it into the periplasm. To release the recombinant ChoX protein from the periplasmic space, the cells were subjected to rapid osmotic shock by resuspending the cells in wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole [pH 8.0]) containing 500 mM saccharose, followed by incubation for 30 min on ice. Insoluble material was removed by centrifugation (20,000 × *g* for 40 min at 4°C). Soluble fractions were applied to nickel-iminodiacetic acid (Ni-IDA) columns, and purification was performed as described by the supplier (Macherey and Nagel). ChoX was eluted from the column with 200 mM imidazole in wash buffer. Protein purity was assessed by Coomassie staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels (12.5%).

Size exclusion chromatography. Size exclusion chromatography was performed using a Superdex 75 10/30 column run with 50 mM NaH_2PO_4 and 50 mM NaCl (pH 8.0) at a flow rate of 0.5 ml/min. The column was calibrated with the globular proteins RNase (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), and albumin (67 kDa), obtaining an R^2 value of 0.99 for the calibration curve. A 250-μl aliquot of protein at a concentration of 0.8 mg/ml was injected. Eluates (0.5 ml) were pooled and concentrated in Amicon Ultra concentrators (molecular-weight cutoff, 10,000; Millipore), and the buffer was exchanged against binding buffer (10 mM Tris-HCl, 200 mM NaCl [pH 7.4]). Protein concentrations were determined from A_{280} values with a calculated extinction coefficient (ε) of 39,545 M^{-1} cm^{-1}.

CD spectroscopy. The circular dichroism (CD) spectra of recombinant ChoX variants were recorded 10 times between 190 and 320 nm with a Jasco 715 spectropolarimeter at 20°C in 50 mM potassium phosphate buffer, pH 8. The final spectra obtained were the average results of the 10 scans, normalized against buffer. Analyses were performed in duplicate using 10 μM enzyme.

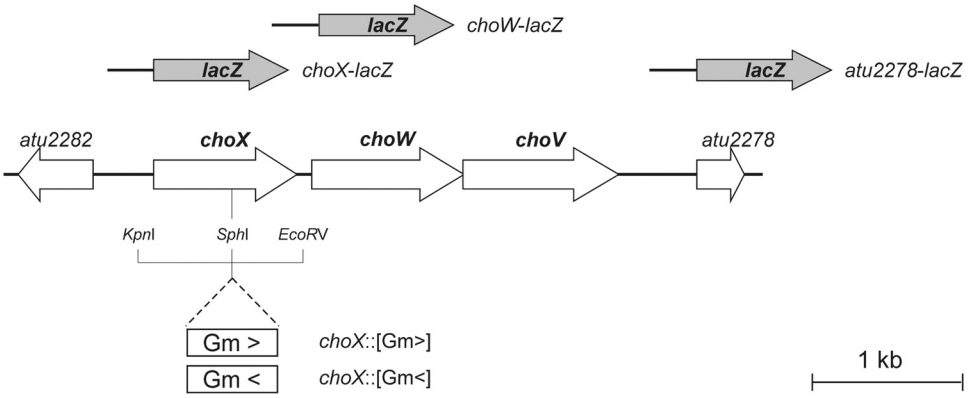


FIG. 1. Genetic organization of the *choXWV* locus. The *cho* genes are flanked by two ORFs: *atu2282*, encoding a putative thymidine kinase, and *atu2278*, a NolR-like regulator. *choX* encodes a periplasmic choline-binding protein, *choW* encodes a permease, and *choV* encodes an ATPase. The positions of the gentamicin (Gm) cassette insertion and corresponding mutants (*choX*::Gm> and *choX*::Gm<) are indicated. The upper part shows the constructed *lacZ* fusions.

***In vitro* [^{14}C]choline-binding studies.** For choline-binding assays, 5 μM recombinant ChoX protein was incubated with 0 to 50 μM [*methyl*-^{14}C]choline chloride (55 mCi/mmol) in binding buffer for 5 min at room temperature ([RT] total assay volume, 50 μl). For competition experiments, unlabeled choline chloride (0 to 50 μM) was added to the samples and incubated for 5 min at 30°C before the addition of 5 μM [^{14}C]choline.

Binding assay mixtures were passed over HAWP 02500 filters on a filtration funnel, and unbound [*methyl*-^{14}C]choline chloride was removed by washing samples four times with 300 μl of binding buffer. Bound [*methyl*-^{14}C]choline chloride was quantified by liquid scintillation spectrometry (Beckman Counter LS-6000 TA). The Michaelis-Menten equation was used for calculation of choline-binding affinities of the wild type and mutated ChoX variants. The data were fitted by nonlinear regression using SigmaPlot, version 9.0.

Fluorescence-based ligand-binding assays. The affinity of recombinant ChoX protein for choline acetylcholine and betaine was determined by intrinsic tryptophan fluorescence spectroscopy according to Oswald et al. (36). The excitation wavelength was set to 295 nm, and fluorescence was monitored from 300 to 450 nm using a Thermo Amicon Bowman II Luminescence Spectrometer at 22°C. Different amounts of ligands (0 to 100 μM) in binding buffer (200 mM NaCl, 10 mM Tris-HCl, pH 7.4) were titrated to ChoX (10 μM; assay volume, 100 μl) in binding buffer, and after equilibration (5 min) fluorescence was measured. To account for background fluorescence, spectra with and without protein were subtracted from each other. Upon ligand titration the emission maximum shifted toward shorter wavelengths (blue shift) (see Fig. 6). The changes in the emission maximum (Δλ, in nm) were plotted against the ligand concentration, and the Michaelis-Menten equation was used for calculation of ligand-binding affinities. The data were fitted by nonlinear regression using SigmaPlot, version 9.0.

