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Heterologous Expression of Colicin Y in Lactic Acid Bacteria

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1. Introduction

Bacteriocins are ribosomally synthesized antibacterial metabolites that are produced by bacteria with inhibitory activity generally against closely-related strains or species. In the past decade, application of bacteriocins as food biopreservatives has received considerable attention because of their ability to prevent growth of foodborne pathogens such as *Listeria* and *Clostridium* species. The major focus of applied research on bacteriocins has been to inhibit the growth of foodborne pathogens, especially *Listeria monocytogenes* because of serious outbreaks of listeriosis that spurred a growing awareness of food safety concerns (Ennahar *et al.*, 1999). Numerous studies on application of pure bacteriocin, bacteriocin-producing cultures or bacteriocin-containing fermentation products from a variety of lactic acid bacteria have been investigated for use in milk and dairy products, vegetable fermentations and meat products (Holzapfel *et al.*, 1995; McMullen and Stiles, 1996). For example, nisin is a bacteriocin produced by some strains of *Lactococcus lactis* subsp. *lactis* that is active against a broad range of Gram-positive bacteria. Nisin has been approved as a food preservative in numerous countries (Delves-Broughton, 1990). Pediocin PA-1/AcH is another bacteriocin of lactic acid bacteria, for which several U.S. and European patents have been issued but it is not yet in commercial use. In addition, two commercial compounds, Microgard and Alta 2341, which are fermentation products of food grade bacteria that impart antibacterial properties to foods, have been licensed for addition to foods. No other bacteriocins have been approved for addition to foods (Buard *et al.*, 2003; Lemay *et al.*, 2002).

A large number of antilisterial bacteriocins have been identified and many of them have been well characterized. Most of these belong to class IIa bacteriocins, which are defined as small, heat resistant peptides that have a YGNGVXC sequence motif near the N-terminus. This group of bacteriocins appears to be the most promising candidates for application in the food industry, not only because of their strong antilisterial activity but also because of their relatively broad activity spectrum against other spoilage and foodborne pathogenic bacteria including spoilage lactic acid bacteria, *Brochothrix* spp. *Clostridium* spp. and *Bacillus* spp. (Eijsink *et al.*, 1998; Ennahar *et al.*, 1999). Unfortunately, the bacteriocins produced by lactic acid bacteria including nisin and pediocin PA-1/AcH are not active against Gram-negative bacteria, such as *E. coli* O157:H7 and *Salmonella, Shigella, Yersinia* spp. and other *Enterobacteriaceae* unless the cells are exposed to an additional sublethal stress (Cutter and Siragusa, 1995; Stevens *et al.*, 1991, 1992).

Bacteriocins such as colicins and microcins produced by Gram-negative bacteria differ from those produced by lactic acid bacteria. Colicins are specifically active against *E. coli* and other closely related strains of *Enterobacteriaceae* (Pugsley and Oudega, 1987). It is, therefore, possible that purified colicin may have potential for application as biopreservatives for control of *E. coli*. Alternatively, well-screened food grade microorganisms such as lactic acid bacteria could be the host strains for production of colicin(s) and then directly applied to foods. Therefore, it should be valuable to develop a powerful bacteriocin expression system that could produce multiple bacteriocins active against Gram-negative and Gram-positive bacteria using food grade lactic acid bacteria that consequently could kill undesirable Gram-negative and Gram-positive bacteria.

