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Citation

Van Tyne D, Gilmore MS. 2014. Virulence Plasmids of Nonsporulating Gram-Positive Pathogens. Microbiol Spectr. 2, no. 5. doi: 10.1128/microbiolspec.PLAS-0002-2013.

Published Version

doi:10.1128/microbiolspec.PLAS-0002-2013

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Published in final edited form as:

Microbiol Spectr. 2014 ; 2(5): . doi:10.1128/microbiolspec.PLAS-0002-2013.

Virulence Plasmids of Nonsporulating Gram-Positive Pathogens

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SUMMARY

Gram-positive bacteria are leading causes of many types of human infection, including pneumonia, skin and nasopharyngeal infections, as well as urinary tract and surgical wound infections among hospitalized patients. These infections have become particularly problematic because many of the species causing them have become highly resistant to antibiotics. The role of mobile genetic elements, such as plasmids, in the dissemination of antibiotic resistance among Gram-positive bacteria has been well studied; less well understood is the role of mobile elements in the evolution and spread of virulence traits among these pathogens. While these organisms are leading agents of infection, they are also prominent members of the human commensal ecology. It appears that these bacteria are able to take advantage of the intimate association between host and commensal, via virulence traits that exacerbate infection and cause disease. However, evolution into an obligate pathogen has not occurred, presumably because it would lead to rejection of pathogenic organisms from the host ecology. Instead, in organisms that exist as both commensal and pathogen, selection has favored the development of mechanisms for variability. As a result, many virulence traits are localized on mobile genetic elements, such as virulence plasmids and pathogenicity islands. Virulence traits may occur within a minority of isolates of a given species, but these minority populations have nonetheless emerged as a leading problem in infectious disease. This chapter reviews virulence plasmids in nonsporulating Gram-positive bacteria, and examines their contribution to disease pathogenesis.

VIRULENCE PLASMIDS IN *STAPHYLOCOCCUS AUREUS*

S. aureus – virulence and pathogenesis

Infection with *S. aureus* can result in a wide variety of diseases, including wound infections, toxic shock, food poisoning, endocarditis, pneumonia, and septicemia (1). Virulence and drug resistance often occur together, as recent outbreak strains of methicillin-resistant *S. aureus* (MRSA) also produce a number of different virulence factors (2). It is perhaps not surprising that a bacterium capable of causing such a wide array of diseases possesses a diverse repertoire of virulence factors. A consequence of this versatility is that the pathogenesis of *S. aureus* is usually multifactorial (3).

S. aureus is capable of producing a number of extracellular toxins, including cytolytic toxins (α -toxin, β -toxin, γ -toxin), enterotoxins, toxic shock syndrome toxin (TSST-1), and exfoliative toxins. Although staphylococcal virulence is seldom attributable to one factor alone, different toxins have been linked to different types of staphylococcal infection. For example, staphylococcal enterotoxins are associated with food poisoning (4, 5), and

exfoliative toxins have been linked to staphylococcal scalded-skin syndrome (SSSS) (6–9). Many of these toxins function as superantigens, causing widespread T-lymphocyte activation and resulting in systemic shock (10–15). Cytolytic toxins, enterotoxins, and TSST-1 have all been shown to function as superantigens, while the association of superantigenic activity with exfoliative toxins is less clear (9, 16). Several staphylococcal antigens have been found to occur on mobile genetic elements, including the plasmid-encoded staphylococcal enterotoxin D (SED), staphylococcal enterotoxin J (SEJ), and exfoliative toxin B (ETB), which are discussed in greater detail below.

Enterotoxin

Staphylococcal enterotoxins (SEs) comprise a large family of related proteins similar to streptococcal pyrogenic exotoxins (15). These heat stable, pepsin resistant proteins include SEA-SEE, SEG-SEI, and SER-SET (4, 5). Staphylococcal food poisoning results primarily from the ingestion of contaminated meat or poultry, and to a lesser degree from contaminated fish, shellfish, and milk (4, 5, 17). Enterotoxin production within different *S. aureus* strains may vary, but food poisoning often results from the ingestion of any one or a combination of preformed enterotoxins (18). Historically, the most common enterotoxin associated with staphylococcal food poisoning is staphylococcal enterotoxin A (SEA), followed by SED and SEB (19, 20).

