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CHAPTER 1.2.6

Enterococcus

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Physiology and Genetics—General Introduction

Studies of enterococcal physiology have been conducted, for the most part, on members of two species, *Enterococcus faecalis* and *Enterococcus faecium*, with the exception of a few reports on *Enterococcus hirae*, which in many earlier publications was identified as *E. faecalis* or *E. faecium*. *Enterococcus faecalis*, recognized as a distinct species, is distinguishable from the *E. faecium* group, to which both *E. faecium* and *E. hirae* have been assigned on the basis of 16S rRNA sequences. Physiologically, *E. faecalis* can be distinguished from members of the *E. faecium* group in that the former can produce acid from glycerol, can ferment pyruvate, can utilize menaquinones as non-cytochrome electron carriers, and does not require exogenous folic acid for growth. *Enterococcus hirae* is physiologically distinguishable from *E. faecium* on the basis of the latter's ability to produce acid from L-arabinose (Devriese et al., 1993).

Most enterococcal genetic studies have involved strains of *E. faecalis*, a small number have involved *E. faecium*, and less than a handful have involved other enterococcal species, mostly antibiotic-resistant clinical isolates. Several plasmids of enterococcal origin have been studied in detail, including two types of conjugative plasmids: those that transfer via solid surface matings only and those that respond to *Enterococcus*-specific pheromones. Numerous nontransmissible plasmids, many of which are mobilizable by conjugative plasmids, also have been described, and some have been shown to be composites of two or more smaller plasmids. Several transposons, including Tn*916* (Franke and Clewell, 1981), the first conjugative transposable element to be described, have been identified in enterococcal isolates, and many have received considerable attention.

Until recently, the physiology and genetics of the enterococci have been totally separate fields. However, because of the recent completion of the nucleotide base sequences of the genomes of strains of two enterococcal species, *E. faecalis* (see The Institute for Genomic Research website) and *E. faecium* (see The Department of Energy Joint Genome Institute Web site), it should soon be possible to identify the genetic basis of all enterococcal physiological traits, including their mechanisms of regulation.

Physiology

General Physiological Traits

The enterococci, unlike the streptococci (once considered members of the same genus), are not confined to carbohydrates for their energy needs. In addition to 15 to greater than 30 different carbohydrates, depending on the species, a variety of other substrates may serve as energy sources, e.g., glycerol, lactic acid, malic acid, citric acid, α-keto acids, and the diamino acids (arginine and agmatine). As with the lactic acid bacteria in general, substrate-level phosphorylation is the primary mechanism of ATP generation. They lack most of the enzymes of the tricarboxylic acid (TCA) cycle, as well as typical electron transport chains. Some species, *E. faecalis* in particular, are not totally dependent on substrate-level phosphorylation for the production of energy, but can also produce a proton motive force via electron transport to drive oxidative phosphorylation.

The absence of catalase as well as a typical electron transport chain among the lactic acid bacteria is attributed to their inability to synthesize porphyrins. However, *E. faecalis* synthesizes a number of enzymes that function to rid the cell of toxic H_2O_2. Some strains of *E. faecalis* have been shown to develop catalase-like activities if grown aerobically in the presence of added hemin, and certain strains of *E. faecalis*, but not *E. faecium*, have also been reported to synthesize cytochromes if provided hemin. Despite the inability to synthesize cytochromes in the absence of added hemin, *E. faecalis* is capable of oxidative phosphorylation. Growth yields of *E.*

faecalis are much better when cultures are grown aerobically, rather than anaerobically. Molar ATP yields under aerobic conditions up to twice those obtained under anaerobic conditions (Whittenbury, 1978) have been reported.

The enterococci possess a variety of ATP-dependent, phosphoenolpyruvate (PEP)-dependent, symport and antiport systems for the transport of nutrients, various ions, and catabolic end products through the cellular membrane.

Carbohydrate Metabolism

All species of enterococci, with the exception of a few strains, produce acid from 15 carbohydrates or carbohydrate-containing compounds. These include *N*-acetyl glucosamine, amygdalin, arbutin, cellobiose, D-fructose, galactose, β-gentiobiose, glucose, lactose, maltose, D-mannose, methyl-β-D-glucopyranoside, ribose, salicin, and trehalose. One or more species have also been reported to produce acid from other sugars, or sugar alcohols, such as adonitol, L-arabinose, D-arabitol, L-arabitol, D-glycodextrin, dulcitol, gluconate, glycerol, inulin, 2-ketogluconate, D-lyxose, mannitol, melizitose, melibiose, α-methyl-D-glucoside, D-raffinose, sorbitol, L-sorbose, and xylitol (Devriese et al., 1993). Despite this large number of potential substrates, glucose and, to some extent, gluconate, mannitol and glycerol, have been the only substrates whose metabolism has received any significant attention.

