Structural Basis for High Specificity of Amadori Compound and Mannopine Opine Binding in Bacterial Pathogens

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Running title: Selection of mannopine opine and Amadori compound by PBPs

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ABSTRACT

*Agrobacterium tumefaciens* pathogens genetically modify their host plants to drive the synthesis of opines in plant tumours. Opines are either sugar-phosphodiesters or the products of condensed amino acids with ketoacids or sugars. They are *Agrobacterium* nutrients and imported into the bacterial cell via periplasmic binding proteins (PBPs) and ABC-transporters. Mannopine, an opine from the mannityl-opine family, is synthesized from an intermediate named deoxy-fructosyl-glutamine (DFG), which is also an opine and abundant Amadori compound (a name used for any derivative of aminodeoxysugars) present in decaying plant materials. The PBP MotA is responsible for mannopine import in mannopine-assimilating agrobacteria. In the nopaline-opine type agrobacteria strain, SocA protein was proposed as a putative mannopine-binding PBP, and AttC protein was annotated as a mannopine binding-like PBP. Structural data on mannityl-opine-PBP complexes is currently lacking. By combining affinity data with analysis of seven X-ray structures at high resolution, we investigated the molecular basis of MotA, SocA and AttC interactions with mannopine and its DFG precursor. Our work demonstrates that AttC is not a mannopine binding protein, and reveals a specific binding-pocket for DFG in SocA with an affinity in nanomolar range. Hence, mannopine would not be imported into nopaline-type agrobacteria strains. In contrast, MotA binds both mannopine and DFG. We thus defined one mannopine-and two DFG-binding signatures. Unlike mannopine-PBPs, selective DFG-PBPs are present in a wide diversity of bacteria, including Actinobacteria, α-, β-, and γ-proteobacteria, revealing a common role of this Amadori compound in pathogenic, symbiotic and opportunistic bacteria.

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*Agrobacterium tumefaciens* pathogens have adapted a strategy of niche construction (called tumour niche or opine niche) inside the plant hosts. Indeed, they mediate the transfer of a portion of their tumour-inducing (Ti) plasmid, (namely T-DNA), to the plant cell (1, 2). The T-DNA becomes incorporated into the plant nuclear DNA. T-DNA gene expression drives the synthesis of plant hormones that leads to the formation of a tumour characteristic of the crown-gall disease. Expression of the T-DNA genes in plant tumours also allows the production of novel organic compounds called opines which are either sugar-phosphodiesters or the products of condensed amino acids with ketoacids or sugars (3–5). Opines are specifically utilized as growth substrates by the inciting agrobacteria and confer upon these pathogens an advantage when in competition with other members of the soil microflora for the colonization of the opine-rich environments (3, 7–11). The genes required for opine catabolism (import and degradation) in the agrobacterial pathogens are coded for by their Ti plasmid, but located in segments outside the T-DNA region. Some opines, designated conjugative opines, such as octopine or agrocinopine (actually only the arabinose-2-phosphate moiety of the agrocinopine is recognized) (12), mediate the horizontal transfer (bacterial conjugation) of the Ti-plasmid (13).
In Agrobacterium-induced plant tumours, over 20 opines are known and classified into distinct families according to their precursors which are sugars, amino acids and ketoacids. Octopine and nopaline are opines synthesized in a one-step conjugation of the amino acid arginine and a ketoacid (14, 15). Unlike octopine and nopaline, mannopine (1-deoxy-mannosyl-glutamine) from the mannityl-opine family is not the result of a one-step formation, since it is formed from a precursor which is also an opine named 1-deoxy-fructosyl-glutamine (DFG) from the chrysopine family (16, 17). Mannopine synthesis requires the Mas2 enzyme which conjugates fructose and glutamine into DFG followed by the activity of the oxydo-reductase Mas1, which reduces DFG into mannopine (Fig. 1) (18). DFG is a peculiar opine, in that this compound is widely available in the environment outside the Agrobacterium-induced plant tumour as a product of the decomposition of organic materials, and belongs to a wide class of molecules known as Amadori compounds, a term used for any derivative of aminodeoxysugars (19).

In A. tumefaciens, the recognition and import of opines is facilitated by periplasmic binding proteins (PBPs) and their associated ATP-binding cassette (ABC) transporters (20, 21). Structural data on mannityl-opine-PBP complexes is currently lacking. In this study, we investigated and compared the structural and biochemical properties of two PBPs sharing 5% sequence identity, MotA and SocA, which have been associated with the assimilation of mannopine using genetic approaches (mapping of gene regions and defective mutants) and opine uptake/transport experiments (22–25). MotA belongs to the mannopine transport system encoded by the Ti plasmid of the A. tumefaciens strain B6 (Fig. 1) (24). In A. tumefaciens strain C58, Ti plasmid genes drive the synthesis (in the plant cells) and assimilation (in the bacterial cells) of the opines agrocinopine and nopaline, but not those of mannopine. Therefore MotA and the mannopine-degrading enzyme MocC are not present in this strain, which cannot grow when mannopine is the sole source of C and N. In contrast, A. tumefaciens strain C58 can grow when DFG is the sole resource (25). Remarkably, the constitutive expression of the mannopine-degrading enzyme MocC in A. tumefaciens C58 conferred upon this strain the capability to grow on mannopine. Baek et al (25) identified the region of the pAt carrying the socA gene (encoding the PBP SocA) and socB gene (encoding the associated ABC transporter) as responsible for both DFG and mannopine import in wild-type strain C58 and its MocC-expressing derivative, respectively.