The gene encoding SED, *sed*, was determined to be present on a 27.6-kilobase penicillinase plasmid designated pIB485, which was isolated from strain KSI1410 and also contains genes for resistance to penicillin and cadmium sulfate (Figure 1) (21). Upon curing the strain of this plasmid, the ability to produce SED was lost. Cloning and expression of SED in *E. coli* from a restriction fragment of pIB485 proved that *sed* was encoded by pIB485. Transcription of *sed* was found to be regulated by the accessory gene regulator system (*agr*), a two-component system which regulates the transcription of virulence factors in a cell density-dependent manner (22–24). Regulation of *sed* by *agr* results in its induction during post-exponential growth (23). A study by Zhang et al. (23) identified a second enterotoxin-like gene within pIB485. This gene was termed *selj* and encodes staphylococcal enterotoxin-like protein J (SEJ) (Figure 1).

More recently, additional plasmid-encoded enterotoxins have been found in *S. aureus*. These include the enterotoxin-like protein SER, which shows sequence similarity to SEG and is encoded by a plasmid similar to pIB485 named pF5, for the Fukuoka 5 strain from which it was isolated (25). pF5 was also found to encode staphylococcal enterotoxins S and T (SES and SET), as well as several accessory genes. The contributions of SEJ, SER, SES, and SET to the pathogenesis of staphylococcal food poisoning, either singularly or in combination with SED, have not yet been addressed.

Exfoliative toxin

S. aureus is capable of producing four exfoliative toxins (ETs): exfoliative toxin A (ETA), ETB, ETC, and ETD. ETs are most common among phage group II isolates of *S. aureus* (26, 27), and cause peeling and blistering lesions of the skin (28). ETA, ETB, and ETD are glutamate-specific serine proteases that cleave desmoglein I, a desmosomal intracellular

adhesion molecule expressed in the superficial layers of the epidermis (29). ETC was first isolated from a horse lesion and appears to affect horses, chickens and mice (30), but its role in human disease, if any, has not been characterized. ETs have been associated with staphylococcal scalded skin syndrome (SSSS), a group of diseases including Ritter's disease, toxic epidermal necrosis, bullous impetigo, and erythema (7, 31). It has been suggested that high nasal carriage rates of *S. aureus* in adults, as well as the apparent protective nature of ET antibodies in SSSS (32–35), account for why SSSS is seen primarily in young children and the immunocompromised (36, 37). Finally, fewer than 5% of *S. aureus* clinical isolates harbor genes for ETs (38, 39), suggesting that these genes are acquired by horizontal gene transfer and can be passed between bacterial strains on mobile genetic elements.

While ETA, ETB, and ETD share similar gene sequences and modes of action, they are found in distinct genetic contexts. The gene encoding ETA is normally localized to the *S. aureus* chromosome (40), however it has also been found within the genome of an integrated temperate phage (41). ETD was identified on a novel *S. aureus* chromosomal pathogenicity island along with a gene for the epidermal cell differentiation inhibitor-B (EDIN-B) (42). ETD appears to act similarly to ETA, but it has also been found in patients with infections other than bullous impetigo or SSSS. Additionally, ETD was found to be present in approximately 10% of *S. aureus* clinical isolates in France (43), suggesting that it may play a role in a wider array of staphylococcal infections than ETA and ETB.

In contrast to ETA and ETD, the locus for ETB is typically plasmid-encoded (Figure 2) (44, 45). Formal evidence that the structural gene for ETB, designated *etb*, occurred on a plasmid was provided by Jackson and Iandolo (46). It was also discovered that the plasmid carries a cadmium sulfate resistance gene, which is part of a putative transposon (47). ETB is found on plasmids that vary in size from approximately 35 to 60 kilobases, yet the genetic organization of these plasmids appears to be conserved (Figure 2B) (48). Interestingly, the exfoliative toxins of *Staphylococcus hyicus*, SHETA and SHETB, which are associated with exudative epidermitis in pigs and chickens, are similar to ETA and ETB – SHETA is produced by plasmid-free strains, while SHETB production is dependent on the presence of a large plasmid (49, 50).

A staphylococcal bacteriocin two-component lantibiotic system, named BacR1/C55, is also located on various ETB-producing plasmids of *S. aureus* (51). BacR1 was initially characterized by Rogolsky (52), and was more extensively examined by Crupper et al. (53). Staphylococcin activity was attributed to the synergistic activity of two peptides, called C55 α and C55 β . The respective structural genes *sacA* and *sac β A*, as well as potential processing genes *sacM1* and *sacT*, are all organized within the lantibiotic operon shown in Figure 2 (51). The *sacA* and *sac β A* genes were detected by PCR only in strains that were also positive for ETB. Although found on different plasmids, C55 and BacR1 appear to constitute the same lantibiotic system, which also resembles the two-component bacteriocin lacticin 3147 from *Lactococcus lactis* (51).