Enterococcus faecalis can express the enzymes of all three of the major carbohydrate catabolic pathways, the Embden-Meyerhof-Parnas (EMP) or glycolytic pathway, the Entner-Doudoroff pathway, and the hexose monophosphate pathway (Sokatch and Gunsalus, 1957). The enterococci very likely ferment sugars primarily, if not exclusively, via the EMP pathway, and the major product of fermentation is lactate. When grown at the expense of glucose, *E. faecalis* also synthesizes at least the oxidative enzymes of the hexose monophosphate pathway, i.e., glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and phosphoketolase. Whether transaldolase is synthesized under the same conditions is not known (Sokatch, 1960). On the basis of results of studies in which labeled glucose was used as the substrate, however, the primary product of glucose fermentation was shown to be lactic acid (Gibbs et al., 1955; Platt and Foster, 1957), suggesting that the EMP is utilized almost exclusively for the metabolism of this substrate. Although lactate is the primary product of glucose fermentation by this enterococcal species, fermentation balances consistently reveal the presence of acetate, formate and ethanol. Production of these three fermentation products increases, at the expense of lactate, under more alkaline growth conditions (Wood, 1961).

The enzymes of the Entner-Doudoroff pathway are induced in the presence of gluconate, but are absent when *E. faecalis* cultures are propagated in the presence of glucose (Wittenberger et al., 1971). Lactic acid dehydrogenase of *E. faecalis* has an absolute requirement for fructose-1,6 *bis*-phosphate for activation, involving a conformational change in the enzyme (Wittenberger and Angelo, 1970). This glycolytic intermediate also regulates the activity of one of two phosphogluconate dehydrogenases synthesized by *E. faecalis*. An nicotinamide adenine dinucleotide phosphate (NADP)-dependent phosphogluconate dehydrogenase is inhibited by fructose-1,-6 *bis*-phosphate, but is unaffected by ATP, whereas a nicotinamide adenine dinucleotide (NAD)-dependent enzyme is insensitive to fructose-1,6-*bis*-phosphate, but is inhibited by ATP (Brown and Wittenberger, 1972).

Carbohydrate Transport—Phosphoenolpyruvate-dependent Phosphotransferase System

The transport of many carbohydrates metabolized by enterococci is mediated by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). These include D-glucose, D-fructose, lactose, maltose, D-mannose, trehalose, sucrose, mannitol and *N*-acetylglucosamine (Huycke, 2002). Although gluconate is transported via a proton motive force (PMF)-driven proton symport mechanism in most Gram-positive bacterial species, this nutrient appears to enter the cell via the PTS in *E. faecalis* (Saier et al., 1993).

Enzyme I of the *E. faecalis* PTS, which is constitutively expressed, has a molecular weight of 70,000, with the native form existing as a dimer of two identical subunits. A single histidine residue is phosphorylated at the expense of PEP (Alpert et al., 1985). Phosphorylated Enzyme I transfers its phosphate to a constitutively expressed histidine-containing protein (HPr), the second phosphocarrier protein of the PTS. Also, HPr has been purified from *E. faecalis* (Deutscher et al., 1986b) and crystallized (Jia et al., 1993). The protein has a molecular weight of 9,438. The structure has been determined at a 1.6 Å resolution, and it has been shown that torsion-angle strain plays a direct role in the functioning of HPr. Like other HPr proteins of Gram-positive bacteria, the *E. faecalis* protein can be phosphorylated at a histidine residue (at position 15) at the expense of phosphorylated Enzyme I, or at serine-46 by an ATP-dependent HPr kinase, the latter abolishing phosphotransfer to sugar-specific Enzyme IIs (Reizer et al., 1989; Heng-