Finally, we used the synthetic opine analogue glucopine which constitutes the sugar part-C2 epimer of mannopine to probe ligand specificity. This work led to the identification of fingerprint sequences that define mannopine- and/or DFG-binding PBPs and provides new insights into the utilization of mannopine and DFG in host-interacting bacteria.

RESULTS

Affinity of MotA, SocA and AttC to Mannopine, DFG and the Synthetic Glucopine—By intrinsic protein fluorescence titration, the dissociation constant (K_D) values between MotA and mannopine, DFG and glucopine, were 4 ± 0.5 nM, 104.5 ± 20 nM and 55.5 ± 9.1 nM respectively (Fig. 2A) showing that MotA is very efficient for mannopine binding. Using isothermal titration calorimetry (ITC), similar K_D values were obtained, i.e. 19.8 ± 3 nM, 99 ± 13 nM and 79.4 ± 3 nM for MotA-mannopine, MotA-DFG and MotA-glucopine respectively (Fig. 2B). For SocA, autofluorescence displayed K_D values of 56.9 ± 10 nM and 2.5 ± 0.4 µM for DFG and glucopine respectively (Fig. 2A). With ITC, they were 74.1 ± 13 nM and 4 ± 0.2 µM respectively, in the same magnitude than those obtained from fluorescence titration (Fig. 2B). No interaction could be measured between SocA and mannopine. The ITC data confirmed the 1:1 binding stoichiometry for both MotA and SocA and indicated a negative enthalpy change for all ligands, suggesting that the binding was enthalpy driven.

Because SocA was not able to bind mannopine, the system transport associated with SocA could not import mannopine in strain C58. Indeed, when no binding of a ligand is observed, there is no import via the system transport of the examined PBP. Therefore, we searched among the annotated PBPs in strain C58, whether another PBP could bind mannopine. The PBP AttC also encoded by the At plasmid of A. tumefaciens C58 was the only PBP annotated as a mannopine binding-like PBP in this strain. Nonetheless, no interaction between AttC and the three ligands was detected by ITC and fluorescence titration measurements.

MotA is a PBP from Cluster D While SocA a PBP from Cluster F—The X-ray
structures of the mature monomeric unliganded MotA and the three liganded MotA with mannopine, DFG and glucopine were determined at 2.55 Å, 1.75 Å, 1.9 Å and 1.8 Å resolution respectively (Table 1). The three liganded forms adopt a similar closed conformation (average root mean square deviation (RMSD) of 0.22 Å for all Ca) whereas the unliganded structure shows an open conformation (Fig. 3A-B) as commonly reported for PBPs from clusters B, C, D and F solved with and without a ligand (12, 26–28, 11). A 36° rotation around the hinge region (formed by 2 short β-strands of residues 127-134 and 256-263) of the C-terminal domain (residues 134-256) was observed once the N-terminal domains (residues 31-127 and 263-354) of the unliganded and liganded structures were superimposed (Fig. 3C).

The unliganded MotA structure shows that each N-terminal sequence containing a His-tag enters the ligand binding site of a symmetric molecule (Fig. 3D). The monomeric MotA possesses a typical fold of cluster D within the PBP structural classification (29) as SSM-EBI (http://www.ebi.ac.uk/msd-srv/ssm) (30) reports: RMSD between Ca atoms of MotA and similar PBP structures are over 2.2 Å with sequence identity around 17-29%. The closest structure PBP structures are over 2.2 Å with sequence identity around 17-29%. The closest structure to MotA is that of (RMSD of 1.8 Å over 301 Cα atoms and 32% sequence identity) that of A. tumefaciens Atu4243 in complex with GABA (PDB code 4EU0) (28).

The two X-ray structures of the mature monomeric SocA liganded with DFG were obtained at 1.93 and 1.5 Å and that with glucopine at 1.84 Å (Table 1). Although SocA with DFG crystallizes in two different cell parameters from the same space group (one monomer versus two monomers in the asymmetric unit), the three SocA-DFG complexes are very similar, displaying an average RMSD of 0.4 Å for all Ca atoms. Moreover, all liganded forms adopt a similar closed conformation (average RMSD for all Ca atoms of 0.46 Å), SocA belongs to cluster F (29) and resembles several PBPs from the same cluster which mainly bind amino acids or derivatives of amino acids such as ArtI from Caldanaerobacter subterraneus and STM4351 from Salmonella enterica, both in complex with arginine (PDB codes 4YMX and 2Y7I with RMSD of 1.14 Å and 1.2 Å over 207 and 211 Cα atoms respectively) (31, 32). The PBP NocT from A. tumefaciens in complex with nopaline (PDB code 4POX) (11) displays with SocA a RMSD of 1.68 Å over 214 Ca atoms, corresponding to a sequence identity of 31%.