In addition to bacteriocin activity attributable to either BacR1/C55, a novel virulence factor has also been linked to ETB-encoding plasmids. Through sequencing, Yamaguchi et al. (48)

identified a protein potentially capable of ADP-ribosylating Rho GTPases, which are members of the Ras superfamily of proteins involved in cytoskeletal network regulation within eukaryotic cells. Inactivation of Rho GTPases has been shown to inhibit the chemotactic and phagocytic activities of immune cells during infection (54), inhibit the differentiation of structural cells (55), and contribute to the dissemination of bacteria through the vasculature and tissues (56). Inactivation of Rho GTPases by *S. aureus* is accomplished by exotoxins of the epidermal cell differentiation inhibitor (EDIN) family (48, 55, 57, 58). The EDIN determinant on ETB-containing plasmids was designated EDIN-C (*ednC*) (Figure 2). EDIN-C has been found in the vast majority of EDIN-carrying *S. aureus* isolates, and was enriched among isolates recovered from deep-seated infections (59). This further suggests that EDIN-C is an important contributor to virulence of *S. aureus* infections in humans.

More recently, next-generation DNA sequencing has allowed for a systematic analysis of virulence factor-encoding plasmids in *S. aureus* (60). This effort has more than doubled the number of known plasmid groups, giving a fuller picture of the dissemination of plasmids among different strains of *S. aureus*, as well as the drug resistance and virulence traits that are associated with particular plasmid groups. The diversity among *S. aureus* plasmids is so great that plasmids can be used to track the spread of drug resistant isolates within a hospital setting (61). Importantly, this effort has identified many new *S. aureus* plasmids, which likely contain additional virulence factors that have yet to be described.

VIRULENCE PLASMIDS IN *ENTEROCOCCUS FAECALIS*

E. faecalis – virulence and pathogenesis

Enterococcus faecalis and *E. faecium* are members of the normal commensal flora of the gastrointestinal tracts of humans and other animals (62). However, *E. faecalis* and *E. faecium* are also leading causes of hospital acquired infection, including urinary tract infections, bacteremia, endocarditis, and intra-abdominal infections (63–68). The treatment of enterococcal infections is particularly challenging because antibiotic resistances enable the bacteria to survive standard therapies (69). Furthermore, the ability of enterococci to survive harsh environments, such as low pH, detergents, and bile salts, enables them to persist in the hospital environment (70–72). Vancomycin has been considered a drug of last resort for many enterococcal infections, but vancomycin-resistant enterococci (VRE), particularly *E. faecium*, are now commonly found in hospital settings (73). Although the majority of VRE infections are due to *E. faecium*, nearly 75% of all enterococcal infections are caused by *E. faecalis* (66, 74). This may be attributable to the additional virulence determinants that *E. faecalis* possesses, some of which are described below.

E. faecalis produces a number of factors that are presumably involved in adhesion to host tissues, and thereby contribute to host-pathogen interactions, as well as to the virulence and pathogenesis of enterococcal infections. These factors include enterococcal surface protein (Esp), aggregation substance, and the cytolysin. *E. faecalis* also expresses a capsular polysaccharide, which helps form the physical interface between bacteria and host. The capsular polysaccharide varies in structure, possibly because of immune pressure (75, 76). Because it is easily detectable on the surface of the bacterial cell and is the target of

complement-mediated opsonophagocytosis, the capsular polysaccharide has been used in typing schemes to classify different serotypes of *E. faecalis* (77). While the structure of the capsular polysaccharide is variable among different *E. faecalis* strains, the genes encoding the capsule have thus far been localized to the bacterial chromosome (78, 79).

The enterococcal surface protein Esp is an adhesin that has been localized to a 150-kilobase pathogenicity island in strains of *E. faecalis* (80, 81). Esp has been associated with outbreaks of VRE in hospitals (82), antibiotic resistance and biofilm formation (83, 84), and adherence of *E. faecalis* in the pathogenesis of urinary tract infections (80, 85). Aggregation substance is a surface protein that has similarly been shown to contribute to disease pathogenesis, also via enhanced adhesion and biofilm formation (86–92). Finally, some *E. faecalis* strains produce a cytolysin with both bacteriocidal and toxin activities (93, 94). The cytolysin operon is found, along with aggregation substance, on both pheromone-responsive plasmids (95), as well as within the chromosomal pathogenicity island on which Esp and is also found (81). Pheromone-responsive plasmids in enterococci are briefly reviewed below, followed by a discussion of the *E. faecalis* pheromone-responsive virulence plasmids that encode for aggregation substance and the cytolysin.