stenberg et al., 1993). The ATP-dependent HPr kinase gene has been cloned from *E. faecalis* (Kravanja et al., 1999) and expressed in *Escherichia coli*. The enzyme has a bifunctional activity, i.e., ATP-dependent HPr kinase activity, expressed at high levels of ATP, as well as a phosphatase activity, hydrolyzing serine 46-phosphorylated HPr (P-Ser-HPr) at low ATP levels. ATP-dependent phosphorylation of HPr by the HPr kinase/phosphatase enzyme is activated by, but does not require the presence of fructose-1,6 *bis*-phosphate. Among Gram-positive bacteria, P-Ser-HPr interacts with a catabolite control protein, CcpA, which in turn binds to operon regulating catabolite response element (CRE) sequences resulting in carbon catabolite repression (CRR). A *ccpA* homolog has been cloned from the chromosome of *E. faecalis* and sequenced (Leboeuf et al., 2000). In addition to being involved in the regulation of carbohydrate metabolism, the PTS is also a major player in the regulation of aerobic glycerol metabolism by the enterococci.

Mannitol is transported among both Gram-positive and Gram-negative bacteria via the PTS, and the mannitol EII/EIII permease components are the only enterococcal sugar-specific PTS components to have been studied in any detail. Generally among lactic acid bacteria, there are two inducible mannitol-specific PTS enzymes involved in the transport and phosphorylation of this carbon and energy source, a soluble Enzyme III^{mtl} ($EIII^{mtl}$) and a membrane-bound Enzyme II^{mtl} (EII^{mtl}). The latter actually is responsible for the transport across the cytoplasmic membrane and phosphorylation of the substrate. The next step in the utilization of mannitol is mannitol-1-phosphate dehydrogenase, which oxidizes the cytoplasmic mannitol phosphate to fructose-6-phosphate, which is further metabolized via the glycolytic pathway. The genes encoding $EIII^{mtl}$ and mannitol-1-phosphate dehydrogenase have been cloned from *E. faecalis* (Fischer et al., 1991). The predicted amino acid sequence of $EII\text{-}I^{mtl}$ was 45% similar to $EIII^{mtl}$ of *Staphylococcus carnosus*, and 41% similar to the $EIII^{mtl}$ domain of the *E. coli* EII^{mtl} enzyme. The predicted amino acid sequence of the *E. faecalis* mannitol-1-phosphate dehydrogenase, when compared to the same enzyme from *S. carnosus* and *E. coli*, was shown to share a high degree of similarity with the respective putative N-terminal NAD^+-binding domains. The mannitol-specific genes of *E. faecalis* appear to be organized in an operon in the order, *mtlA*, *orfX*, *mtlF*, *mtlD*, with *mtlF* and *mtlD*, encoding $EIII^{mtl}$ and mannitol-1-phosphate dehydrogenase, respectively. The predicted protein of the *E. faecalis mtlA* gene shares some similarity to the C-terminal portion of the *E. coli* EII^{mtl}, but this similarity does not include the active center cysteine of the latter.

Pyruvate Metabolism

The final product of the oxidation of any carbohydrate via glycolysis is pyruvate, which in *E. faecalis* may be further metabolized via several enzymes or combinations of enzymes, dependent on environmental conditions. Under anaerobic conditions, the pyruvate is reduced primarily to lactate by lactic dehydrogenase (LDH). The LDH of *E. faecalis*, as well as *E. faecium*, is activated by fructose-1,6-*bis*-phosphate, owing to an alteration in the conformation of LDH, which results in a lowering of its apparent Km for both pyruvate and reduced nicotinamide adenine dinucleotide (NADH; Wittenberger and Angelo, 1970). Even under strictly fermentative conditions, although the enterococci are homofermentative, always some acetate, formate and ethanol are formed, the proportions of which increase with rising pH (Wood, 1961). Following a transition from anaerobic to aerobic conditions, the products of glucose metabolism change from predominantly lactate to acetate and CO_2 (Snoep et al., 1992b). The production of acetate may be the result of the activity of pyruvate formate lyase (PFL), pyruvate dehydrogenase (PDH), or possibly even a third alternative, an uncharacterized PDH-like activity (Yamazaki et al., 1976). The carboxyl of pyruvate may end up in CO_2 via PDH or in formic acid (HCOOH) via PFL. There is no CO_2 formed from HCOOH by enterococci because of the absence of formate-hydrogen lyase enzymes (Lindmark et al., 1969). The PFL of *E. faecalis* has been purified and shown to exchange formate with the carboxyl group of oxalacetate, α-ketoglutarate, and α-ketobutyrate, as well as with pyruvate (Lindmark et al., 1969). The enzyme is unstable in the presence of oxygen, and the only co-enzyme required for its activity is thiamine-pyrophosphate (thiamine-PP). Acetyl-CoA, formed via the activities of either PFL or a PDH, may be converted to acetate or ethanol, dependent on the oxidation reduction (O/R) ratios and energy needs of the cell, which in turn are controlled by environmental conditions and available nutrients and cofactors.