Development of a homology model for ChoX. The three-dimensional structure of ChoX was predicted by the threading method using the I-TASSER online server (41, 56, 57). Structures of proteins with the following Protein Data Bank identifiers (PDB IDs) were chosen by I-TASSER as the templates in the modeling procedure: 2rf1A, 2rinA, and 2regA (*S. meliloti* ChoX); 1r91A (*E. coli* ProX); and 3L6gA (lactococcal OpuAC). This server produced five possible models for ChoX. The first model with the best quality of prediction (confidence [C] score, −0.33; template-modeling [TM] score, 0.67 ± 0.13; and root mean square deviation [RMSD], 7.0 ± 4.14 Å) was used here.

RESULTS

Identification of a ChoXWV-like transporter system in *A. tumefaciens*. Using the Transporter Protein Analysis Database (40), we identified *atu2281*, *atu2280*, and *atu2279* encoding a putative choline ABC-transporter similar to the ChoXWV system from *S. meliloti*. Atu2281 is highly similar in the amino acid sequence (85% similarity and 80% identity) to the periplasmic choline-binding protein ChoX. Atu2280 and Atu2279 are highly similar (87% similarity and 76% identity) to ChoV (permease) and ChoW (ATPase), respectively (13). Accordingly, *atu2281*, *atu2280*, and *atu2279* were tentatively designated *choXWV* (Fig. 1).

Genetic organization of the *choXWV* genes. The *choXWV* genes are located on the circular chromosome of *A. tumefaciens* C58. They are flanked by two open reading frames (ORF), namely, *atu2282*, transcribed in the opposite direction, and *atu2278*, oriented in the same direction as the *cho* genes (Fig. 1). The ORF *atu2282* encodes a putative thymidine kinase, and *atu2278* codes for a putative NolR regulator. NolR is present in species belonging to the *Rhizobium* and *Sinorhizobium* genera and belongs to the AsrR family of regulators. In *S. meliloti* NolR is a global regulatory protein involved in nodulation, bacterial growth and survival, and conjugative transfer of plasmid (9, 10, 22, 25). The putative *A. tumefaciens* NolR protein had no significant effect on *choXWV* expression in the absence or presence of choline (data not shown).

Within the *cho* locus, *choX* and *choW* are separated by a 109-bp noncoding region while the translational start codon of *choV* overlaps with the translational stop codon of *choW*, suggesting translational coupling between these two genes.

Expression of the *cho* operon. To examine whether *choXWV* was expressed, transcriptional *lacZ* fusions were constructed (Fig. 1). A *choW-lacZ* fusion was used to test whether *choWV* expression was originated from a separate promoter or was driven from the *choX* promoter. The resulting reporter plasmids, pBO2017 (*choX-lacZ*) and pBO890 (*choW-lacZ*), were electroporated into wild-type *A. tumefaciens*. To examine the effect of choline on *choXWV* expression, all reporter strains were grown in AB minimal medium in the presence or absence of 1 mM choline. Clear *choX* expression was detected in both the absence and presence of choline. Expression was moderately but reproducibly enhanced when cells were grown with choline (Fig. 2). To test whether this effect of choline was specific, an *atu2278-lacZ* fusion was measured as a control. In contrast to *choX*, expression of *atu2278* was not affected by choline at all (Fig. 2).

The *choW-lacZ* fusion was not expressed under all conditions tested (Fig. 2), excluding a possible promoter in the intergenic region upstream of the *choW* gene. The conclusion

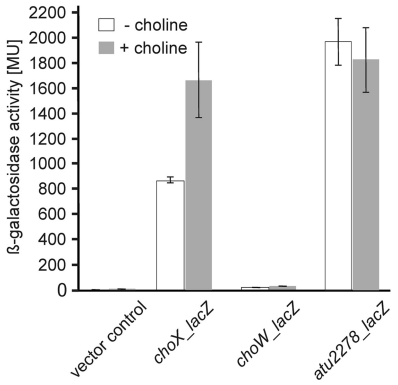


FIG. 2. β-Galactosidase activities of plasmid-encoded transcriptional *choX-lacZ*, *choW-lacZ*, and *atu2278-lacZ* fusions in *A. tumefaciens* C58. Cells were grown in AB minimal medium in the absence or presence of 1 mM choline at 30°C. The plasmid pAC01 containing the promoterless *lacZ* gene was used as a negative control. Values are the mean of three independent experiments ± standard deviations.

