A STRATEGY FOR REDUCING AMMONIA IN ANIMAL PRODUCTION

J.B. Koger and T. van Kempen

Summary
Ammonia is one of the gases produced in greatest volume from swine waste. It constitutes a serious environmental threat due to its potential for ground water nitrification and adverse effects on animal and human health. Engineering microorganisms to be more efficient in removing ammonia from their environment and in converting this ammonia to stable, non-odorous compounds is one way to reduce the ammonia problem. These engineered microbes can be added to feed as probiotics (direct fed microbials) or used in biofiltration systems. In order to reduce ammonia levels from animal production, a novel chimeric enzyme has been created to efficiently utilize ammonia and fix it in stable organic compounds. The chimera consists of the carbamoyl phosphate synthetase/aspartate transcarbamoylase cDNA except that the portion encoding the aspartate binding domain has been replaced by the ornithine binding domain sequence of porcine ornithine transcarbamoylase in order to alter substrate specificity. The chimera has been synthesized, cloned, and identified by plasmid size and restriction pattern. By creating the chimera, we hope to avoid the inhibition normally exercised by pyrimidine pathway metabolites thereby establishing a system for continual ammonia removal and conversion to useful, stable compounds.

Introduction
With the rapid growth of high-density confinement hog operations in North Carolina, management of animal waste has become one of the state’s top environmental and public policy priorities. Ammonia, a very pungent and irritating gas, is emitted in large quantities from production facilities when urine is mixed with fecal microbes containing urease. The fecal nitrogen load itself makes a negligible contribution as it is bound up in the relatively stable form of bacterial proteins and amino acids. Ammonia can be incorporated into polyamines, such as putresine and cadaverine that are major components of the odor problem. In addition, ammonia can contribute to ground water nitrification. Finally, ammonia, at 10-25 ppm, in animal housing is irritating to the lungs of both humans and pigs. In humans, this irritation results in lung damage, greater susceptibility to opportunistic infections, loss of productivity (increased sick leave), and even disability. In pigs, the effects are similar but appear as “failure to thrive” and result in a low market weight and, hence, lower profitability.

In mammals, excess nitrogen is eliminated by two routes: as urea in urine and as fecal nitrogenous compounds, principally bacterial amino acids and proteins. However, as
much as 40% of plasma urea can be recycled through the digestive tract where it is hydrolyzed to ammonia by bacterial urease. Ammonia is absorbed from the large bowel for re-synthesis of urea in the liver. This urea is transported by the blood to the kidneys for excretion or back to the gut where the cycle repeats itself. This “futile cycle” is an energy expenditure for the animal and a potential contributor to fecal ammonia and odor. The ability to interrupt this cycle, by dietary manipulation, and to fix ammonia in bacterial protein, which is excreted in feces, has been shown to reduce urinary nitrogen secretion by as much as 25%. Manipulation of the microbial flora, by contrast, offers the possibility of establishing a semi-permanent ammonia-fixing “machinery” within the colon that can effectively redistribute excess nitrogen into the relatively stable fecal pathway. While this machinery exists to some extent in the native flora, it is controlled by regulatory mechanisms that prevent over-accumulation of metabolites.

Ammonia can be incorporated into biological molecules by three distinct enzymes: glutamate dehydrogenase, glutamine synthetase, and carbamoyl phosphate synthetase. This last enzyme catalyzes the first reactions of the arginine and pyrimidine biosynthetic pathways. Carbamoyl phosphate, however, is labile and must be utilized quickly. In vivo, it is incorporated into citrulline by ornithine transcarbamoylase (OTCase) or carbamoyl aspartate by aspartate transcarbamoylase (ATCase). The two transcarbamoylases are members of the same enzyme family and have been structurally conserved throughout the phylogenetic spectrum. Each has two substrate binding "domains": the first binds carbamoyl phosphate, the second, either aspartate or ornithine (ATCase and OTCase, respectively). Their apparent homology has been further confirmed by the previous creation of functional chimeras (Houghton et al., 1989 and Serre et al., 1994). Native OTCase is always monofunctional, but ATCase can be found as part of bi- and trifunctional polypeptide chains that include carbamoyl phosphate synthetase (CPSase) as the first enzymatic activity. This arrangement permits efficient channeling of carbamoyl phosphate from the site of synthesis to the site of utilization (Penverne et al., 1994).

The homology of the transcarbamoylases and the multifunctional organization of eukaryotic ATCase make these proteins good candidates for formation of a chimeric enzyme complex that will be efficient in trapping excess ammonia. The cDNA for the novel enzyme will consist of CPSase/ATCase activities encoded by the Saccharomyces cerevisiae URA2 gene with the ornithine binding domain of porcine OTCase replacing the aspartate binding domain of ATCase. The carbamoyl phosphate synthetase activity present with ATCase in a single polypeptide guarantees that the ammonia captured in carbamoyl phosphate will be channeled to the active site of the chimeric transcarbamoylase and rapidly incorporated into a more stable compound. The domain substitution will convert a bifunctional protein active in pyrimidine biosynthesis to one participating in the arginine biosynthetic pathway. Substituting the ornithine binding domain of OTCase for the aspartate binding domain of ATCase changes the substrate specificity of the reaction so that citrulline is the amine product obtained (rather than carbamoyl aspartate). This molecule continues down the pathway to arginine biosynthesis, but no further, as prokaryotes lack the arginase enzyme necessary for subsequent conversion to urea and ornithine.
Materials and Methods