MotA Recognizes Mannopine, DFG and the Synthetic Glucopine: The Mannopine/DFG-Binding Signature—Mannopine, DFG and glucopine bound between the two closed lobes of MotA are surrounded by Ser37, Trp41, Gln67, Gln87, Gly89, Asp92, Ser128, Tyr130, Trp235, Arg238, Asp261 and Thr297 residues. The glutamine moiety of the three ligands, sandwiched by Trp41, Tyr130 and Trp235 aromatic residues, makes identical interactions with MotA involving its glutamine side chain with Ser128, Asp261 and Thr297 side chains, its carboxylate part with Arg238 and Ser37 side chains and finally its amino group with the carboxyl group of Asp261 (Figures 4A-C). The sugar moiety of the three ligands makes slightly different interactions implying Ser37, Gln67, Gln87, Gly89, Asp92 and Asp261 (Fig. 4A-C). Upon superposition of the three MotA complexes, the glutamine moiety and the C1 atom of all ligands overlap in contrast to the rest of the ligand. Indeed, the deoxyfructosyl part of DFG adopts an α-pyranosyl conformation cyclized in C6-C2 and the glucopine, although linear, follows the conformation of the DFG with its C2 and C3 carbons and their respective bound hydroxyls at a similar position to those of the sugar part of DFG (Fig. 4D). Though the C2 hydroxyl of the mannopine is shifted by 1.56 Å in DFG and glucopine, it forms a hydrogen bond with Asp261 side chain. Therefore, Asp261 is a critical protein residue as it maintains the glutamine side chain, the main amino atom and the OH of the C2 atom in all ligands. The C2 hydroxyl also interacts with the side chain of Tyr130 in MotA-mannopine complex while it interacts with the side chain of Gln87 in DFG and glucopine complexes. The conformation of the sugar moiety of both DFG and glucopine induces a 1.48 Å shift of Asp92 residue toward the ligand compared to its position in the mannopine complex where the mannopine adopts a linear conformation (Fig. 4D).

The ligand binding sites between MotA and its closest structural homolog (the GABA-binding Atu4243) share four conserved residues only: Tyr130, Trp235, Arg238, and Asp261, which are Tyr123, Trp222, Arg225, Asp248 in Atu4243 (Fig. S1). Atu4243 presents a narrow pocket adapted for a selective GABA-binding. Indeed, both the tryptophan and glutamate at positions 30 and 82 in Atu4243 replacing Ser37 and Gly89 in MotA respectively clash with the
sugar moiety of mannopine thus preventing the binding of any longer ligand in Atu4243. Ser128 and Thr297 in interaction with the glutamine moiety of mannopine correspond to Phe121 and Tyr284 in Atu4243, respectively. Both aromatic residues block access to any ligand in this region of Atu4243. Although the large binding site of MotA might accept a GABA molecule which could be held by the four conserved residues with Atu4243, no interaction between MotA and GABA was observed with intrinsic fluorescence and ITC assays, confirming the requirement of aromatic residues contacts restricting the size of the ligand binding site to accommodate a GABA. In contrast, the apparent $K_D$ value of 2.2 ± 0.3 μM for MotA towards glutamine shows that MotA can bind glutamine (Fig. S2). We were thus able to propose two signatures: $S_2$W$D_4$S$D_8$W$_{235}$R$_{238}$D$_{261}$T$_{297}$ for glutamine binding, and $Q_8$G$_{92}$Y$_{130}$D$_{261}$ for mannose binding with Asp261 being involved in both. Together, they form the mannopine binding signature.

**AttC is a PBP from Cluster D: Fold Comparison with MotA**—The structure at 2.49 Å resolution (Table 1) of the mature monomeric unliganded AttC belongs to the cluster D (29) and adopts an open conformation (Fig. S3A). A model of its closed conformation based on MotA-mannopine structure shows that AttC and MotA share four residues in the ligand binding site: Gly99 (Gly89 in MotA), Trp244 (Trp235 in MotA) involved in the ligand stacking, Arg247 (Arg238 in MotA) involved in the binding of the ligand carboxylate group and Asp270 (Asp261 in MotA) which interacts with an amino-group of the ligand. The rest of the mannopine-binding signature is not conserved in AttC in line with the fluorescence and microcalorimetry results. The ligand binding sites of AttC and MotA exhibit almost the same size. However, that of AttC contains several charged and polar residues such as Arg, Asp, Gln, Thr (Fig. S3B).

**SocA Specifically Recognizes DFG: The DFG-binding signature**—Similarly to what is observed in MotA, the deoxyfructosyl part of the bound DFG in SocA is also an α-pyranosyl conformation cyclized in C6-C2 (Fig. 5A). The DFG bound between the two closed lobes of SocA is surrounded by Phe96, Ala113, Ala114, Gly116, Arg121, Gly134, Leu163, Asp201, Ser223 and Ala226 residues (Fig. 5A). By adopting a bent conformation, the synthetic glucopine shares with DFG the same binding mode involving several Van der Walls contacts and ten protein main chain polar interactions (Figures 5B-C). The position of all their atoms excluding the C6 hydroxyl overlaps (RMSD of 0.22 Å) when both complexed SocA structures are superimposed. Their glutamine moiety interacts with the carbonyl of Ala113 and stacks against Phe96. Their carboxylate group is held by the guanidinium group of Arg121 and two NH of Gly116 and Leu163. Their amino group is maintained by Ala114 main chain and Asp201 side chain. The sugar moiety of both ligands makes four hydrogen bonds with protein main chains and two with side chains (Asp201 and Ser223). We were thus able to discriminate the residues involved in ligand binding via their main chain from those via their side chain, with Ser223 involved in both. Therefore, $A_{111}A_{114}G_{116}G_{134}L_{163}A_{226}$ defines the main-chain-involved binding signature while $M_{52}F_{96}R_{121}D_{201}S_{223}$ the side chain-involved binding signature. Together, they form the DFG binding signature.