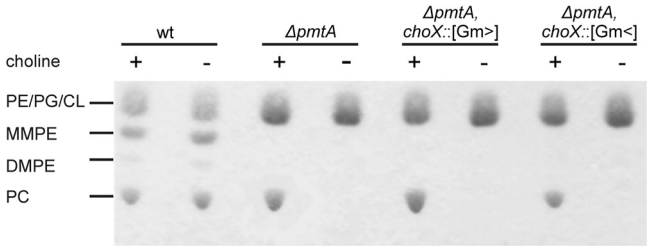


FIG. 3. PC formation in *A. tumefaciens* wild-type (wt) and mutant strains. Cells were grown in M9 minimal medium in the absence (−) or presence (+) of 1 mM choline for 24 h. Lipids were extracted, separated by one-dimensional TLC, and visualized by Molybdenum Blue staining. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; MMPE, monomethyl-PE; DMPE, dimethyl-PE; PC, phosphatidylcholine.

that *choXWV* genes are organized in an operon is supported by recent RNA sequencing data from our laboratory (data not shown).

Construction and growth phenotype of *choX* mutants. To study the role of the *choXWV* operon in choline uptake, we constructed *choX* mutants by insertion of a gentamicin (Gm) resistance cassette into the *choX* coding region, as described in Materials and Methods. The Gm resistance cassette was inserted in either the same (Gm>) or the opposite (Gm<) orientation of the *choX* gene, resulting in the two *A. tumefaciens choX*::Gm> and *choX*::Gm< mutants, respectively. As shown previously (29), Gm cassettes induce polar or nonpolar mutations depending on their orientations. Thus, the *choX*::Gm< mutant is expected to be defective for *choX*, *choW*, and *choV*, whereas expression of *choW* and *choX* should be driven by the Gm promoter in the *choX*::Gm> mutant.

To test for obvious phenotypic defects, the growth behavior of the *choX* mutants was investigated under different conditions. Growth of the mutants was indistinguishable from that of the *A. tumefaciens* wild type at 30°C in minimal medium under low- and high-osmolarity conditions (without added NaCl or with NaCl concentrations ranging from 0.2 to 0.8 M) in the presence of 0.1 mM or 1 mM choline. Likewise, the strains showed no differences when they were cultivated in AB minimal medium without choline (data not shown), demonstrating that *choX* mutants of *A. tumefaciens* grow normally in liquid culture in either the absence or presence of choline. Choline enhanced growth of wild-type and mutant strains in the presence of 0.2 to 08 M NaCl, suggesting that choline has an osmoprotective role in *A. tumefaciens* C58 and that this effect is not dependent on the Cho system.

ChoXWV is not required for choline-dependent PC biosynthesis. PC formation in an *A. tumefaciens pmtA* mutant occurs via the Pcs pathway, which is dependent on choline in the growth medium (53). To address whether the ChoXWV transporter is necessary for PC biosynthesis, we analyzed PC production in *pmtA choX* double mutants grown in minimal medium in the presence or absence of 1 mM choline by TLC. As expected, wild-type *A. tumefaciens* accumulated phosphatidylethanolamine (PE), monomethyl-PE (MMPE), dimethyl-PE (DMPE), and PC under both tested conditions (Fig. 3). The *pmtA* mutant contained PC produced via the Pcs pathway in the presence of choline. However, in the absence of choline, none of the methylated lipids were present. The *pmtA choX*::Gm> mutant strain produced PC amounts comparable to those of the parental strain, indicating that the Pcs pathway is efficiently supplied with its substrate choline. The *pmtA choX*::Gm< mutant (expected to be deficient for *choX*, *choW*, and *choV*) accumulated slightly reduced PC amounts compared to parental strain levels.

***A. tumefaciens choX* mutants are severely impaired in choline uptake.** To analyze the role of ChoXWV in *A. tumefaciens* choline uptake, radioactively labeled choline transport assays were carried out. For this purpose *A. tumefaciens* cells were incubated with 0.1 mM [^{14}C]choline, and initial choline uptake at different time points was analyzed as described in Materials and Methods. Choline uptake by the *A. tumefaciens* wild type increased with incubation time (Fig. 4A). The *choX*::Gm> mutant strain (expressing *choWV* from the Gm promoter) retained some residual choline uptake activity (~25%). Since *choW* and *choV* are still expressed in this strain from the Gm promoter, other ChoX-like choline-binding proteins might partially compensate for the loss of ChoX. The *choX*::Gm< mutant (deficient for the entire *choXWV* system) was completely defective in choline transport (Fig. 4A).

Choline uptake deficiency of the *choX*::Gm> mutant was completely restored by expression of vector-encoded *choX* (pVSBAD_*choX*) (Fig. 4B). As expected, complementation of the choline uptake deficiency of the *choX*::Gm< mutant was not possible with plasmid-encoded *choX* (Fig. 4B) since this strain still lacks the corresponding permease and ATPase (ChoWV).