2. Colicins and their potential use in food systems

Researchers have isolated and characterized a diverse group of colicins from *E. coli*, but their potential for practical use is in question. The research has been narrowly focused on the biochemical, physiological and molecular aspects of colicins. Regardless of their exact role, it is obvious that colicins occur in relatively high frequencies in *E. coli* populations and that colicin-producing cells have competitive advantage over members of a microbial community by killing other organisms (Riley and Gordon, 1999). The most obvious advantage of colicins is their potential use to inhibit the growth of pathogenic strains of *E. coli* O157H7 and *Salmonella.*

E. coli and *Salmonella* are of major concern in a wide variety of foods. Foodborne disease caused by these pathogens has caused considerable clinical and public health concern and significant economic loss. Among the five major groups of pathogenic *E. coli* that are currently associated with foodborne illness, Enterohemorrhagic *E. coli* (EHEC) strains that include *E. coli* O157:H7 are an important pathogens. They cause serious illness in humans in the form of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). EHEC infections have most often been associated with outbreaks of foodborne illness from undercooked ground beef, contaminated vegetables and contaminated water. Other acidic foods such as mayonnaise, apple cider and yogurt have also been implicated in outbreaks of HC (Tsai and Ingham, 1997). *Salmonella* spp. can cause typhoid or enteric fever and severe gastroenteritis. Although animal-based foods are mostly involved in outbreaks of salmonellosis, apple cider has also been implicated (Leyer and Johnson, 1993; Tsai and Ingham, 1997). These Gram-negative foodborne pathogens are not sensitive to nisin or other classes of bacteriocins from lactic acid bacteria. In contrast, colicins, the classical bacteriocins of Gram-negative bacteria, differ from those of the lactic acid bacteria. They are specifically active against *E. coli* and other closely-related strains of *Enterobacteriaceae* (Pugsley and Oudega, 1987). The inhibitory activity of several colicins against *E. coli* O157:H7 and other pathogenic *E. coli* has been reported. Bradley *et al.* (1991) showed that colicins G, H, E2 and V strongly inhibit the growth of strains of EHEC. Murinda *et al.* (1996) showed similar results. This report indicated that colicins with strong effectiveness against strains of *E. coli* O157:H7 may have the potential for application in the control of this undesirable organism. In contrast, research on bacteriocins from lactic acid bacteria has received enormous attention and their

practical application as food biopreservatives has been studied in great detail. As a consequence of the intense investigation on bacteriocins of lactic acid bacteria, a large number of bacteriocins have been discovered and characterized, and the genetic mechanisms behind bacteriocin production have been determined. This has led to new preservation strategies for application in food systems.

2. Colicin Y and it's genetic characteristics

A colicin-producing *E. coli* KB101 was isolated from ground beef and determined the characteristics of its colicin. Colicin Y is a non-SOS-inducible proteinaceous antibacterial substance. Colicin Y exhibited a strong inhibitory effect against the growth of strains of *E. coli* including serotype O157:H7 and *Salmonella* and it was also active against strains of *Shigella, Klebsiella* and *Citrobacter* spp. Colicin Y is stable at temperatures from 70℃ to boiling and over a wide pH range from pH 3 to 10. These advantageous characteristics suggest that it would be a good candidate for use in foods with the potential for application as an effective method for controlling *E. coli* O157:H7 and other *Enterobacteriaceae* in food preservation and processing. Unlike most other known colicins, it has a small molecular size of 8,776.6 Da. The genetic determinants for its production, immunity and export were identified and nucleotide analysis revealed that colicin Y was a natural variant of colicin V with differences in two amino acids. Its leader peptide was identical to that of colicin V.