Around the glutamine moiety of DFG and glucopine, the ligand binding site of SocA resembles those of amino acid-binding PBPs from cluster F with four conserved residues corresponding to Phe96, Arg121, Ala114 and Asp201 in SocA. As suggested, SocA binds glutamine with an apparent $K_D$ value of 2.1 ± 0.3 μM (Fig. S2). In contrast, longer residues such as arginine will create a steric hindrance with the Met52 side chain in line with the lack of detectable interaction between SocA and arginine by ITC and autofluorescence. Both opine-binding SocA and NocT are distinguishable from their homologs in cluster F due to their binding site accommodating bulkier ligands possessing an amino acid. However, they differ in their ligand binding mode. SocA cannot accept a nopaline molecule due to the presence of the arginine moiety and the presence of the first carboxylate group of its ketoacid moiety which create an additional steric clash with Asp201 (Fig. S4). NocT does not bind DFG by autofluorescence likely due to the absence of the equivalent Asp201 in SocA which is Ser207 in NocT.

**Comparison between MotA and SocA Structures**—The selective DFG binding site of SocA shows no similarity with the non-selective one of MotA. Hence, the conformation and orientation of DFG bound in the two ligand binding sites are different (Fig. S5A). Notably, DFG exhibits opposite orientation when SocA and MotA structures are superimposed.
Comparison according to the superposed ligands shows no structurally conserved protein residue. However, Arg238 and Asp261 in MotA, and Arg121 and Asp201 in SocA conserve the same function: the arginine guanidinium group makes a salt-bridge with the carboxylylate group of the DFG glutamine part while the aspartic acid side chain makes a hydrogen bond with the main amino group of the DFG glutamine part (Fig. S5B). The glutamine moiety of each ligand shares a similar conformation. In contrast, the sugar moiety of DFG in SocA differs in MotA by a 70° rotation around the C1-C2 bond and the compact conformation of the sugar moiety of glucopine in MotA corresponds to a bent conformation in SocA (Fig. S5C).

**MotA is Present in a Few Bacteria—**About 40 bacterial MotA-homologous PBPs with a threshold set at at least 40% identity were recovered using blastP from NCBI. All redundant sequences were removed and three additional homologous sequences found in *Agrobacterium* genomes of the AgrobacterScope genome library (Genoscope, France) as well as the sequence of AttC were added. The relation tree constructed from 17 sequences reveals different subgroups (Fig. 6). Members of the MotA subgroup sharing more than 89% sequence identity possess the mannopine/DFG binding signature. S$_{97}$W$_{41}$Q$_{85}$G$_{60}$D$_{92}$S$_{129}$Y$_{130}$W$_{235}$R$_{238}$D$_{261}$T$_{297}$. They belong to four *A. tumefaciens* octopine/mannityl-opine type (B6, AF242881, TT111 and Ach5) and one agrocinopine/mannityl-opine type (Bo542) strains and to one strain of the plant symbiont *Sinorhizobium fredii*. In addition, two MotA-like deduced proteins from *Rhizobium leguminosarum* and remarkably the β-proteobacterium *Burkholderia* sp. strain PAMC26561 (80% and 74% sequence identity with MotA, respectively) might bind mannopine. Indeed, their binding site only differs by S$_{89}$/A$_{89}$ instead of G$_{89}$ and E$_{92}$ instead of D$_{92}$, and modelling revealed that this would not affect the mannopine/DFG binding. All members of the other subgroup in which mannopine/DFG-signature is degenerated are annotated as putative putrescine/spermidine-binding PBPs. The binding signature is also strongly degenerated in AttC in line with the observed 31% sequence identity with MotA.

**SocA is Present in Numerous Bacteria—**SocA relatives search in the bacterial kingdom (protein database at NCBI) and subsequent phylogenetical analysis reveals over 240 PBPs with conserved DFG binding signature. To refine this analysis, we reconstructed a phylogeny with the SocA-relatives extracted from the complete genome NCBI database only (Fig. 7). The SocA-clade delineated with a solid bootstrap value (100%) encompasses PBPs from α-proteobacteria such as Rhizobiales (*Agrobacterium, Aureimonas, Rhizobium* and *Sinorhizobium*), γ-proteobacteria such as Enterobacteriales (*Klebsiella, Pantoea, Erwinia*) and Actinobacteria (*Arthrobacter*) which all possess the DFG binding signature. Outside the SocA-cluster, the DFG-signature degenerates, but modelling indicates that only the E$_{201}$/D$_{201}$ side chains variation in the *Micromonaspora* sub-branch may affect the DFG binding.

**DISCUSSION**

This work establishes a structural basis for the high mannopine specificity of the PBP MotA, encoded by the Ti-plasmid of octopine/mannityl-opines-type and agrocinopine/mannityl-opines-type *Agrobacterium* strains. Mannopine is synthesized from the intermediate DFG, which is a naturally abundant Amadori compound present in decaying plant materials. To date, no DFG transporter has been characterized in a mannopine-assimilating agrobacteria strain.