Pyruvate, in addition to being the product of glycolysis, or an intermediate in the metabolism of gluconate via the hexose monophosphate pathway, may also serve as an energy source for the growth of *E. faecalis*, but not *E. faecium* (Deibel and Niven, 1964c). Pyruvate fermentation may occur under both anaerobic and aerobic conditions, and when present as an energy source, lipoic acid is required for growth (Deibel, 1964a). Lipoic acid is an essential cofactor of activity of the PDH complex (Snoep et al., 1993). In addition to being required for growth at the expense of pyruvate as the sole energy source, lipoic acid added to a chemostat culture of *E.*

faecalis growing under limiting glucose + pyruvate concentrations results in a change in fermentation pattern that suggests activation of PDH (Snoep et al., 1993). Under glucose-limited conditions, a change from aerobic to anaerobic conditions results in a change in end products from acetate plus CO_2 to predominantly lactate. Addition of excess glucose to the culture also causes a switch to homolactic fermentation. However, addition of excess pyruvate to the limited glucose chemostat culture results in increases in both PDH and PFL activities (Snoep et al., 1992a). The PDH complex of anaerobically grown *E. faecalis* has been purified and characterized (Snoep et al., 1992b). It was found to resemble PDH complexes of other Gram-positive bacteria and eukaryotes, consisting of four polypeptide chains. Functions assigned to the chains include pyruvate dehydrogenase (E1α and E1β), dihydrolipoyl transacetylase (E2), and lipoamide dehydrogenase. Anaerobic growth of *E. faecalis* on pyruvate involves the activity of LDH, as well as PDH (Snoep et al., 1990). Under these conditions, the pyruvate is functioning as both an electron donor and an electron acceptor. The crystal structure of the 60-subunit dihydrolipoyl acyltransferase core of the *E. faecalis* PDH complex has been determined (Izard et al., 1999).

Alpha Keto Acid Metabolism

A lipoic acid-dependent α-keto acid dehydrogenase complex, separate from PDH, has been described in *E. faecalis*. This complex, designated "BKDH," was purified and shown to catalyze the oxidative decarboxylation of the branched-chain α-keto acids, α-ketoisocaproic acid (KIC), α-ketoisovaleric acid (KIV), and α-keto-β-methylvaleric acid (KMV) to the corresponding branched-chain acyl-CoAs (Rudiger et al., 1972). Subsequently, the *bkd* gene cluster, which encodes BKDH (*bkdDABC*), an acylphosphotransferase (*ptb*), and an acyl kinase (*buk*), was cloned from *E. faecalis* and characterized (Ward et al., 1999). The *bkdD*, *A*, *B* and *C* genes correspond to the E3, E1α, E1β, and E2 polypeptide chains, respectively, by analogy to the PDH complex. The acylphosphotransferase and acyl kinase convert the branched-chain acyl-CoAs to their corresponding free acids, isovalerate, isobutyrate, and methylbutyrate, with the generation of ATP via substrate level phosphorylation. Results of gene knockout studies showed that the *bkd* gene cluster is required for utilization of α-keto acids as energy sources. The *bkd* gene cluster is an operon controlled via a single promoter 5' of the *ptb* gene (Ward et al., 2000). Expression of the operon is inducible by KIC, KIV or KMV, with the latter being the strongest inducer. Expression is inhibited by the presence of carbohydrates metabolized via the EMP, such as glucose, lactose, or fructose, but not by gluconate, which is metabolized via the hexose monophosphate pathway (HMP). Such results suggest that repression of the *bkd* operon is due to phosphorylation of HPr at serine-47, which is dependent on fructose-1-6 *bis*-phosphate. An additional indication that the *bkd* operon is subject to catabolite repression via the PTS is the presence of a putative catabolite response element (CRE) in the promoter region (Ward et al., 2000). The α-ketoacid pathway is functional under both anaerobic and aerobic conditions, but under the former, a suitable electron acceptor, such as fumarate, must be present for the oxidation of NADH. In the absence of such an electron acceptor, the α-ketoacid itself may serve as a temporary electron acceptor, as evidenced by the presence of an extracellular reduced α-ketoacid intermediate during incubation under anaerobic conditions. Under aerobic conditions, oxygen serves as the electron acceptor.

