Mutagenesis on an ancestral background elucidates new determinants of the xanthine specificity in the NAT/NCS2 family of transporters

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NAT/NCS2 (Nucleobase-Ascorbate Transporter/ Nucleobase-Cation Symporter-2) is a family of transporters with wide evolutionary distribution and diversity in substrate preferences. To date, research on this family has been based on the study of present-day homologs. Herein, we present an evolutionary strategy, based on ancestral reconstruction, that is applied for the first time, to analyze structure-function and specificity relationships in this family. We performed phylogenetic analysis of the bacterial NAT/NCS2 homologs and reconstructed AncXanQ, the putative common ancestor of all homologs comprising a clade represented by the well-studied xanthine-specific permease XanQ of *E. coli*. In contrast to XanQ, AncXanQ transports both xanthine and guanine and recognizes a wide range of analogs. Homology modelling showed that AncXanQ conserves all key binding-site residues of XanQ. We subjected both homologs to rationally designed mutagenesis and found that 5 amino acid residues outside the predicted binding-site are involved in the specificity change. In particular, we reveal Ser377 of XanQ (Gly in AncXanQ) as a major determinant. Replacement of this Ser to Gly enlarges the specificity of XanQ towards an AncXanQ-phenotype. Orthologs from *Neisseria meningitidis* retaining Gly at this position are also xanthine/guanine transporters with an extended substrate profile like AncXanQ. The specificity effect of S377G is masked by G27S or other mutations through epistatic interactions.

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