Although only little or no initial choline uptake was detected in *A. tumefaciens choX* mutants, they still produced PC via the Pcs pathway (Fig. 3). Two scenarios of how Pcs is provided with choline are possible. First, choline might be taken up by a thus far unidentified low-affinity transporter(s). Second, Pcs itself might bind choline from the periplasm before it converts it to PC. In this case, the Pcs enzyme would be independent from choline uptake systems. To test whether alternative choline uptake systems might exist in *A. tumefaciens*, we analyzed the accumulation of [^{14}C]choline in the *choX* mutants by incubat-

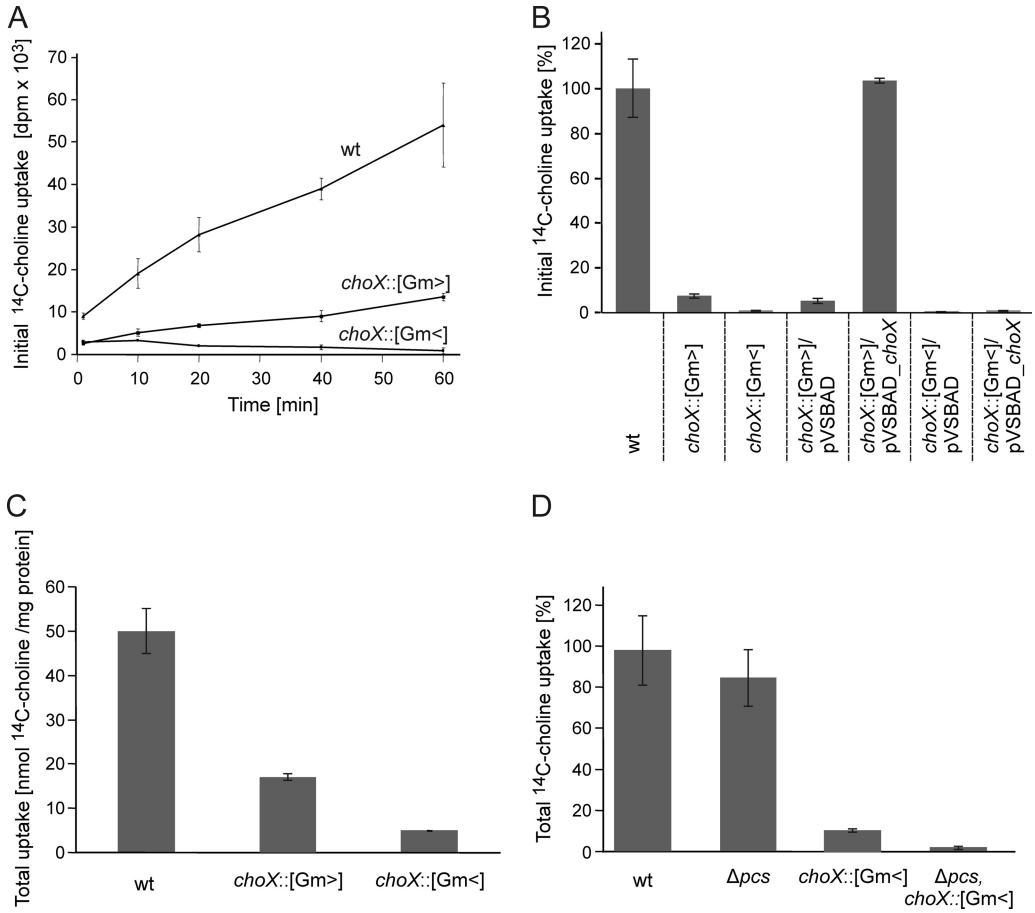


FIG. 4. [^{14}C]choline uptake analyses of the *A. tumefaciens* wild type (wt) and *choX* mutants. (A) Initial choline uptake of the *A. tumefaciens* wild type and *choX* mutants with a final choline concentration of 0.1 mM [*methyl*-^{14}C]choline chloride (55 mCi/mmol) at the time indicated. The points shown are the mean values of three individual experiments. (B) Complementation of the *choX* mutants by plasmid (pVSBAD)-encoded *choX* expression. Uptake was assayed over 10 min with a final [^{14}C]choline concentration of 5 μM. Total [^{14}C]choline uptake of the *A. tumefaciens* wild type and *choX* mutants (C) and the Δ*pcs choX*::[Gm<] double mutant (D) was assayed using a final choline concentration of 10 μM [^{14}C]choline. The accumulation of radioactivity was quantified after a 24-h incubation. The values shown are the means of two individual experiments.

ing the cells overnight with 10 μM radioactively labeled choline. Compared to the wild type, approximately 35% and 10% choline uptake was measured in the *choX*::Gm> and *choX*::Gm< mutant strains, respectively (Fig. 4C). To differentiate whether this long-time accumulation of [^{14}C]choline was due to PC synthesis via Pcs or low-affinity uptake systems, we analyzed [^{14}C]choline accumulation in a *pcs choX*::Gm< double mutant. Only marginal amounts of [^{14}C]choline were taken up by the *pcs choX*::Gm< double mutant (Fig. 4D), demonstrating an involvement of Pcs in [^{14}C]choline accumulation.

Purification of *A. tumefaciens* ChoX. C-terminally His_6-tagged ChoX was produced in *E. coli* BL21(DE3) cells and purified to homogeneity by nickel chelate chromatography and size exclusion chromatography. As judged by SDS-polyacrylamide gel electrophoresis (PAGE), ChoX protein of ~95% purity was obtained after size exclusion chromatography (Fig. 5A, inset). Consistent with the calculated mass of the His-tagged protein (~32.2 kDa, without its N-terminal signal peptide), recombinant ChoX migrated as a band with an apparent molecular mass of ~32 kDa. The major ChoX peak after gel filtration corresponded to a molecular mass of about 30 kDa (Fig. 5A), indicating that ChoX is a monomer.

