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Hemolysin of *Streptococcus faecalis* Subspecies *zymogenes* Contributes to Virulence in Mice

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The conjugative plasmid pAD1 (56.7 kilobases) in *Streptococcus faecalis* confers hemolysin-bacteriocin (Hly-Bcn) expression and a mating response to the sex pheromone cAD1 excreted by recipient cells. We examined the contribution of hemolysin to pathogenicity in intraperitoneally infected mice by using Tn*916* and Tn*917* insertion mutants altered in hemolysin expression. Strains exhibiting the normal hemolysin phenotype were significantly more virulent than the nonhemolytic insertion mutants. A mutant plasmid with an increased copy number which gave rise to a larger-than-normal zone of hemolysis on blood agar rendered host strains more virulent than the wild-type streptococci in mice.

Hemolysin production is common among gram-positive and gram-negative bacteria; however, the extent to which this property contributes to virulence is, in most cases, not clear. There have been reports that urinary tract infections caused by *Escherichia coli* are more likely to involve hemolytic strains (7, 8, 18), and a cloned *E. coli* hemolysin determinant has been shown to enhance virulence in an animal model (21). *Streptococcus faecalis*, a normal inhabitant of the gut, can also cause urinary tract infection. In addition, it can be involved in endocarditis and root canal infection (4). *S. faecalis* subsp. *zymogenes* is distinguished from other *S. faecalis* strains by its production of a cytotoxin which is active in the lysis of human, rabbit, and horse erythrocytes. Strains producing this beta-hemolysin also produce a bacteriocin which is active against a wide variety of gram-positive bacteria, including most *Streptococcus* spp. (3). It is believed that hemolysin and bacteriocin are mediated by the same genetic determinant (1, 2, 4, 14). To date, the hemolysin-bacteriocin (Hly-Bcn) activity of *S. faecalis* strains has been shown to reside in a conjugative plasmid (4). The plasmids usually confer a sex pheromone response (4) and transfer to recipient cells at a relatively high frequency (10^{-3} to 10^{-1} per donor cell) in broth culture (10, 17). It has not been shown that the hemolytic property contributes to pathogenicity in human infection or to virulence in experimental animal infection.

Cloned fragments of DNA carrying portions of the Hly-Bcn determinants of plasmid pAD1 (56.7 kilobases) originally identified on *S. faecalis* var. *zymogenes* DS16 (5, 19), have recently been shown to share extensive homology with DNAs of other Hly-Bcn plasmids isolated from *S. faecalis* var. *zymogenes* strains of diverse geographical origin (17). These results suggest that the Hly-Bcn trait has been disseminated among *S. faecalis* strains in natural environments. In this communication, we report that the Hly-Bcn determinant of pAD1 enhanced virulence in intraperitoneal infections in mice.

The expression of pAD1-mediated Hly-Bcn activity in certain *S. faecalis* derivatives can be altered by the insertion of the tetracycline (Tc) resistance transposon Tn*916* (11–13) and the erythromycin (Em) resistance transposon Tn*917* (5, 20) into specific regions of the plasmid. A physical map of the plasmid and the location of the Hly-Bcn determinant, revealed by insertion mutagenesis, have been reported (5). Insertion mutants altered in hemolysin expression were used to examine effects on virulence. The derivatives of pAD1 and the *Eco*RI fragments on which Tn*916* and Tn*917* are located are shown in Table 1. Plasmid pAM714 expresses normal hemolysin in its host strain (6, 16), whereas derivatives pAM307 and pAM211 do not express hemolysin (5, 12). Derivative pAM710 is a plasmid copy number mutant which gives rise to larger-than-normal zones of hemolysis for pAM714 on blood agar (15). The copy number was increased to 4.8 times that of pAM714 (one to two copies per chromosome) in a strain OG1RF1 background (15). We used *S. faecalis* OG1RF1 (9), carrying the various pAD1 derivatives, to test for virulence.

Figure 1 shows representative results on the lethality of the various strains harboring the pAD1 derivatives after intraperitoneal injection. Table 1 shows the 50% lethal dose and the time to death of the experimental mice. These data show a significant increase in mouse mortality when pAM714 was present in OG1RF1, suggesting that the plasmid has a determinant contributing to virulence. The strains harboring pAM307 or pAM211, which do not express hemolysin, did not exhibit the effect seen with pAM714. This implies that the Hly-Bcn expression encoded by pAD1 is responsible for the increased lethal behavior. The copy mutant derivative pAM710 conferred a higher degree of virulence than did pAM714, and all of the OG1RF1-(pAM710)-infected mice died 1 to 2 h after injection. An increase in the gene dosage of hemolysin is presumed to be the cause.