Using two different biophysical approaches, we showed that the PBP MotA can bind both mannopine and DFG with high affinity (nanomolar range), and with a slightly higher preference for mannopine. With an affinity in the micromolar range, MotA also binds the amino-acid glutamine. Thus, MotA mainly contributes to the importation of mannopine as a specific nutrient and could also contribute to that of DFG. Phyllogenetical and structural data revealed the presence of a single copy of the *motA* gene among α-proteobacteria that are *Rhizobiales* (*Agrobacterium, Sinorhizobium* and *Rhizobium*). Notably, a *motA* gene orthologue was identified in the β-proteobacterium *Burkholderia* sp. PAMC 26561 indicating that this strain could also take up mannopine and DFG. To our knowledge, mannopine is only produced by T-DNA transformed plant cells, suggesting that non-pathogenic bacteria which have acquired mannopine-importation and assimilation may live in host plants infected by *A. tumefaciens*, which is in agreement with observations in engineered plants producing opines (33). However, in tumours, these non-pathogenic bacteria would probably not outcompete the
pathogenic agrobacteria due to the production of a wide variety of opines in the tumour niche. As previously shown (11), binding and assimilation of all these opines contribute to the fitness of pathogenic agrobacteria in plant tumours.

Using ITC and autofluorescence, we clearly showed that SocA cannot bind mannopine. Conversely, Baek et al. (25) observed that the genomic region of the Agrobacterium tumefaciens strain C58 coding for the PBP SocA and its membrane transporter mediates mannopine import, suggesting that SocA may bind mannopine. However, the strain C58 lacks the gene encoding the enzyme MocC that degrades mannopine into DFG. Within the frame of the concept of the opine niche (11), the selective pressure that leads to the ability of strain C58 to assimilate mannopine without using it as a nutrient remains elusive, especially considering that this strain is also lacking the mannopine synthesis genes on the T-DNA of the Ti plasmid. A possible explanation for this discrepancy lies in the sources of mannopine. Mannopine used in the present study was checked by mass spectrometry and from the same lab-made, synthetic batch used in previously reported, mannopine uptake experiments (22, 23). Mannopine used in Baek et al. (25) was at this time of commercial origin, and possibly contaminated by residual amount of precursors, as no control of its purity was reported. In contrast to mannopine, we observed that SocA binds DFG with a higher affinity (nanomolar range) than previously reported (micromolar range) (34). The SocA ligand pocket seems pre-formed to selectively accommodate DFG via several protein main chains interactions. The rigid binding site architecture is not appropriate for mannopine or any extended ligand, which would create steric clashes with the sugar in a modelled mannopine. Furthermore, to be accommodated in SocA, the bound synthetic opine analogue glucopine must adopt a bent conformation that is not possible for mannopine. We propose the structure of SocA as a reference model to understand the selective DFG-binding mode among bacterial PBPs. Phylogenetic and structural data have shown that SocA is conserved (between 70 and 100% sequence identity) in different bacterial phyla (Rhizobiales, Enterobacteriales and Actinobacteria), and that PBPs belonging to other clusters could bind DFG with a similar binding mode to SocA. This wide occurrence of SocA PBPs correlates with the abundance of DFG in nature. It is tempting to speculate that DFG is associated with a selective pressure towards the acquisition of binding, transport and degradation functions in micro-organisms. Bacteria that have evolved a SocA-like PBP could therefore benefit from a marked selective advantage in DFG-rich environments. Although the octopine/mannityl-opines type strains, such as B6 or Ach5, possess MotA to bind DFG and maybe import it, they also possess SocA on their plasmid. The DFG-binding capability of MotA is likely to be a relatively promiscuous event as the motA gene is located in a Ti plasmid region exclusively involved in mannopine degradation. Nevertheless, MotA and SocA both display a strong affinity for the Amadori compound. The DFG sugar moiety can adopt linear and cyclic conformations, the latter being the common form observed in both PBPs.

Although the structure of the PBP AttC from the nopaline-type C58 strain belongs to the same structural cluster as MotA, we could not measure any interaction between AttC and mannopine or DFG. Moreover, AttC appears in the MotA phylogenetic tree in an evolutionarily remote branch with respect to the MotA clade and does not harbor the mannopine-binding motif. Taken together these data should lead to a revision of the automatic annotation of AttC which has been predicted as a mannopine-binding like protein. This is in line with the fact that the A. tumefaciens strain C58 is not able to catabolize mannopine. The AttC binding site which is rich in arginine, aspartate and polar residues suggests that AttC may be able to bind a ligand possessing branched carboxylate and amino groups. Precise ligand identification will enable an understanding of the role of this PBP and provide new insights on the diversity of ligands bound to PBPs from cluster D (29).