ChoX is a high-affinity choline-binding protein. In order to demonstrate that *choX* encodes a choline-binding protein, filter-binding assays were performed with radioactively labeled choline and recombinant ChoX protein. Without ChoX, only negligible background radioactivity was detected on the filter (Fig. 5B, Control −ChoX). In the presence of ChoX, significant amounts of [^{14}C]choline were bound, demonstrating that ChoX is a choline-binding protein. A 10-fold molar excess of unlabeled choline (50 μM) efficiently competed with [^{14}C]choline (Fig. 5B, +Unlabeled choline excess). About 50% of the radioligand was exchanged in the presence of equal molar amounts (5 μM) of unlabeled choline (Fig. 5C). A 10-fold molar excess displaced the radioligand to less than 15%.

To determine the kinetics for choline binding of ChoX, we performed [^{14}C]choline-binding experiments with various choline concentrations and calculated a binding constant for choline in the low-micromolar range (2.4 μM) (Fig. 5D). Thus, we concluded ChoX is as a high-affinity choline-binding protein.

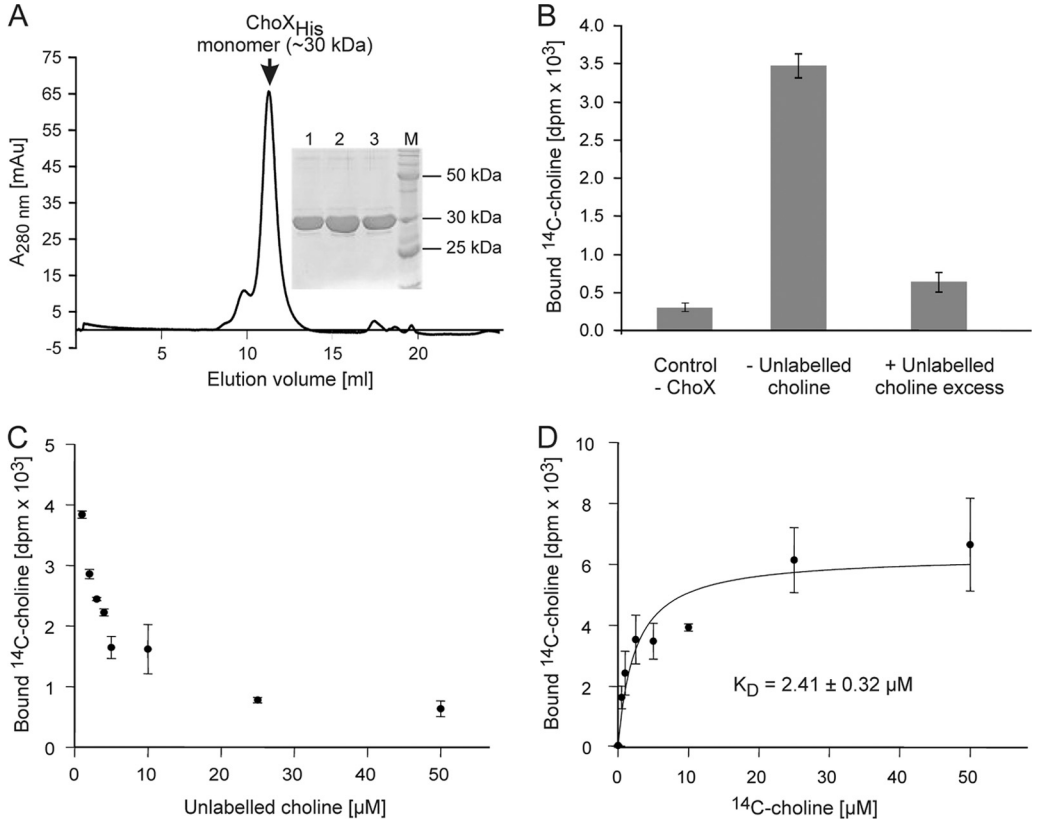


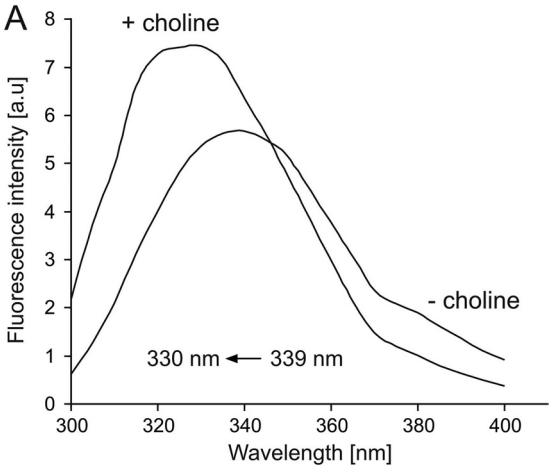
FIG. 5. Purification and *in vitro* choline-binding properties of recombinant ChoX. (A) Size exclusion chromatography of Ni-IDA-purified PmtA. The peak fractions 1, 2, and 3 were analyzed by SDS-PAGE as depicted on the right. AU, absorbance units. Lane M (inset), BenchMark protein standard (Invitrogen). (B) Radioactive choline-binding activity of recombinant ChoX. Choline-binding activity was analyzed with 5 μM ChoX and 5 μM [^{14}C]choline over 5 min. The displacement assay (+unlabeled choline excess) contained, in addition, 10-fold unlabeled choline. (C) Competitive displacement of radioactively labeled choline by unlabeled choline (0 to 50 μM). The assay mixture contained 5 μM recombinant ChoX and 5 μM [^{14}C]choline. (D) Plots of choline-binding data for wild-type ChoX. A reaction volume of 50 μl contained 5 μM recombinant ChoX and 0 to 50 μM [^{14}C]choline. Changes in bound [^{14}C]choline (*y* axis) were plotted against choline concentration (*x* axis). All data sets were fitted to the equation for one-site binding (see Materials and Methods) by nonlinear regression using SigmaPlot, version 9.0. The points shown are the mean values of three individual experiments.