These results indicate that hemolysin is toxic to mice. We examined the question of whether the OG1RF1(pAM710) culture supernatant alone was responsible for the lethality. The OG1RF1(pAM710) suspensions of 2×10^9 or 2×10^{10} cells per ml were centrifuged for 10 min at 10,000 rpm at 4°C in a Kubota RA-3 rotor, and 0.5 ml of these supernatants was intraperitoneally injected into mice by the method described in the legend to Fig. 1. None of the mice died within 7 days of the injection. These results imply that either (i) the hemolysin may be unstable and become inactivated soon after the

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TABLE 1. 50% Lethal dose of *S. faecalis* harboring pAD1 derivatives and time to death of mice after infection

Strain[a]	Phenotype	50% Lethal dose[b]	Time to death (h) of mice after infection of 10^9 cells
OG1RF1(pAM710)	Hyperhemolytic	1.6×10^8	1–2[c]
OG1RF1(pAM714)	Normal hemolytic	2.6×10^8	4–5[c]
OG1RF1(pAM307)	Nonhemolytic	$>3.0 \times 10^9$	—[d]
OG1RF1(pAM211)	Nonhemolytic	$>3.0 \times 10^9$	—[d]
OG1RF1	Nonhemolytic	$>3.0 \times 10^9$	—[d]

[a] pAM710, pAM714, pAM307, and pAM211 are representative derivatives of pAD1 which were altered in hemolysin expression by Tn*917* or Tn*916* transposon insertion. Tn*917* was inserted into *Eco*RI fragment B in pAM710 and pAM714 and into *Eco*RI fragment H in pAM307. Tn*916* was inserted into *Eco*RI fragment F in pAM211.

[b] 50% lethal dose was derived from the data of Fig. 1.

[c] All of the mice died within this time.

[d] None of the mice died within 7 days of injection of 10^9 cells. All of the mice died between 18 and 72 h after injection of 10^{10} cells.

excretion, and the continuous excretion by cells is necessary to provide enough toxin to be lethal; or (ii) the hemolysin may be stable, but it requires some additional substance of bacterial cell origin to become lethal.

We examined the question of whether the death of the mice was caused by hemolysin lysis of erythrocytes. Just before the death of mice inoculated with 10^9 cells of OG1RF1(pAM710), blood samples were obtained from the orbital venous plexes with hematocrit capillary tubes and centrifuged for 5 min at 12,000 rpm with Kubota hematocrit KH-120A. The separated sera showed no reddish discoloration from hemolysis. These results indicate that the death of mice was not simply caused by hemolysin lysis of erythrocytes.

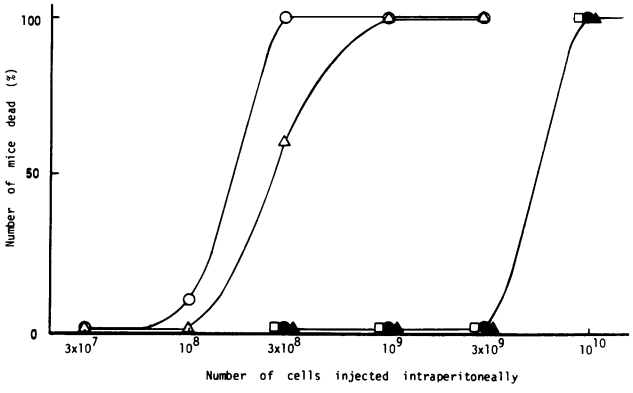

FIG. 1. Virulence of *S. faecalis* strains harboring different pAD1 derivatives. A 0.05-ml sample of an overnight culture of bacteria in Penassay broth (Difco) was plated out onto Penassay broth agar plates (Difco). The plates were incubated for 18 h at 37°C. The cells grown on the agar plates were suspended in 2 ml of phosphate-buffered saline, dilutions into phosphate-buffered saline were made, the optical density at 530 nm was checked, and the viable cells were counted on AB3 broth agar. Samples (0.5 ml) of appropriate dilutions containing the number of cells indicated in the figure were intraperitoneally injected into each mouse. Ten female ICR mice (weight, 25 to 27 g) were used for each inoculum size of bacteria. The mice were observed for 7 days. Symbols: ○, OG1RF1 (pAM710); △, OG1RF1(pAM714); ●, OG1RF1(pAM307); ▲, OG1RF1(pAM211); □, OG1RF1.

Although the mode of action of *S. faecalis* hemolysin in pathogenicity is still not known, our data provide the most definitive evidence that hemolysin contributes to virulence in *S. faecalis* infection in mice. The role of hemolysin in human *S. faecalis* infection remains obscure; to our knowledge, no epidemiological data showing a correlation of the hemolytic trait with a specific type of infection have been reported.

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