EXPERIMENTAL PROCEDURES

Synthesis and purification of opines—Mannopine was synthesized using L-glutamine and D-mannose as precursors in the presence of sodium hydrogen carbonate as described by Tate and al. (35) while glucopine was synthesized using L-glutamine and D-glucose. DFG was synthesized using L-glutamine and D-glucose as described by Chilton and al. (19) Solutions of mannopine and DFG were checked by mass spectrometry (Fig. S6). Cloning, expression and purification of MotA, SocA and AttC—The primers 5’-CCCCCATATGGATGTGGTTATTCGCTCT-3’ (86), 5’-CCCCATATGGATGTGGTTATTCGCTCT-3’ (86), and 5’-CCCCATATGGATGTGGTTATTCGCTCT-3’ (86), respectively, were used to clone the genes using the primers. The primers were designed to amplify the coding sequences of MotA, SocA and AttC. The amplified DNA was cloned into pET22b(+) using the NdeI and BamHI restriction sites. The plasmid was transformed into E. coli BL21 (DE3) cells and the expression of the encoded proteins was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside for 4 h at 20°C. The expression levels were determined by Western blot analysis using the antibodies specific to MotA, SocA and AttC. The proteins were purified using Ni-NTA column affinity chromatography. The purified proteins were validated by mass spectrometry and verified by Western blot analysis. The validation of protein expression and purification was performed using SDS-PAGE and Western blot analysis.
TCAGGCG and 5'-CCCCCGGCGGCGCTTAAGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGTA were used to PCR-amplify the motA gene from A. tumefaciens B6 without the signal sequence. The amplicon was cloned into pET-28b. The synthetic genes (Genscript) coding for the mature SocA and AttC were inserted into pET-28b and pET-29a, respectively. E. coli BL21 pLysS were transformed either by pET-28b-motA or pET-28b-socA and E. coli Rosetta pLysS by pET-29a-attC. Cells were grown at 37°C in LB broth supplemented with 0.5 mM IPTG for protein production. Cells were centrifuged, resuspended in 50 mM Tris-HCl pH 8.0, 20 mM imidazole, 500 mM NaNCl buffer, and disrupted by sonication. After centrifugation at 20 000 g for 30 min at 4°C, the supernatant was loaded onto a 5 mL Ni-Trap column (GE Healthcare). Protein elution was performed with 50 mM Tris-HCl pH 8.0, 300 mM imidazole and 500 mM NaNCl. Protein fractions were loaded onto a gel filtration column (HiLoad 26/60 Superdex 200 prep grade, GE Healthcare). Protein elution was performed with 50 mM Tris-HCl pH 8.0, 200 mM imidazole and 500 mM NaNCl. Protein fractions were loaded onto a gel filtration column (HiLoad 26/60 Superdex 200 prep grade, GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8.0 and 150 mM NaNCl.

**Crystallization and structure determination**—Crystallization conditions (Table 1) for liganded MotA and SocA (molar ratio of protein:ligand used was 1:5), unliganded MotA and AttC were screened using the QIAGEN kits and manually optimized. Crystals were transferred to a cryo-protectant solution (paraffin oil or 22% (w/v) PEG 400) and flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K on the PROXIMA I and II beamlines at SOLEIL synchrotron (Saint-Aubin, France). Data collection and processing statistics are given in Table 1. All structure determinations were performed by molecular replacement (MR) with PHASER (36). The coordinates of the N-terminal (residues 31 to 128 and 260 to 310) and the C-terminal (residues 131 to 258 and 324 to 352) domains of a model from @TOME-2meta-server (37) was used for MotA-mannopine structure. Structures of the other liganded forms and the unliganded-form of MotA were determined using MotA-mannopine coordinates. Structure determination of both SocA-DFG complexes and SocA-glucopine complex was performed using the coordinates of the N-terminal (residues 43 to 133 and 227 to 270) and the C-terminal (residues 138 to 222) domains of a SocA model from @TOME-2 (37). AttC structure was determined using the N-terminal and the C-terminal domains of MotA as a search model. Refinements were performed with BUSTER-2.10 (38) with NCS restraints when necessary and TLS groups. All electron density maps were evaluated using COOT (39). Refinement details are shown in Table 1. Molecular graphics images were generated using PYMOL (http://www.pymol.org).

**K_D measurements by fluorescence titration and microcalorimetry**—Ligands bound to MotA, SocA and AttC were monitored by autofluorescence by excitation the proteins at a wavelength of 295 nm and monitoring the quenching of fluorescence emission of tryptophans at 335 nm. All experiments were performed at 24°C in triplicates in 50 mM Tris-HCl pH 8 and 150 mM NaNCl with a fixed amount of proteins (1 μM) and increasing concentrations of ligand used a Tecan infinite M1000 PRO microplate reader (Tecan Group SA). Each ligand has no emission signal at 335 nm. The data were analyzed using Origin 7 software (ORIGIN) and fitted to the equation \( f = \Delta \text{Fluorescence}_{\text{max}} \times \text{abs}(x) / (K_D + \text{abs}(x)) \).

Isothermal titration microcalorimetry experiments were performed with an ITC200 isothermal titration calorimeter from MicroCal Llc (Northampton, MA). The experiments were carried out at 20°C in 50 mM Tris-HCl pH 8 and 150 mM NaNCl (same buffer as fluorescence titration). Protein concentration in the microcalorimeter cell (0.2 ml) was 50 μM. 19 injections of 2 μl of the ligand solution at 500 μM, were performed at 180 s intervals while stirring at 1000 rpm. The experimental data were fitted to theoretical titration curves with the software supplied by MicroCal (ORIGIN). This software uses the relationship between the heat generated by each injection and \( \Delta H \) (enthalpy change in Kcal.mol\(^{-1}\)), \( K_a \) (the association binding constant in M\(^{-1}\)), n (the number of binding sites), total protein concentration and free and total ligand concentrations.

**Phylogenetic analysis**—Sequences were analyzed using blastP from NCBI (http://blast.ncbi.nlm.nih.gov/). Alignments of MotA/SocA and related sequences were conducted using the ClustalW software. Relationship tree was build using the MEGA software, Version 5. The phylogeny was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The
evolutionary distances are in units of the number of amino acid substitutions per site.
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The atomic coordinates and structure factors have been deposited at the Protein Data Bank (PDB) under accession codes 5L9P (free-ligated MotA), 5L9G (MotA with mannopine), 5L9I (MotA with DFG), 5L9L (MotA with glucopine), 5L9M (SocA with DFG at 1.93 Å resolution), 5LOM (SocA with DFG at 1.5 Å resolution), 5L9O (SocA with glucopine) and 5L9S (free-ligated AttC).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: YD provided the mannopine, DFG and glucopine compounds and information on mannopine and DFG metabolism. LM and SM performed all the crystallography work. LM and AV performed the fluorescence assays. MAN performed the microcalorimetry experiments. LM, DF and SM performed the phylogenetic analysis. SM wrote the manuscript. All the authors discussed the results and contributed to the writing of the manuscript.