The structural gene (*cyaC*) for colY encodes 103 amino acid residues and that the secreted translation product of *cyaC* is processed to remove its N-terminal leader peptide containing a specific Gly-Gly cleavage site. This so-called double-glycine-type leader peptide is common for class IIa bacteriocins of lactic acid bacteria and it is associated with a dedicated bacteriocin export system (Fath *et al.*, 1994; Nes *et al.*, 1996). Bacteriocins in this group are produced as precursors with a N-terminal leader peptide of 18 to 24 amino acids (Håvarstein *et al.*, 1994; Klaenhammer, 1993; Van Belkum *et al.*, 1997). These dedicated export systems require at least two membrane-bound proteins: a transporter protein of the **A**TP **B**inding **C**assette (ABC) superfamily and an accessory protein (Franke *et al.*, 1996; Van Belkum and Stiles, 1995). The ABC transporters are responsible for cleavage of the N-terminal leader peptide and they are involved in transportation of the mature peptide out of the cell. The function of the accessory protein remains unclear in bacteriocins from Gram-positive bacteria (Fath and Kolter, 1993; Håvarstein *et al.*, 1995). The dedicated protein export system has also been shown to be involved in the secretion of *E. coli* α-hemolysin (HlyBD) and *Erwinia* protease (PrtDEF). Both proteins of the ABC superfamily (CvaA, HlyB, and PrtD) and an accessory protein referred as the MFP family (CvaB, HlyD, and PrtE) in Gram-negative bacteria are structurally and functionally related. In Gram-negative bacteria, an outer membrane protein such as TolC in colicin V and α-hemolysin export and PrtF in *Erwinia* protease export is required for the extracellular secretion of protein or peptide (Fath and Kolter, 1993; Skvirsky *et al.*, 1995). The export of colicin V was shown to be mediated by substitution with HlyBD and PrtDEF proteins (Fath *et al*, 1991).

The 15-amino-acid double-glycine-type leader peptide of colicin Y is identical to the leader peptide of colicin V. Differences between colicin Y and colicin V involved replacements of two amino acids in

positions 49 (Ile^{49} to Val) and 86 (Ala^{86} to Asp). The CyaAB/TolC dedicated export protein complex is essential for secretion of colicin Y. The ABC transporter, CyaB has six potential transmembrane domains located in the inner membrane with a typical ATP-binding domain in a C-terminal cytoplasmic region. CyaB is believed to interact with CyaA and its translation level may be reduced with the absence of CyaA translation by preventing ribosome loading because of co-translation with CyaA.

3. Heterologous expression of colicin Y by lactic acid bacteria

Several studies have shown that construction of a multiple bacteriocin producing system and heterologous expression of bacteriocins by various food grade lactic acid bacteria strains is possible (McCormick *et al.*,, 1999; Stiles, 1996; Van Belkum *et al.*, 1997). This may offer an excellent tool to extend the application of bacteriocins in food preservation. Part of the research in our laboratory has focused on these strategies with special interest in the development of colicin-producing lactic acid bacteria. It is obvious that colicins that have strong activity against Gram-negative spoilage and pathogenic microorganisms have great potential for application in control of spoilage and pathogenic organisms in foods. It may be possible to apply well-characterized, non-toxic and purified colicins as food preservatives. It may even be possible to design a useful tool incorporating bacteriocins from lactic acid bacteria with colicins from *E. coli* to control both undesirable Gram-positive and Gram-negative spoilage and pathogenic species using well-screened, food grade lactic acid bacteria as host strains.

Previously, colicin V was produced by lactic acid bacteria by replacing the 15 amino acid N-terminal leader peptide of colicin V with the signal peptide of divergicin A (McCormick *et al.*, 1999). Heterologous expression of colicin Y using signal peptide of divergicin A in lactic acid bacteria was also achieved in this study. Divergicin A is a bacteriocin of *Carnobacterium divergens* LV13 that is produced as a prepeptide and it is exported from the cell by the general protein secretion (*sec*) pathway. The prepeptide consists of a classical N-terminal signal peptide of 29 amino acids and a mature peptide of 46 amino acids (Van Belkum *et al.*, 1997; Worobo *et al.*, 1995). Using the heterologous expression system, we confirmed production of colicins Y and V by heterologous lactic acid bacteria. All strains of *E. coli* O157:H7 tested were effectively inhibited by colicin Y and colicin V produced by LAB.