Citrate Metabolism

Citrate utilization is initiated by a C_2-C_4 cleavage to acetate and oxaloacetate, catalyzed by citrate lyase. Acetyl-CoA is not produced in the C_2-C_4 cleavage. Pyruvate is produced by the decarboxylation of oxaloacetate. When citrate is the only source of energy, lipoate is required (Deibel and Niven, 1964c). The lipoate requirement has been interpreted as an indication that the energy-producing step involves the fermentation of pyruvate, possibly via PDH. *Enterococcus faecalis* strain FAIR-E 229, isolated from Cheddar cheese, metabolizes citrate in skim milk but does not utilize citrate if either glucose or lactose is also present in the growth medium (Sarantinopoulos et al., 2001). Thus, citrate is utilized when it is the sole source of energy, yielding acetate and formate as the primary end products, suggesting activity of PFL.

Malate Utilization

Under anaerobic conditions, *E. faecalis* grows slowly at the expense of malate as the sole source of energy (Deibel, 1964a). Growth may be enhanced by the addition of fumarate to an anaerobically growing culture, or by a switch to aerobic conditions, suggesting that either fumarate or oxygen can serve as an electron acceptor for the utilization of malate. A decarboxylating malate:NAD oxidoreductase (malic enzyme) has been purified from *E. faecalis* (London and Meyer, 1969a) and characterized (London and Meyer, 1969b). Synthesis of the enzyme is

unaffected by the presence of glucose; however, its activity is inhibited by fructose-1,6 *bis*-phosphate, 3-phosphoglycerate, and ATP. On the other hand, induction of the malate transport system (malate permease) has been shown to be subject to catabolite repression (London and Meyer, 1970). It has been speculated that malate carbon enters the EMP pathway at the level of pyruvate following decarboxylation (London and Meyer, 1970).

Glycerol Metabolism

Most species of enterococci, including *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. dispar*, are able to ferment glycerol under aerobic or microaerophilic conditions (Gunsalus and Sherman, 1943; Charrier et al., 1997; Huycke, 2002). Glycerol enters the cell via facilitated diffusion under all conditions, and lactate is the final product of its fermentation after entrance into the EMP pathway. However, the route to lactate is different in anaerobic versus aerobic or microaerophilic environments (Jacobs and VanDemark, 1960). Aerobically, glycerol is phosphorylated by glycerol kinase to α-glycerophosphate. Since the glycerol is phosphorylated immediately upon entrance into the cell, a concentration gradient is formed constantly, such that any glycerol in the external environment can continuously enter the cell. The α-glycerophosphate is oxidized to dihydroxyacetone-phosphate via L-α-glycerophosphate oxidase, a flavin adenine dinucleotide (FAD)-linked enzyme. The enzyme has been purified and characterized from a strain of *E. faecium* that produces it in high yield (Esders and Michrina, 1979). The enzyme exists as a dimer and contains FAD as a tightly bound cofactor, at two moles of FAD per mole of enzyme. Fructose-1-phosphate and fructose-6-phosphate, but not glucose-1-phosphate, glucose-6-phosphate, or fructose-1,-6 *bis*-phosphate, are inhibitory. Dihydroxyacetone-phosphate is further oxidized via the bottom half of the EMP pathway to pyruvate. Under aerobic conditions, lactate is the final end product accompanied by an accumulation of hydrogen peroxide.

Among the enterococci, only *E. faecalis* can utilize glycerol as a source of energy under anaerobic conditions (Gunsalus and Sherman, 1943). The first step in the anaerobic utilization of glycerol by *E. faecalis*, following facilitated diffusion into the cell, is oxidation to dihydroxyacetone, a reaction catalyzed by an NAD-linked glycerol dehydrogenase. Dihydroxyacetone kinase then catalyzes the phosphorylation of dihydroxyacetone to dihydroxyacetone-phosphate, which is further oxidized via the bottom half of the HMP pathway. It was noted early (Gunsalus, 1947; Jacobs and VanDemark, 1960) that fumarate can accept electrons from the NADH formed during the anaerobic utilization of glycerol, with the production of succinate. Subsequently, this was shown to involve a simple electron transport chain comprised of glycerol dehydrogenase, a *b*-type cytochrome, and fumarate reductase. In this system, fumarate serves as the terminal electron acceptor, reduced to succinate by fumarate reductase (Gunsalus and Shuster, 1961). Recently, a strain of *E. faecalis*, RKY1, was described that could not ferment glycerol anaerobically unless fumarate was added to the growth medium (Ryu et al., 2001). This strain produced no detectable lactate from the fermentation of glycerol, instead producing succinate, acetate, CO_2 and formate. The strain also produced high amounts of succinate from the fermentation of glucose if grown in the presence of added fumarate.