As a second approach, intrinsic tryptophan fluorescence-based binding assays were used to quantitate substrate binding. A representative fluorescence spectrum in the absence and presence of choline is shown in Fig. 6A. Binding of choline and acetylcholine to ChoX resulted in a blue shift of the emission spectrum of 9 nm (Fig. 6A) and 5 nm (data not shown), respectively. Binding of betaine to ChoX induced only a marginal blue shift (3 nm) of the emission spectrum (data not shown). These shifts were used to determine the binding affinity constants according to the Michaelis-Menten equation (Fig. 5B). These data demonstrate that ChoX recognizes choline, acetylcholine, and betaine with high, medium, and low affinity, respectively. The equilibrium dissociation constant (K_D) of 3.3 μM for choline is in good agreement with the binding constant of 2.4 μM determined via the [^{14}C]choline-binding assay.

Site-directed mutagenesis of residues forming the predicted ligand-binding pocket. To gain more insights into residues forming the ligand-binding pocket, we generated a model of ChoX using the I-TASSER server. *A. tumefaciens* ChoX exhibits a fold highly similar to that of *S. meliloti* ChoX (Fig. 7). The overall structure resembles the well-known substrate binding protein (SBP) fold composed of two domains, which are connected via two β-strands. The predicted choline-binding site is formed by an aromatic box, which is located in a deep cleft between the two domains. This box is composed of four aromatic amino acids, W41, W88, Y117, and W203 (Fig. 7A and B). To examine the role of these amino acid residues for ligand binding, two of these amino acids (W41 and W88) were exchanged to alanine, and the effect of the mutation on choline binding was analyzed. Both individual mutations resulted in a nearly complete loss of choline binding and the double mutant (W41A W88A) was completely unable to bind choline (Fig. 8A and B). As a control, point mutations in two tryptophan residues outside the binding region (W283 and W294) (Fig. 7A) had no influence on choline binding. The structural integrity of the mutated ChoX variants was ascertained using CD spectroscopy. Like the wild-type protein, all mutated variants were structurally intact (Fig. 4C).

DISCUSSION

A. tumefaciens contains PC as a major phospholipid in both its inner and outer membranes (21). PC synthesis via the Pcs pathway is dependent on exogenous choline, which is a posi-



B

Ligand	K_D [μM]
Choline	3.27 ± 1.31
Acetylcholine	82.36 ± 18.77
Betaine	470.40 ± 234.07

FIG. 6. Fluorescence-based ligand-binding analysis of ChoX. (A) Emission spectra of ChoX (10 μM) in the absence or presence of 100 μM choline. au, arbitrary units. (B) Binding affinities (K_Ds) of ChoX for choline, acetylcholine, and betaine were determined via intrinsic tryptophan quenching after addition of the ligands (0 to 100 μM) to ChoX protein (10 μM). Changes in the emission maximum (Δλ in nm) were plotted against the ligand concentration (data not shown), and the Michaelis-Menten equation was used for calculation of ligand-binding affinities. The data were fitted by nonlinear regression using SigmaPlot, version 9.0. Values are the means of three independent experiments ± standard deviations.