4. Application of multiple bacteriocin expression system in food bio-preservation

There has been interest in the potential to apply bacteriocins including colicins in food systems. Researchers in our laboratory have constructed multiple bacteriocin expression systems for lactic acid bacteria that have the potential to offer an excellent tool for enhanced overall effectiveness against target organisms. It is noteworthy that expression of bacteriocins with a double glycine leader peptide can possibly be achieved by a heterologous system in a heterologous host (Allison *et al.*, 1995; McCormick *et al.*, 1997; Van Belkum *et al.*, 1997). For example, colicin V could be secreted in *L. lactis* via the lactococcin A dedicated export machinery. In addition, divergicin A under the control of the *sec*-dependent

general secretion pathway could be secreted by substitution of the leader peptides of either leucocin A, lactococcin A or colicin V. On the other hand, it was also shown that the *sec*-dependent secretion system also allows secretion of the double glycine leader dependent bacteriocins by replacing their leaders with a signal peptide. The signal peptide of divergicin A can direct the secretion of carnobacteriocin B2 (McCormick *et al.*, 1997) and enterocin B (Franz *et al.*, 1997) that are generally exported by a dedicated export system. Replacement of the colicin V leader peptide with a signal peptide of divergicin A could also direct colicin V export in lactic acid bacteria (McCormick *et al.*, 1999). We also accomplished colicin Y production by *sec*-pathway in lactic acid bacteria through divergicin A signal peptide. Strategies for development of colicin-producing lactic acid bacteria would enable the inhibitory spectrum of lactic acid bacteria to be extended to Gram-positive and Gram-negative organisms.

5. Conclusion

Colicin Y from *E. coli* KB101 could be produced in lactic acid bacteria via the general secretion pathway. In this case, the host lactic acid bacteria do not need to produce immunity protein because colicins from *E. coli* are not active against Gram-positive bacteria. Even though the secretion level by the heterologous system and host was decreased, strains of *E. coli* O157:H7 were efficiently inhibited. These results raise the interesting possibility of further development and extension of potential bacteriocin applications in foods. Because bacteriocins from Gram-positive bacteria do not have antibacterial activity against Gram-negative bacteria and the most common causes of foodborne illness are Gram-negative bacteria, strategies using colicin-producing lactic acid bacteria that produce defined bacteriocins of interest will be the great advantage to ensure the food safety.

6. References.

1. Allison, G. E., C. Ahn, M. E. Stiles, and T. R Klaenhammer, 1995. Utilization of the leucocin A export system in *Leuconostoc gelidum* for production of a *Lactobacillus* bacteriocin. FEMS Microbiol. Lett. 131:87-93.
2. Bradley, D. E., S. P. Howard, and H. Lior. 1991. Colicinogeny of O157:H7 enterohemorrhagic *Escherichia coli* and the shielding of colicin and phage receptors by their O-antigenic side chains. Can. J. Microbiol. 37:97-104.
3. Buard. A., B. D. Carlton, F. Floch, and G. S. Simon. 2003. Subchronic toxicity, mutagenicity and allergenicity studies of a cultured dextrose food product. Food Chem. Toxicol. 41:689-94
4. Cutter, C., and G. R. Siragusa. 1995. Population reductions of Gram-negative pathogens following treatment with nisin and chelators under various conditions. J. Food Prot. 58: 977-983.
5. Delves-Broughton, J. 1990. Nisin and its uses as a food preservative. Food Technol. 44(11):100-117.
6. Ennahar, S., K. Sonomoto, and A. Ishizaki. 1999. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. J. Biosci. Bioeng. 87:705-716.