Abbreviations: periplasmic binding proteins, PBPs; deoxy-fructosyl-glutamine, DFG.

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Selection of mannopine opine and Amadori compound by PBPs

FIGURE LEGENDS

FIGURE 1. Metabolism of deoxyfructosylglutamine (DFG, orange) and mannopine (green) in transformed plant cells and the two A. tumefaciens B6 (up) and C58 (down) strains. In plant tumour cells, the genes mas2 (dark red) and mas1 (blue) responsible for the biosynthesis of DFG and mannopine respectively, are located on the T-DNA transferred from the A. tumefaciens B6 Ti plasmid. In A. tumefaciens B6, the genes (motA-D) coding for mannopine import on the Ti plasmid are shown in purple. Mannopine is converted into DFG which in turn is deconjugated into glutamine and fructose by the reverse synthesis reactions. No DFG transporter has been identified yet. In A. tumefaciens C58 strain, mannopine and DFG were proposed to be both transported by the products of socAB (slate) located on the At plasmid. DFG is degraded by the products of socCD into glutamine and fructose. AttC (gene in light brown) was annotated as a mannopine-binding like protein.

FIGURE 2. ITC and fluorescence K_D measurements. The top graphs of MotA (A) and SocA (B) microcalorimetry experiments show heat differences upon injection of ligand (top panel) and integrated heats of injection with the best fit (solid line) to a single binding model using Microcal ORIGIN (low panel). The lower graphs show fluorescence monitoring upon titration with each ligand and fit (solid line) to a single binding model using Origin 7. Measures were done in triplicates. (C) Calculated parameters for each experiment are indicated.

FIGURE 3. Ribbon representation of MotA structures: (A) with mannopine in green, (B) unliganded. Lobes I and II are shown in deep-purple and pink, respectively, and the hinge region in red. (C) Comparison between the open unliganded (deep-purple) and the closed liganded (green) forms of MotA. (D) Packing of the unliganded MotA crystal: the N-terminus His_6-tag (shown in sticks) of a molecule (deep purple) of the asymmetric unit enters the ligand binding site of its symmetric molecule (green).

FIGURE 4. Mannopine (A), DFG (B) and glucopine (C) bound to the binding site of MotA are shown in grey/green, grey/orange and grey/deep-blue sticks respectively and in their annealing Fo-Fc omit map contoured at 4σ. Hydrogen bonds between MotA and each ligand are shown as dashed lines in black (distance below 3.2 Å) and in green (distance between 3.2 and 3.4 Å). (D) Superposition of the three ligands in the binding site of MotA. The residues in green interact with mannopine, in orange with DFG and in deep-blue with glucopine.

FIGURE 5. DFG (A) and glucopine (B) bound to the binding site of SocA are shown in grey/orange and grey/deep-blue sticks respectively and in their annealing Fo-Fc omit map contoured at 4σ. Hydrogen bonds between SocA and each ligand are shown as dashed lines in black (distance below 3.2 Å) and in green (distance between 3.2 and 3.4 Å). (C) Superposition of both ligands in the SocA binding site. The residues in orange interact with DFG and in deep-blue with glucopine.

FIGURE 6. MotA phylogeny and mannopine-binding signature. The displayed tree was rooted with AttC sequence. For each protein or protein cluster, the residues which are identical to (black) and different from (red) those involved in the binding of the glutamine part (purple box) and sugar part (green box) of mannopine are indicated.

FIGURE 7. SocA phylogeny and DFG-binding signature. The branches are collapses when the clade is composed of proteins from a same genus and are colored in red for α-proteobacteria, purple for β-proteobacteria, blue for γ-proteobacteria and green for Actinobacteria. For each protein or protein cluster, the residues which are identical to (black) and different from (red) those involved in the binding of DFG via their main chain (orange box) and via their side chain (purple box) are indicated.
### TABLE 1. Crystallization conditions, crystallographic data and refinement parameters