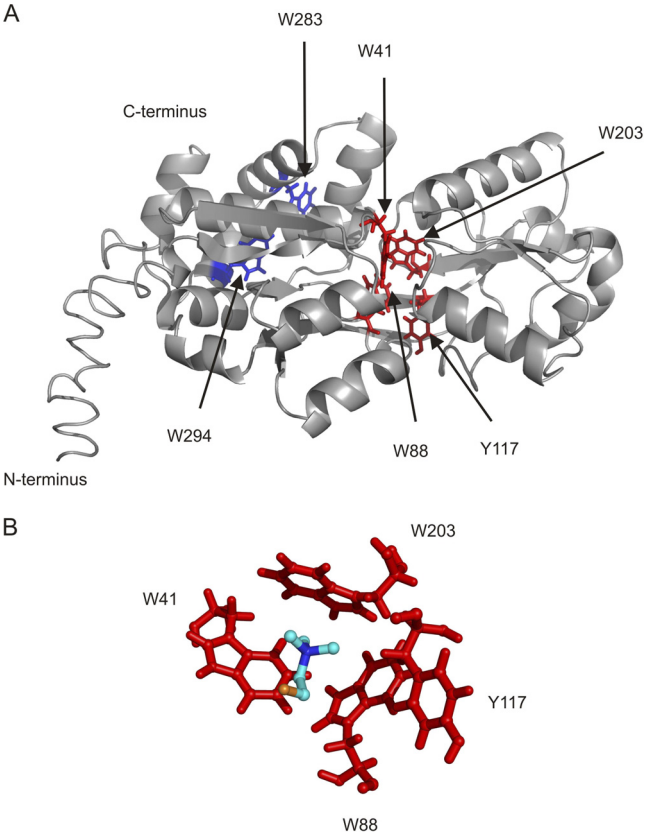


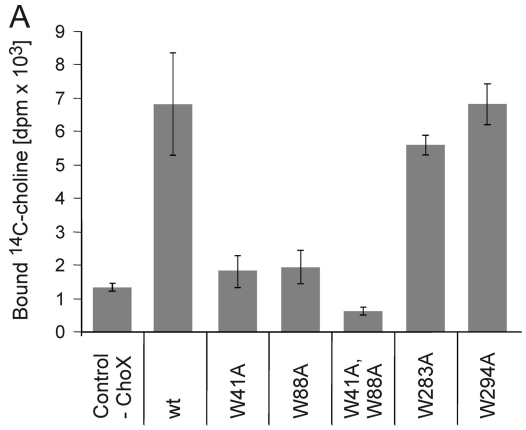
FIG. 7. Homology model of *A. tumefaciens* ChoX. (A) ChoX model was generated using the online server I-TASSER (41) and visualized by PyMOL (http://www.pymol.org). The fold is characterized by a bilobal organization into two lobes connected via a linker region. The proposed ligand-binding site is located within the two lobes. Proposed choline-binding residues are shown in red, and two aromatic residues (W283 and W294) outside the binding pocket, depicted as negative controls, are shown in blue. (B) Detailed view of the ligand-binding site of *A. tumefaciens* ChoX. Proposed residues participating in ligand binding and the ligand choline are shown in stick and in ball-and-stick representation, respectively.

tively charged quaternary amine and requires a protein-mediated mechanism to effectively pass the membrane lipid barrier (31). In this study, we have characterized a *cho*-like operon involved in the high-affinity uptake of choline in *A. tumefaciens*. This system belongs to the ABC-transporter family involving multiple components: a periplasmic choline-binding protein (ChoX), an integral inner membrane protein (ChoW), and an ATPase (ChoV).

The purified *A. tumefaciens* ChoX is a high-affinity choline-binding protein with a medium affinity for acetylcholine. Binding affinities in the low-micromolar range are typical for periplasmic substrate-binding proteins in Gram-negative bacteria. The determined affinity constants for choline (2.4 μM and 3.3 μM) and for acetylcholine (82.4 μM) are in good agreement with the K_D values of 2.7 μM and 2.3 μM for choline and 100 μM for acetylcholine reported previously for the *S. meliloti* ChoX protein (13, 36). In contrast to the *S. meliloti* ChoX protein, which binds betaine with a K_D of 77 μM, *A. tumefaciens* ChoX bound betaine with very low affinity (470 μM). Thus, *A. tumefaciens* ChoX is a substrate-binding protein with a preference for choline.

In silico predictions backed up by site-directed mutagenesis revealed a ligand-binding pocket and mode of substrate binding of *A. tumefaciens* ChoX highly similar to the *S. meliloti* ChoX and other glycine/betaine/proline substrate-binding proteins (28, 35, 36, 39). The high-resolution crystal structure of the *S. meliloti* ChoX protein, with its ligands (choline and acetylcholine) and a ligand-free state, was determined recently (35, 36). Choline is bound via cation-π interaction of the trimethylammonium moiety and four aromatic residues (W43, W90, Y119, and W205) and hydrogen bonds between the hydroxyl group and N156 and D157 (35, 36). These residues are also present in the *A. tumefaciens* ChoX protein, and as shown here, the conserved aromatic residues W41 and W88 located in the predicted ligand-binding pocket are essential for choline binding of *A. tumefaciens* ChoX.

Two *choX* mutants generated via insertion of a Gm cassette in the same or opposite orientation to the *cho* operon showed a wild-type-like growth behavior in minimal medium in the absence or presence of choline. Interestingly, two different choline uptake phenotypes were observed due to polar effects of the Gm cassette. The *choX*::Gm> mutant (expression of *choWV* from Gm promoter possible) transported marginal



B

ChoX variant	Choline K_D [μM]
wt	2.41 ± 0.32
W41A	No binding
W88A	No binding
W41A,W88A	No binding
W283A	2.54 ± 1.26
W294A	2.55 ± 1.02

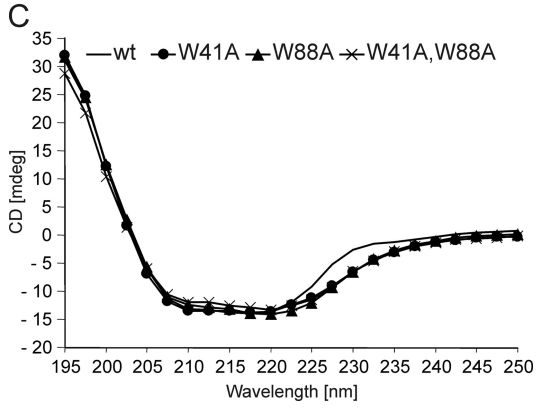


FIG. 8. *In vitro* choline-binding properties of point-mutated ChoX proteins. (A) [^{14}C]choline binding was assayed with 5 μM ChoX and 5 μM [^{14}C]choline. (B) Choline affinity constants (K_Ds) of ChoX variants. To calculate affinity constants, 5 μM ChoX protein was titrated with 0 to 50 μM [^{14}C]choline, and bound [^{14}C]choline (in disintegrations per minute) was plotted against the [^{14}C]choline concentration (data not shown). The Michaelis-Menten equation was used for calculation of ligand-binding affinities. The data were fitted by nonlinear regression using SigmaPlot, version 9.0. Values are the means of three independent experiments ± standard deviations. (C) Circular dichroism analysis of the wild type and inactive ChoX variants. The spectrum of each protein with a concentration of 10 μM is plotted as ellipticity in millidegrees (mdeg) versus wavelength.