Aerobic metabolism of glycerol by enterococci is highly regulated at the level of glycerol kinase, as is true for glycerol metabolism among Gram-positive and Gram-negative bacteria, in general. However, whereas allosteric inhibition of glycerol kinase by fructose-1,6 *bis*-phosphate is common among both Gram-positive and Gram-negative microorganisms (Deutscher and Sauerwald, 1986a; deBoer et al., 1986; Liu et al., 1994), as is regulation by the PTS, the actual components of the latter system involved in glycerol kinase regulation differ among the two groups of bacteria (deBoer et al., 1986; Deutscher and Sauerwald, 1986a; van der Vlag et al., 1994). Glycerol kinase is phosphorylated at the N-3 position of a histidyl residue by Enzyme I and HPr of the PTS at the expense of PEP, rendering it ten times more active than the unphosphorylated enzyme (Deutscher and Sauerwald, 1986a). The reaction is reversible in that phosphoglycerol kinase can transfer the phosphate group to HPr, which may occur if a PTS substrate, metabolized in preference to glycerol, is added to the medium, since the addition of such a substrate results in a predominance of the less active unphosphorylated glycerol kinase (Deutscher et al., 1993). The gene (*glpK*) encoding glycerol kinase has been cloned from *E. faecalis* and from *E. casseliflavus* (Charrier et al., 1997). Recombinant enzyme from both species, purified from *E. coli*, was phosphorylated in vitro at the expense of PEP in the presence of Enzyme I and HPr, resulting in a ninefold increase in activity. Both Enzyme I and HPr were required for phosphorylation. The site of phosphorylation on the glycerol kinase from *E. casseliflavus* was identified as His-232. Replacement of this residue by alanine, glutamate, or arginine eliminated the ability of the enzyme to be phosphorylated. The activity of the enzyme purified from

E. casseliflavus was inhibited by fructose-1,6 *bis*-phosphate.

Aerobic Lactate Utilization

A strain of *E. faecium*, with an ability to utilize lactate as a source of carbon and energy, has been described (London, 1968). Aerobic growth on lactate alone is concentration dependent, with a growth yield that is approximately 5% of that obtained with equivalent amounts of glucose. The presence of glucose and lactate in the growth medium results in enhanced growth yields relative to glucose alone. No lactate-specific growth factors could be identified. Lactate could not be utilized for growth under anaerobic conditions, even in the presence of added fumarate. A lactate-oxidizing enzyme, or enzyme system (lactate oxidase), is present in cultures grown aerobically on a number of hexoses, pentoses and trioses but appears to be repressed during growth in the presence of fructose. Lactate oxidase is also repressed during anaerobic growth. The enzyme(s) converts lactate to acetate and CO_2 with the consumption of oxygen. No hydrogen peroxide is produced in the reaction. *Enterococcus faecalis* oxidizes lactate 10–20 times faster in the presence of hematin than in its absence, and this increased rate of lactate oxidation is inhibited by the respiratory uncouplers carbonyl cyanide chlorophenylhydrazone (CCCP) and gramicidin D (Pritchard and Wimpenny, 1978). Such hematin-grown cells contain a membrane-bound cytochrome system (Ritchey and Seeley, 1976).

Amino Acid Catabolism

Only *E. faecalis* among the enterococci can utilize arginine, agmatine, and L-serine as sources of energy for growth (Deibel, 1964a). All enterococci can hydrolyze arginine, and approximately 50% of *E. faecium* strains can hydrolyze agmatine. Although not used as sources of energy, tyrosine and phenylalanine can be decarboxylated by the majority of enterococci.