Pheromone-responsive plasmids in enterococci

Plasmid-free enterococci secrete over a dozen distinct peptide pheromones of 7–8 amino acids in length, which induce a mating response from donors carrying plasmids that specifically respond to each of these peptides (96–101) (Figure 3). Upon pheromone peptide secretion by recipient cells, donor cells containing the corresponding pheromone-responsive plasmid will express factors that promote physical interaction between donor and recipient cells, and thereby allow for conjugal DNA transfer. Aggregation substance on the surface of donor cells mediates clumping between donors and recipients via binding substance, a constituent of the cell wall of both plasmid containing and plasmid-free enterococci (102). It is believed that binding substance consists in part of lipoteichoic acid (103–105). Aggregation provides the initial cell-cell contact required for conjugal transfer of plasmid from donor to recipient. In broth, the mating potential of donor cells upon exposure to peptide pheromones is at least 100,000 times greater than that exhibited by non-induced donor cells (106). Transfer dynamics likely differ between bacteria growing in biofilms as compared to broth (107), but in both cases plasmid transfer depends upon cells sensing that they are in close proximity to one another. More than a dozen distinct pheromone-responsive plasmids have been identified within *E. faecalis* (108); the most well characterized of these include pAD1, pCF10, and pPD1. The mechanistic details of pheromone-responsive plasmid transfer in enterococci is covered in considerable detail elsewhere. However, the mechanism underlying transfer of pAD1 is also covered below.

Aggregation substance

The gene encoding aggregation substance (*asa1*) is often found on pAD1, a 60-kilobase, pheromone-responsive, transmissible plasmid (98, 109). Aggregation substances of other pheromone-responsive plasmids are homologous to the aggregation substance of pAD1 (108), although these other plasmids respond to different pheromones. The aggregation substance found on pAD1 is a 137-kDa surface protein, and has often been implicated in the

adherence and virulence of enterococci (86, 87, 89, 110, 111). Aggregation substance also appears to contribute to the spread of cytotoxin and antibiotic resistance determinants, via its role in the conjugal transfer of pheromone-responsive plasmids (112). The pheromone eliciting a response from pAD1-containing donor cells is designated cAD1 (113).

Genes encoding aggregation substance, functions involved in pheromone sensing and response, maintenance of plasmid pAD1, and the cytotoxin operon, are shown in Figure 4. The *asa1* gene is located adjacent to *seal*, which encodes a surface exclusion protein that inhibits transfer between cells containing similar plasmids and thereby limits redundant plasmid transfer (Figure 4A) (108). The *asa1* and *seal* genes are adjacent to the Tra regulon, which encodes a number of ORFs involved in pheromone sensing and regulation. Products of the *traA*, *traB*, *traD*, and *traE1* genes are involved in the regulation of the pheromone response (106, 114–118), while TraC is a surface protein involved in pheromone sensing, and functions as a specific binding factor (119). The *iad* gene encodes the pheromone inhibitor iAD1, which prevents self-induction of the pheromone response by inhibiting it at lower pheromone concentrations (120). Low concentrations of pheromone may arise from plasmid-containing or plasmid-free cells, at such a distance that an interaction allowing for conjugation is unlikely to occur.

Adjacent to genes associated with the transfer of pAD1 lies the *par* locus, a post-segregational killing mechanism (PSK, Figure 4B) (121). A region exhibiting a high degree of homology to the *par* determinant of pAD1 has also been identified in pCF10, another pheromone-responsive plasmid of *E. faecalis* (121). The primary function of PSKs is to maintain low-copy plasmids within a population of bacteria by killing plasmid-free segregants. The *par* locus within pAD1 encodes a toxin, Fst, which is believed to target chromosomal separation and/or cell division. Transcript RNA I contains the ORF believed to encode Fst toxin, a 33 amino acid peptide (122). Transcript RNA II is capable of occluding the 5' end of RNA I via antisense transcription of direct repeats within RNA I, as well as the 3' end of RNA I via the interaction of complementary transcriptional terminator stem-loops (122). RNA II is less stable than RNA I, therefore cells that do not maintain plasmid will not be able to inhibit toxin activity through the synthesis of antidote RNA II. PSKs are discussed in more detail elsewhere. The gene products of *repA* and *repB* are also involved in plasmid replication and maintenance (123, 124). RepA is responsible for initiation of replication, while RepBC, along with flanking repeat sequences, most probably represents a ParAB/SopAB partitioning system, which is covered in detail elsewhere.