7. Eijsink, V. G. H., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64:3275-3281.
8. Fath, M. J., and R. Kolter. 1993. ABC transporters: bacterial exporters. Microbiol. Rev. 57:995-1017.
9. Fath, M. J., R. C. Skvirsky, and R. Kolter. 1991. Functional complementation between MDR-like export proteins: Colicin V, α-hemolysin and *Erwinia* protease. J. Bacteriol. 173:7549-7556.
10. Fath, M. J., L. H. Zhang, J. Rush, and R. Kolter. 1994. Purification and characterization of colicin V from *Escherichia coli* culture supernatants. Biochemistry 33:6911-6917.
11. Franke, C. M., K. J. Leenhouts, A. J. Haandrikman, J. Kok, G. Venema, and K. Venema. 1996. Topology of LcnD, a protein implicated in the transport of bacteriocins from *Lactococcus lactis*. J. Bacteriol. 178:1766-1769.
12. Franz, C. M. A. P. 1997. Characterization and genetic analysis of enterocin B production and immunity in *Enterococcus faecium* BFE 900. Ph.D. thesis. University of Alberta.
13. Håvarstein, L. S., H. Holo, and I. F. Nes. 1994. The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by Gram-positive bacteria. Microbiology 140:2383-2389.
14. Håvarstein, L. S., B. D. Diep, and I. F. Nes. 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol. Microbiol. 16:229-240.
15. Holzapfel, W. H., R. Geisen, and U. Schillinger. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24:343-362.
16. Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12:39-85.
17. Lemay M. J., J. Choquette, P. J. Delaquis, G. Claude, N. Rodrigue, and L. Saucier. 2002. Antimicrobial effect of natural preservatives in a cooked and acidified chicken meat model. Int. J. Food Microbiol. 78:217-26.
18. Leyer, G. J., and E. A. Johnson. 1993. Acid adaptation induces cross-protection against environmental stress in *Salmonella typhimurium*. Appl. Environ. Microbiol. 59:1842-1847.
19. McCormick, J. K., T. R. Klaenhammer, and M. E. Stiles. 1999. Colicin V can be produced by lactic acid bacteria. Lett. Appl. Microbiol. 29:37-41.
20. McCormick, J. K., R. W. Worobo, and M. E. Stiles. 1996. Expression of the antimicrobial peptide carnobacteriocin B2 by a signal peptide-dependent general secretory pathway. Appl. Environ. Microbiol. 62:4095-4099.
21. McMullen, L. M., and M. E. Stiles. 1996. Potential for use of bacteriocin-producing lactic acid bacteria in the preservation of meats. J. Food Prot. 58:Suppl:64-71.
22. Murinda, S. E., R. T. Roberts, and R. A. Wilson. 1996. Evaluation of colicins for inhibitory activity against diarrheagenic *Escherichia coli* strains, including serotype O157:H7. Appl. Environ. Microbiol. 62:3196-3202
23. Nes, I. F., D. B. Diep, L. S. Håvarstein, M. B. Brurberg, V. Eijsink, and H. Holo. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antionie van Leeuwenhoek 70:113-128.

24. Pugsley, A. P., and B. Oudega. 1987. Methods for studying colicins and their plasmids. p. 105-161. *In* K. G. Hardy (ed.), Plasmids: a practical approach. IRL Press, Washington, D. C.
25. Riley, M. A. and D. M. Gordon. 1999. The ecological role of bacteriocins in bacterial composition. Trend. Microbiol. 7:129-133.
26. Skvirsky, R., S. Reginald, and X. Shen. 1995. Topology analysis of the colicin V export protein CvaA in *Escherichia coli*. 177:6153-6159.
27. Stevens, K. A., B. W. Sheldon, N. A. Klapes, and T. R. Klaenhammer. 1991. Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl. Environ. Microbiol. 57:3613-3615.
28. Stevens, K. A., B. W. Sheldon, N. A. Klapes, and T. R. Klaenhammer. 1992. Effect of treatment conditions on nisin inactivation of Gram-negative bacteria. J. Food Prot. 55:763-766.
29. Stiles, M. E. 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70:331-345.
30. Tsai, Y. W., and S. C. Ingham. 1997. Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in acidic condiments. J. Food Prot. 60:751-755.
31. Van Belkum, M. J., and M. E. Stiles. 1995. Molecular characterization of genes involved in the production of the bacteriocin leucocin A from *Leuconostoc gelidum*. Appl. Environ. Microbiol. 61:3573-3579.
32. Worobo, R. W., M. J. van Belkum, M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1995. A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. J. Bacteriol. 177:3143-3149.