The complete pathways for the utilization of arginine, the arginine deiminase (ADI) pathway, and the agmatine deiminase (AgDI) pathway, by *E. faecalis* have been elucidated (Simon and Stalon, 1982a; Simon et al., 1982b; Cunin et al., 1986). The pathways are similar, and each requires three enzymes. The first reaction is a deimination of the substrate to citrulline (in the ADI pathway) or carbamoylputrescine (in the AgDI pathway), by a substrate-specific deiminase, each yielding NH_3. The next step is phosphorolysis of citrulline by ornithine carbamoyltransferase, yielding carbamoylphosphate plus ornithine (in the ADI pathway), or of carbamoylputrescine by putrescine carbamoyltransferase, producing carbamoylphosphate plus putrescine (in the AgDI pathway). Finally, there is a carbamate kinase specific for each pathway that catalyzes the transfer of the carbamoylphosphate high-energy phosphate bond to ADP, yielding ATP, CO_2 and NH_3. The net energy yield of each pathway is 1 mole of ATP per mole of substrate. Induction of these enzymes is pathway specific, i.e., enzymes of the ADI pathway are induced only in the presence of arginine, and those of the AgDI pathway are induced only in the presence of agmatine. Whereas synthesis of the ADI pathway enzymes is repressed in the presence of glucose or fumarate, as well as during growth under anaerobic conditions (Simon et al., 1982b), synthesis of the AgDI pathway enzymes is repressed by either glucose or arginine (Simon and Stalon, 1982a).

Arginine enters the *E. faecalis* cell via an energy independent, arginine-inducible, arginine/ornithine antiporter (Poolman et al., 1987). Similarly, agmatine is transported into the cell by an agmatine-inducible agmatine/putrescine antiporter (Driessen et al., 1988). While the former system is specific for monovalent, positively charged amino acids, such as arginine, homoarginine and lysine, the latter appears to be specific for divalent positively charged diamines such as agmatine, and possibly also homoagmatine and cadaverine (Driessen et al., 1988).

Energy Metabolism

The enterococci derive most of their energy when grown anaerobically via substrate level phosphorylation. Sources of ATP are primarily carbohydrates, which are oxidized through the glycolytic pathway, and NAD is regenerated via the reduction of pyruvate to lactate. Growth conditions can alter the amounts of lactate produced, with concomitant changes in the amounts of acetate, ethanol and CO_2 made. Whereas at an acidic pH, the primary product of glycolysis is lactic acid, under alkaline conditions more ethanol, CO_2 and acetate are produced, with concomitantly greater yields of ATP from the production of acetate (Gunsalus and Niven, 1942; Graham and Lund, 1983). Unlike the streptococci, which rely exclusively on carbohydrate fermentation for their energy needs, the enterococci may generate ATP through the metabolism of a number of other substrates. The ATP produced by substrate-level phosphorylation is hydrolyzed by F_0F_1-ATPase to produce a proton potential for maintenance of cytoplasmic pH and for a variety of proton-coupled transport reactions. The maximum size of the proton potential that is generated is generally between -130 and -150 mV, while a potential of at least -200 mV is required for the reverse reaction, i.e., the synthesis of ATP by the F_0F_1-ATPase (Kakinuma, 1998). Thus,

among the enterococci, as is true of nonrespiratory bacteria, this ATPase does not appear to be a major contributor of cellular ATP.

The F_0F_1-ATPase of *E. hirae* was the first of the bacterial ATPases to be described (Abrams et al., 1960). The primary physiological role of this enzyme in enterococci appears to be the maintenance of cytoplasmic pH under acid environmental conditions. It also provides proton potential for proton-linked secondary transport systems. The operon encoding F_0F_1-ATPase activity has been cloned and sequenced (Shibata et al., 1992), and although the enzyme itself appears evolutionarily close to that of *Bacillus megaterium*, the order of genes appeared to be identical to that found in the corresponding operon of *E. coli* and nearly identical to that in *Streptococcus mutans*. The enzyme has an optimal activity at pH 6.0–6.5, has minimal activity at alkaline pHs, and unlike many other bacterial proton ATPases, is not inhibited by azide (Kakinuma, 1998). Not only is the activity of the enzyme optimized at acid pH, but also the amount of enzyme appears to increase. This was shown not to be due to increased transcription or to the synthesis of new enzyme subunits, but rather to an increase in the amounts of functional enzyme assembled on the cell membrane, suggesting that regulation is primarily post-translational. *Enterococcus hirae* was shown to adapt to acid stress within a single generation in continuous culture, but de-adaptation occurred over several generations (Belli and Marquis, 1991).