Although the exact occurrence of events, as well as the interplay of the various Tra regulon factors in the coordination of the pheromone response, continue to be somewhat controversial topics, a generalized scheme is presented in Figure 5. In the uninduced state, TraA and a small RNA molecule resulting from the transcription of *traD* both negatively regulate levels of TraE1 (125). TraA accomplishes this negative regulation through DNA binding upstream of *iad* and *traE1*. When levels of the pheromone cAD1 surpass inhibition by iAD1, cAD1 binds directly to TraA, which loses its affinity for DNA and allows for transcription of *traE1* (126). TraE1 then goes on to activate production of other factors involved in conjugation (127).

Apart from its role in conjugal plasmid transfer, aggregation substance has also been linked to the virulence of *E. faecalis*. Aggregation substance was postulated to have a role in the adherence of enterococci to host cells, based on the presence of two Arg-Gly-Asp (RGD) motifs within its protein sequence. These motifs were hypothesized to mediate interactions between fibronectin and integrins on mammalian cell surfaces (128). *E. faecalis* expressing aggregation substance was shown to more readily bind renal epithelial cells in culture, and this enhanced adherence was blocked by the addition of a synthetic RGD peptide, but not by a peptide of divergent sequence (86). Mutating the RGD motifs in the pCF10-encoded aggregation substance gene (*prgB*) affected the virulence of *E. faecalis* in a rabbit endocarditis model (129), but not the invasion of human intestinal epithelial cells *in vitro* (130). The latter study focused instead on the aggregation domain as being critical for internalization. Finally, expression of aggregation substance has been reported to be induced *in vivo*, via an interaction between plasma components and the inhibitor peptide pheromone, which renders the latter unable to inhibit endogenous pheromone (91).

Few studies of the role of aggregation substance in the pathogenesis of infection have distinguished between the ability of aggregation substance to promote bacterial clumping, versus its potential contribution to the direct interaction between bacteria and specific types of human or animal cells (131). The aggregation domain of aggregation substance has been shown to mediate internalization of bacteria by enterocytes (130, 132). Expression of aggregation substance also correlates with enhanced uptake of enterococci by intestinal epithelial cells, but this increase in uptake did not result in an increase in bacterial translocation across the intestinal epithelium *in vitro* (89). Expression of aggregation substance was also found to affect the function of polymorphonuclear neutrophils (PMNs) and macrophages, by promoting the phagocytosis of enterococci by these cells (133, 134). Interestingly, aggregation substance was observed to promote the intracellular survival of enterococci within PMNs by inhibiting acidification of the phagolysosome (110) and within macrophages by inhibiting respiratory burst (134). In addition, the peptide pheromones involved in the induction of AS expression are also potentially able to alter the host immune response, in that the pheromones appear to be chemotactic for PMNs (135).

Aggregation substance does not appear to contribute to the virulence of *E. faecalis* in either a rabbit model of endophthalmitis (136), nor in *Caenorhabditis elegans* infection (137). However, in a rabbit model of endocarditis, a disease in which vegetations of platelets and fibrin resulting from bacterial infection are associated with inflammation of the heart valves and lining, aggregation substance from both pAD1 and pCF10 caused an increase in the size of cardiac vegetations (88, 90, 129). No effect of aggregation substance on vegetation size was observed in a rat endocarditis model (138), suggesting that the effects of aggregation substance on pathogenesis may be species or model-specific. The role of aggregation substance in an infection may be to promote the formation of a quorum of cells in a localized microenvironment through clumping, which could affect the expression of factors now known to be quorum regulated, such as the cytolysin (139). Aggregation substance in the absence of cytolysin expression was shown to increase the size of vegetations in endocarditis (88). When expressed from a plasmid that also encodes the cytolysin, such as pAD1, vegetations were observed to be smaller in mass but much more toxic. Thus

aggregation substance, by increasing the number of bacteria at a site of infection, could enhance production of cytolysin, though this has not yet been proven directly.