*Origin of cDNA Clones.* A cDNA library constructed from adult male pig liver mRNA in λgt10 (Clontech) was used for isolating the porcine ornithine transcarbamoylase clone. The clone was selected by the PCR method of Israel (1993). Sequencing was by the Thermo Sequenase method (Amersham) using [$\alpha$-³²³P]dideoxy nucleotide triphosphates. The *URA2* cDNA, encoding the yeast CPSase/ATCase polypeptide chain, was generously provided by J.L. Souciet and S. Potier (Institute de Botanique, Strasbourg, France).

*Materials.* pRS315 (Stratagene) was used as the common vector for each transcarbamoylase cDNA sequence during the construction of the hybrid. Restriction enzymes were from Boehringer-Mannheim or Promega. Methods were generally those of *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) except as noted.

Results and Discussion

By screening a commercially available library with the PCR method of Israel (1993), a clone was identified that encoded the entire mature sequence of porcine OTCase. Screening and subsequent identification of the reading frame and N-terminal amino acid was greatly facilitated by knowledge of the initial amino acid sequence known from Edman degradation of the purified protein (Koger *et al.*, 1994). The clone identified encodes a protein of 322 amino acids. The nucleotide sequence and the deduced amino acid sequence have been published elsewhere (Koger and Jones, 1997).

In order to construct the chimera, a unique *Bst*E II restriction site was created, by site directed mutagenesis, at the junction between the carbamoyl phosphate binding domain and the ornithine binding domain in the OTCase cDNA. The mutated site matched a naturally occurring *Bst*E II site in ATCase at the corresponding position. This strategy made it possible to join the two cDNAs without disturbing the reading frame. Each sequence was subcloned into a common vector, pRS315 to facilitate chimera construction. The construction strategy, shown in Figure 1, again utilized PCR. After synthesis of the two fragments, products were restricted and ligated at the *Bst*E II site and restricted at each end (*Stu* I and *Not*I) for insertion into the vector. Clones were identified on the basis of plasmid size and restriction endonuclease pattern.
Fig. 1 Construction strategy for the hybrid cDNA encoding a bifunctional polypeptide chain with carbamoyl phosphate synthetase and ornithine transcarbamoylase-like activities.

Work remains to be done on this chimera before it can be put to use. First, the entire sequence must be confirmed since PCR fidelity is not perfect. Then the construct must be placed in an appropriate vector for expression. Once expression is confirmed, levels of enzyme activity and kinetic constants need to be evaluated. These studies can be facilitated by isolation of the enzyme to separate it from native OTCase and ATCase. Purification of the enzyme, if active, should be possible by affinity chromatography using phosphonacetyl-L-ornithine (PALO), a transition-state analog, as the affinity ligand, and size exclusion chromatography to separate it from the much smaller, native OTCase. ATCase will not bind to PALO. Alternatively, a mutant host, lacking OTCase, may be used for expression and enzymatic studies. If the activity of the chimera is suitable and in vivo data confirm that ammonia is efficiently taken up from the environment and made available to the enzyme, then application as a probiotic or biofilter organism can be investigated. While the prospects are exciting, funding is not currently available to pursue these studies.

Implications
Reduced emission of malodorous amines from swine and from their housing can be accomplished by reducing ammonia levels in feces or in swine buildings. Microorganisms can be engineered to be efficient ammonia traps. Use of these microorganisms as probiotics or in biofiltration units may result in improved animal
health and weight gain, as well as increased farm profitability. Positive effects on
human health may also be realized. The environmental benefits include a reduction in
ammonia and odor levels in the air and a decreased potential for ground water
nitrification.

**Literature Cited**

*Current Protocols in Molecular Biology* (1994), F.M. Ausubel, R. Brent, RE.Kingston,
and Sons, Inc.

"Structure of the human ornithine transcarbamylase gene." *J. Biochem.* (Tokyo)
103, 302-308.

by domain substitution effectively switches substrate specificity." *Nature* 338,
172-174.


Porcine OTCase cDNA." *J. of Animal Sci.* 75, 3368.

Penverne, B., M. Belkaid, and G. Herve (1994) "*In situ* behavior of the pyrimidine
pathway enzymes in *Saccharomyces cerevisiae*" *Arch. Biochem. Biophys.* 309,
85-93.

Serre, V., B. Penverne, H. Guy, D. Evans, and G. Herve (1994) "Regulation of
*Saccharomyces cerevisiae* carbamyl phosphate synthetase-aspartate
transcarbamylase complex: Allosteric regulation and channeling in yeast URA2
domains and mammalian-yeast chimeric proteins." *XIVth International Arginine
Workshop*, Paris, France, Abstr. 11.

Souciet, J.L., S. Potier, J.C. Hubert and F Lacroute (1987) "Nucleotide sequence of the
pyrimidine specific carbamoyl phosphate synthetase, a part of the yeast
multifunctional protein encoded by the URA2 gene." *Mol. Gen. Genet.* 207, 314-
319.