| Protein | MotA-mannopine | MotA-DFG | MotA-glucopine | MotA | SocA-DFG | SocA-glucopine | AttC |
|---------|----------------|----------|----------------|------|----------|----------------|------|
| Crystallization conditions | 28% PEG 4K, 0.1 M Tris pH 8.5, 0.2 M CaCl₂ | 24% PEG 4K, 0.1 M MES pH 6.5, 0.2 M CaCl₂ | 30% PEG 4K, 0.1 M Tris pH 8.5, 0.2 M CaCl₂ | 2.08 M (NH₄)₂SO₄, 0.1 M sodium acetate pH 4.6, 1.25% isopropanol | 30% PEG 4K, 0.1 M Tris pH 8.5 | 30% PEG 4K, 0.1 M Tris pH 8.5 | 36% PEG 4K, 0.1 M HEPES pH 7.5 | 40% PEG 4K, 0.1 M MES pH 6.5, 0.2 M ammonium acetate |
| PDB code | 5L9G | 5L9I | 5L9L | 5L9P | 5L9M | 5LOM | 5L9O | 5L9S |
| Space group | P4₁ | P4₁ | P4₁ | P2₂,2 | P2₁ | P₂₁ | P₂₁ | P2₂,2 |
| Cell parameters (Å,°) | a = 71.2 b = 71.2 c = 134.8 | a = 71.4 b = 71.4 c = 134.1 | a = 70.8 b = 70.8 c = 133.2 | a = 64.4 b = 119 c = 111.2 | a = 39.7 b = 62.3 c = 43.4 β = 105.6 | a = 39.6 b = 62 c = 84.9 β = 101.3 | a = 39.6 b = 62 c = 84.2 β = 101.4 | a = 48.0 b = 118.4 c = 121.0 |
| Resolution (Å) | 50.1-1.75 (1.91-1.75) | 50.1-1.9 (2.01-1.9) | 50.1-1.8 (1.9-1.8) | 50.2-5.5 (2.69-2.55) | 50.1-1.93 (2.05-1.93) | 50.1-1.5 (1.59-1.5) | 50.1-1.84 (1.95-1.84) | 50.1-2.49 (2.64-2.49) |
| No. of observed reflections | 305979 (45863) | 236219 (37973) | 269966 (42154) | 163732 (24714) | 45521 (7095) | 431996 (66626) | 163279 (17975) | 124479 (18717) |
| No. of unique reflections | 150781 (20449) | 152821 (8407) | 80610 (9499) | 28279 (4446) | 14862 (2278) | 64331 (10179) | 35762 (4597) | 24641 (3745) |
| R_sym (%) | 8.3 (68) | 6.4 (68.8) | 9.3 (72.1) | 17 (119.8) | 6.1 (51) | 10.4 (70.6) | 7.2 (53) | 11.5 (125.9) |
| Completeness (%) | 99.8 (99.5) | 99.7 (99) | 99.4 (96.6) | 99.3 (96.7) | 96.5 (92.2) | 99.2 (97.2) | 96.3 (81.4) | 99 (95.2) |
| I/σ | 9.4 (1.7) | 11.9 (1.3) | 8.6 (1.2) | 7.3 (1.1) | 11.1 (2.4) | 11.5 (2.1) | 12.7 (1.9) | 11.6 (1.4) |
| CC₁/₂ | 99.5 (67.2) | 99.8 (57.6) | 99.4 (60.4) | 99.3 (82.4) | 99.7 (77.7) | 99.7 (85.6) | 99.7 (90.2) | 99.7 (80.6) |
| R cryst (%) | 17.7 | 17.7 | 20.5 | 19.2 | 16.2 | 19 | 21.8 | 19 |
| Rfree (%) | 20.8 | 20.8 | 24. | 22.9 | 19.5 | 22.3 | 25.2 | 23.3 |
| rms bond deviation (Å) | 0.01 | 0.009 | 0.01 | 0.01 | 0.01 | 0.01 | 0.009 | 0.01 |
| rms angle deviation (°) | 0.96 | 1.0 | 1.06 | 1.14 | 1.04 | 1.0 | 1.02 | 1.15 |
| Average B (Å²) | protein | 46.9 | 52.6 | 38.9 | 60.2 | 42.4 | 18.7 | 42.8 | 64.9 |
| ligand | 31.5 | 44.7 | 33.6 | 29.5 | 10.8 | 35.7 | 44.1 | 33.6 |
| solvent | 50.6 | 52.6 | 43 | 71.8 | 53.9 | 30.2 | 44.1 | 33.6 |

Values for the highest resolution shell are in parentheses

CC₁/₂ = percentage of correlation between intensities from random half-dataset (P. A. Karplus, K. Diederichs, Science 2012, 336, 1030–1033).
Figure 2

Selection of mannopine opine and Amadori compound by PBPs

A

MotA-mannopine

MotA-DFG

MotA-glucopine

B

SocA-DFG

SocA-glucopine

C

|            | FLUORESCENCE | ITC              |
|------------|--------------|-----------------|
|            | Kd (nM) | Bmax | R²   | Kd (nM) | N | Enthalpy (ΔH) (cal/mol) | Entropy (ΔS) (cal/mol/deg) | Entropic contribution (ΔTS) (cal/mol) | Free enthalpy (ΔG) (cal/mol) |
| MotA-MOP   | 4 ±0.5 | 28   | 0.98 | 19.8 ±3 | 0.845 | -22620 ±78.3 | -41.9 | -12282.9 | -10337 |
| MotA-DFG   | 104.5 ±20 | 189.8 | 0.974 | 99.0 ±13 | 0.916 | -47350 ±463.6 | -129 | -37816.3 | -9533.6 |
| MotA-GOP   | 55.5 ±9.1 | 146.9 | 0.985 | 79.4 ±3 | 0.963 | -13780 ±31.7 | -14.5 | -4250.6 | -9529.3 |
| SocA-DFG   | 56.9 ±10 | 125.5 | 0.982 | 74.1 ±13 | 0.972 | -7208 ±62.9 | 8.03 | 2353.9 | -9562 |
| SocA-GOP   | 2485 ±410 | 110.5 | 0.986 | 4000 ±200 | 0.849 | -12250 ±130.8 | -17.1 | -5012.8 | -7237.1 |
Selection of mannopine opine and Amadori compound by PBPs
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Figure 5
Selection of mannopine opine and Amadori compound by PBPs

Figure 6
Selection of mannopine opine and Amadori compound by PBPs

Figure 7
Structural Basis for High Specificity of Amadori Compound and Mannopine Opine Binding in Bacterial Pathogens
Loïc Marty, Armelle Vigouroux, Magali Aumont-Nicaise, Yves Dessaux, Denis Faure and Solange Moréra

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