Apart from its potential to aid in achieving a quorum, the ability of aggregation substance to spread pheromone-responsive plasmids throughout a bacterial population could also enhance the virulence of *E. faecalis*, via the concomitant spread of cytolysin and antibiotic resistance determinants. In a study by Huycke et al. (140), an *in vivo* model was used to examine pheromone-responsive plasmid transfer between *E. faecalis* in the intestine. This study found that pAD1 and its derivatives were transferred between bacteria at high frequency, independent of antibiotic selection. While investigating the contribution of aggregation substance to endocarditis in a rabbit model, Hirt et al. (91) found that pCF10 was also transferred at high frequency *in vivo*. Although the conjugative spread of pheromone-responsive plasmids *in vivo* has been well documented, the precise consequence of the spread of these plasmids and their determinants during infection is not well understood.

Cytolysin

The cytolysin produced by *E. faecalis* is structurally and functionally unique (141, 142). It is capable of lysing erythrocytes, eukaryotic cells, and bacteria, and may also function as a signaling molecule to communicate with other bacteria and probe the environment (143, 144). Since its identification by Todd in 1934 from group D streptococci (*Streptococcus faecalis* subspecies *zymogenes* has since been reclassified as *E. faecalis*) (93), it has been characterized genetically and shown to contribute to virulence in virtually all models of *E. faecalis* infection tested (64, 88, 137, 145, 146). The cytolytic phenotype has also been reported to be more prevalent among infection-derived isolates of *E. faecalis* compared to non-infection isolates (147–149).

The determinant for cytolysin was originally suspected to be plasmid-encoded, because the hemolytic phenotype was observed to be a variable trait of *E. faecalis* and this trait could be transferred to plasmid-free recipients (150, 151). Clewell noted that a transposon insertion into pAD1 correlated with the loss of hemolysin/bacteriocin activity (152). Since then, a highly conserved cytolysin determinant has been found within plasmids of various *E. faecalis* isolates, including the pheromone-responsive plasmids pAD1 (152–154), pJH2 (151, 155), pOB1 (155, 156), pAM81 (157), pX98 (158), and pIP964 (159). Plasmids encoding cytolysin have been determined to fall within the same plasmid incompatibility group (160).

The organization of the cytolysin operon was elucidated through transposon mutagenesis of pAD1 and extracellular complementation, showing that eight genes are involved in the production of the toxin (Figure 4C) (95, 139, 161–163). A schematic of cytolysin expression, including posttranslational modifications, is depicted in Figure 6. Cytolysin activity is attributed to two structural components, designated CylL_L and CylL_S. Both peptides are post-translationally modified within *E. faecalis* by CylM (164), resulting in CylL_L* and CylL_S*. After modification, the subunits are secreted by CylB, an ATP-binding transporter (161, 164). While both CylL_L* and CylL_S* require CylB for secretion, only CylL_L* secretion is dependent upon ATP hydrolysis (161, 164). During secretion, the serine protease domain of CylB removes the leader sequences associated with the modified CylL_L*

and CylL_S* peptides (165), resulting in extracellular CyL_L' and CylL_S'. Finally, CyL_L' and CylL_S' are further cleaved by CylA, an extracellular serine protease, to generate the active toxin subunits CyL_L'' and CylL_S'' (162, 165). Expression of cytolysin is tightly regulated within *E. faecalis*. Its production was determined to be density dependent, with CylL_S'' serving as a signaling molecule for the induction of cytolysin expression (139, 166). Two genes involved in autoinduction by CylL_S'', *cylR1* and *cylR2*, have also been identified (Figure 4C) (139). CylR2 appears to repress expression of the operon in the absence of autoinducer via direct binding to the cytolysin promoter region (167, 168). The precise role of CylR1 is still undetermined.

Although the exact mechanism of action of the cytolysin bacteriocin has yet to be determined, cytolysin structural components resemble lantibiotics, a class of bactericidal peptides produced by Gram-positive bacteria (164, 165, 169). The composition, size, and presence of lanthionine linkages (post-translational condensation of cysteine and serine or threonine side chains) are characteristics shared with other lantibiotics (170–173). Cytolysin was found to kill a wide variety of Gram-positive species including staphylococci, streptococci, clostridia and enterococci (174, 175). It has been shown that CylII, a putative membrane metalloprotease encoded within the cytolysin operon, confers immunity to the cytolysin-producing cell (163), although the precise mechanism of immunity conferred by CylII has not yet been determined.