amounts of choline, whereas the *choX*::Gm< mutant, expected to be deficient in *choXWV*, was dramatically reduced in choline uptake. A possible explanation for the residual choline uptake in the *choX*::Gm> mutant may be that other ChoX-like binding proteins can partially compensate for the loss of ChoX in delivering choline to the core transporter ChoWV. *A. tumefaciens* encodes two candidates, namely, Atu2060 and Atu4647 sharing 64% similarity to ChoX. Cross talk between ABC transporters via their substrate-binding proteins is not uncommon (8, 51). Recently, a choline-betaine-carnitine (Cbc) transporter in *Pseudomonas syringae* and *Pseudomonas aeruginosa* was identified that recruits multiple substrate-binding proteins. In addition to CbcX, the core CbcWV also interacts with the carnitine-specific binding protein CaiX and the betaine-specific binding protein BetX (8, 51). The CbcXWV transport system shares approximately 70% similarity with the *A. tumefaciens* ChoXWV system. The histidine ABC transporter (HisQMP) from *Salmonella enterica* serovar Typhimurium is able to recognize two distinct SBPs (17). Furthermore, the oligopeptide transporter (Opp) from *Borrelia burgdorferi* uses multiple SBPs whose genes are differentially expressed under various environmental conditions (52). The observation that small amounts of choline are taken up when the *choX*::Gm< mutant is incubated with choline overnight (Fig. 4C) suggests that *A. tumefaciens* contains at least one further pathway for a low-affinity choline uptake system. At least one of the five remaining putative quaternary amine transporters (40, 54) encoded in *A. tumefaciens* can be considered for this activity in the *choX* mutants. Multiple choline uptake systems are common in bacteria. *P. aeruginosa* encodes two betaine-choline-carnitine transporters ([BCCT] BetT1 to BetT3) and an ABC-type choline transporter (CbcXWV) with different roles (27). In *P. syringae* one BCCT-type transporter (BetT) and two ABC-type choline transporters (CbcXWV and OpuC) were identified (6–8).

Choline transport systems in *A. tumefaciens* may be important for transport of choline as a carbon and nitrogen source or for use in osmoregulation as in many other bacteria. Although it was reported that the *A. tumefaciens* strains can use choline or betaine as a sole N or C source (5, 47), attempts to grow *A. tumefaciens* C58 in minimal medium with choline as the sole N and/or C source failed in our laboratory (data not shown). Thus, choline seems not to be an efficient N and/or C source for *A. tumefaciens* C58. Does choline instead act as a precursor for the osmoprotectant glycine betaine in *A. tumefaciens* C58? Osmoprotection by choline and evidence for a choline dehydrogenase and betaine aldehyde dehydrogenase activity (for two-step enzymatic oxidation of choline to betaine) in *A. tumefaciens* GMI 9023 crude extracts have been reported (5). In *A. tumefaciens* C58, the strain used in this study, mannosucrose is the major osmolyte, but a slight osmoprotective effect of betaine was also observed (47). In the absence of osmotic stress, betaine is metabolized by *A. tumefaciens* C58, but in stressed cultures betaine accumulates and confers enhanced osmotic stress tolerance (47). A putative choline dehydrogenase (Atu0830) and a betaine aldehyde dehydrogenase (Atu0829) are present in the *A. tumefaciens* C58 genome (14, 54). An osmoprotective effect of choline in this strain was also observed in our laboratory (data not shown). Since this effect was similar in both the wild type and the *choX*::Gm< mutant (data not shown), the osmoprotective effect of choline seems not to be dependent upon the Cho system.

Remarkably, the ChoXWV system is not essential for choline-dependent PC biosynthesis in *A. tumefaciens* since both

choX mutants were still able to produce PC when choline was supplied in the growth medium. The Δ*pmtA choX*::Gm> mutant produced wild-type-like PC amounts and the Δ*pmtA choX*::Gm< mutant showed only slightly reduced PC synthesis (Fig. 3) compared to the parental strain. Thus, both strains are supplied with sufficient amounts of choline for PC synthesis. It is unclear whether Pcs obtains choline from the cytoplasm or from the periplasm. Our data suggest that Pcs obtains choline from the periplasm, and, thus, PC biosynthesis is independent from choline uptake systems in *A. tumefaciens.* Preliminary experiments with the choline uptake-deficient *E. coli* MKH13 strain (15, 20) demonstrated that plasmid-encoded *pcs* is sufficient to enable this strain to produce PC when choline is supplied in the growth medium (data not shown). These lines of evidence suggest that the Pcs enzyme obtains choline from the periplasmic face of the inner membrane. Future biochemical studies on this integral membrane protein will solve this open question.

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