The contribution of the cytolysin to the virulence of *E. faecalis* has been well demonstrated in a number of infection models. Cytolysin-expressing bacterial strains exhibit a significantly lower LD₅₀ in a murine intraperitoneal challenge model than strains deficient in cytolysin (145, 146). In a rabbit model of endocarditis, lethality attributable to *E. faecalis* expressing aggregation substance in the absence of cytolysin was only 7% (88). However, 55% lethality was observed in *E. faecalis* infections where the bacteria expressed both aggregation substance and cytolysin (88). Cytolysin was also determined to contribute to the virulence of *E. faecalis* in a rabbit model of endophthalmitis, in which significant retinal damage and loss of vision was attributable to cytolysin expression (176, 177). Furthermore, cytolysin was shown to increase the lethality of *E. faecalis* in *C. elegans* infection (137), a model currently used as an initial rapid screen for potential virulence factors.

The ability of cytolysin to contribute to enhanced colonization of the intestine by pathogenic *E. faecalis*, as well as the spread of enterococci into the bloodstream, has been addressed in several studies. The potential role of cytolysin in competition with other enterococci within the intestinal niche was examined by Huycke et al. (178). In the same study, cytolytic strains were also able to outcompete non-cytolytic strains *in vitro*. However, in mice that were fed a mixture of cytolytic and non-cytolytic *E. faecalis*, no significant difference in the proportion of cytolytic and non-cytolytic isolates was observed in stool samples. Although a cytolytic strain has been shown to be capable of translocation across the intestinal epithelium (179), this activity has not been conclusively determined to be dependent upon expression of cytolysin. The exact contribution of cytolysin to *E. faecalis* bacteremia has yet to be determined, but cytolytic *E. faecalis* were found to spread from the peritoneum into the bloodstream of mice more readily than non-cytolytic isogenic mutants (66). It has also been suggested that cytolysin-expressing bacteria may be able to acquire exogenous heme,

resulting in enhanced growth within the bloodstream, although this hypothesis has yet to be tested (180).

It is clear that plasmids encoding cytolysin contribute to the virulence of *E. faecalis*. However, the precise contributions of cytolysin to the pathogenesis of infection remain to be described.

VIRULENCE PLASMIDS IN OTHER NONSPORULATING, GRAM-POSITIVE PATHOGENS

Rhodococcus equi

R. equi, a facultative intracellular pathogen which can persist and multiply within macrophages, is associated with severe equine pneumonia in foals, and is also capable of causing pneumonia in humans (181). It has been shown that equine and human clinical isolates of *R. equi* contain large plasmids (182, 183), and these plasmids have been determined to be essential for the ability of *R. equi* to survive within macrophages (184). In the absence of these plasmids, the virulence of *R. equi* is significantly attenuated (184–186).

Virulence-associated proteins (VapA-M) encoded by large plasmids (>50 kb) from *R. equi* clinical isolates are localized to the bacterial cell surface and appear to be critical for virulence (182, 185, 187–190). VapA in particular appears to play a major role in *R. equi* pathogenesis (184, 191). Expression of VapA was found to be dependent on both temperature and pH (192, 193), a feature shared with proteins encoded by virulence plasmids of *Yersinia pestis* and *Shigella flexneri* (194–196). VapB is encoded by a separate plasmid from the one encoding VapA (197, 198), and is associated with less virulent *R. equi* infections in humans (199). VapA-containing plasmids also encode additional Vap proteins VapC-H (200), while VapB-containing plasmids encode Vap proteins VapI-M (198). A map of the VapA-containing plasmid p33701 is shown in Figure 7 (200). The plasmid also contains genes that are associated with conjugation and maintenance functions (Figure 7; red ORFs). The region containing the genes encoding VapC-H (Figure 7; blue ORFs) is bounded by two transposon resolvases. p33701 also contains a putative two-component regulator (*tcr*) and a putative lysyl-tRNA synthetase (Figure 7; yellow ORFs) that both appear from G+C content to be foreign to *R. equi* (200). All Vaps resemble each other in sequence, and Vap homologs have not yet been identified in other organisms. The specific roles of the different Vap proteins in *R. equi* infection remain unknown.

Lactococcus garvieae

Lactococcus garvieae is a widely distributed lactic acid bacteria that is a well-known pathogen of fish, but is also occasionally found as an opportunistic pathogen of humans (201, 202). *L. garvieae* is considered an emerging zoonotic pathogen, and the number of human cases of infection has increased in recent years (203). A recently isolated human strain of *L. garvieae* was found to contain five plasmids, one of which appears to encode putative virulence factors such as toxins and possible surface proteins (204). These proteins may aid the bacteria in adhering to host tissue; future studies should aim to determine if these genes do in fact play a role in *L. garvieae* virulence.

CONCLUDING REMARKS

In general, virulence plasmids are uncommon among nonsporulating Gram-positive pathogens, with notable exceptions in staphylococci and enterococci, as well as the opportunistic pathogens *R. equi* and *L. garvieae*. Staphylococci and enterococci are leading causes of antibiotic resistant, hospital-acquired infections (205). Although these organisms are major causes of nosocomial infection, numerically only a miniscule population of cells is involved in infection, with the vast majority of bacteria occurring in peaceful coexistence with the host as part of the commensal flora. Virulence plasmids may therefore represent “selfish DNA” (206), taking advantage of the otherwise stable association between commensal and host to propagate throughout a population. Alternatively, virulence plasmids may represent a recent acquisition by these species, which at this point has failed to reach a deep penetrance in the population, potentially because of niche sub-specialization and isolation. The occurrence of clonal lineages possessing virulence traits in these species argues for the latter (207, 208), but these two prospects are not mutually exclusive.

Acknowledgments

The authors thank Chris Pillar, Philip Coburn, and Wolfgang Haas for their contributions to the prior edition of this chapter. Portions of this work were funded by NIH grant AI072360, and the Harvard-wide Program on Antibiotic Resistance (NIH grant AI083214). D.V.T. is supported by Grant EY007145 from the National Institutes of Health.

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FIGURE 1.

Plasmid map of pIB485, encoding staphylococcal enterotoxins SED (*sed*) and SEJ (*selj*). Toxin genes are colored red. Genes encoding resistance to cadmium sulfate (*cad*) as well as resistance to beta-lactams (*bla*) are colored dark blue.

FIGURE 2.

(A) Plasmid map of a representative staphylococcal enterotoxin B (ETB)-expressing plasmid, TY825 pETB. The outer circle shows genes that are transcribed clockwise, and genes in the inner circle are transcribed counterclockwise. Genes in red are pathogenic factors; genes in green encode antibiotic resistances; genes in blue are involved in DNA replication, recombination and repair; genes in light blue are transcriptional regulators; genes in purple are transposases; genes in yellow are involved in conjugal transfer; genes in orange encode the BacR1/C55 lantibiotic operon; and genes in grey are conserved ORFs. (B) Structural comparison of TY4 pETB and TY825 pETB plasmids of *S. aureus*. Shading indicates homologous regions. Figure is adapted from (209).

FIGURE 3.

Schematic showing the principal events during pheromone-responsive plasmid transfer between *E. faecalis* cells.

FIGURE 4.

Pheromone-response regulation and virulence factors encoded on plasmid pAD1 (A) Genes encoded by pAD1 that are involved in plasmid transfer and pheromone sensing. Blue genes are involved in replication and maintenance, red genes are negative regulators of pheromone response, and green genes are positive regulators of pheromone response. (B) Detail of the post-segregation killing (PSK) *par* locus of pAD1, which encodes the Fst toxin. (C) Detailed schematic of individual genes within the cytolysin operon.

UNINDUCED: **INDUCED:** **FIGURE 5.**

Regulation of pheromone sensing and plasmid transfer by the enterococcal Tra regulon. Genes encoding surface exclusion protein (*sea*) and aggregation substance (*asaI*) are shown in dark blue. Positive regulators are shown in green, and negative regulators are shown in red. Straight arrows below the genes indicate transcripts detected in the uninduced and induced states, and arrow thickness indicates relative transcript abundance. Figure is adapted from (87) and (117).

FIGURE 6.

Schematic of *E. faecalis* cytolysin expression, post-translational modification, processing and export. (1) Cyl_L and Cyl_S precursor peptides are synthesized, (2) and are intracellularly modified by CylM to create Cyl_L* and Cyl_S*. (3) Cyl_L* and Cyl_S* are secreted and further modified by CylB, resulting in Cyl_L' and Cyl_S', (4) which are cleaved extracellularly by CylA to form the active cytolysin components Cyl_L'' and Cyl_S''. (5) Cyl_L'' and Cyl_S'' are capable of forming aggregates, and are prevented from lysing cytolysin-expressing cells via CylI (6).

FIGURE 7.

Plasmid map of the virulence plasmid p33701 of *R. equi*. Genes believed to be involved in plasmid maintenance and conjugation are shown in red, genes encoding Vaps are shown in blue, and putative genes within the proposed pathogenicity island are depicted in yellow. Figure is adapted from (200).