Proton motive force can be generated in *E. faecalis* by excretion of lactic acid, the major end product of fermentation. Synthesis of ATP at the expense of this PMF was demonstrated with inside-out membrane vesicles. An influx of lactic acid into the inside-out vesicles resulted in the synthesis of ATP outside of the vesicles, which was inhibited by CCCP and by *N*-4, *N*-1-dicyclohexylcarbodiimide (DCCD; Simpson et al., 1983). However, more recent evidence indicates that in *E. faecalis* the concentration of free cytoplasmic lactic acid is balanced precisely with the proton electrochemical potential gradient over a wide range of pHs. At high external pH, there is a pool of tightly bound intracellular lactate. These data would suggest that transport of lactate to the outside of the cell is not an ATP-yielding event (Hockings and Rogers, 1997).

The first evidence for oxidative phosphorylation by enterococci was obtained when it was shown that the ATP could be synthesized via the oxidation of NADH by cell-free extracts of aerobically grown *E. faecalis* (Gallin and VanDemark, 1964). Subsequently, it was proposed that oxidative phosphorylation occurred in this species on the basis of molar growth yields (Smalley et al., 1968). From the demonstration of flavins and naphthoquinones in this species (Baum and Dolin, 1963), it was postulated that oxidative phosphorylation would be at the level of NADH/flavin or via the oxidation of reduced naphthoquinone (Gallin and VanDemark, 1964). Earlier, the presence of an FAD-dependent NADH oxidase had been detected in extracts of aerobically grown *E. faecalis* that catalyzed the 4-electron reduction of oxygen to water (Hoskins et al., 1962). The NADH oxidase was purified (Schmidt et al., 1986; Ahmed and Claiborne, 1989), and it was shown that this enzyme was more closely related to flavoprotein NADH peroxidase of *E. faecalis* than to the flavoprotein monooxygenases, which also catalyze the four-electron reduction of oxygen (Ahmed and Claiborne, 1989).

Although the enterococci do not synthesize cytochromes because they cannot synthesize porphyrins, and were generally considered to be anaerobes, they do synthesize cytochromes if provided with hemin (Whittenbury, 1964). A naphthoquinone was isolated from a strain of *E. faecalis* and identified as 2-solanesyl-1,4-naphthoquinone, or demethylmenaquinone (Baum and Dolin, 1965). The naphthoquinone content of aerobically grown *E. faecalis* was approximately 1.6 times that of anaerobically grown cells. The presence of demethylmenaquinone in *E. faecalis* has also been demonstrated in *E. casseliflavus* and *E. galinarum*, but not in either *E. faecium* or *E. durans*. In addition to the demonstration that *E. faecalis* could make cytochromes in the presence of added hemin, it was also shown that oxygen could serve as an electron acceptor, allowing for higher growth yields and the synthesis of more ATP from glucose under aerobic conditions (Ritchey and Seeley, 1974). Subsequently, it also was shown that most strains of *E. faecalis* grown in the presence of hemin contained cytochrome-like respiratory systems, and it was proposed that they would have cytochrome-like NADH oxidase (Ritchey and Seeley, 1976). Strains of *E. faecium*, however, did not contain any cytochrome-like respiratory systems. The ability of *E. faecalis* to synthesize, in the presence of hematin, a functional cytochrome system in the cellular membrane was confirmed (Pritchard and Wimpenny, 1978). Membrane preparations from cells grown in the presence of hematin had higher levels of NADH oxidase activity, a *b*-type cytochrome, and two possible oxidase components (a cytochrome d_{630} and a CO-binding cytochrome). Evidence was also provided that the transport of electrons to oxygen by the hematin-dependent cytochrome system is coupled to proton translocation. A gene cluster has been identified in the genome sequence of *E. faecalis*, which shares similarity to the cytochrome *bd*-type respiratory oxidase operon, *cydABCD* of

Bacillus subtilis (Winstedt et al., 2000). Membranes from *E. faecalis* cells grown aerobically in the presence of hemin had absorption difference spectra characteristic of cytochrome *bd*. The *cydABCD* gene cluster of *E. faecalis* was cloned and expressed in *B. subtilis*, and the recombinant *B. subtilis* clone formed a spectroscopically detectable cytochrome *bd*. Furthermore, the cloned *cydABCD* gene cluster was shown to complement a cytochrome *bd*-deficient mutant of *B. subtilis*.

Fumarate appears to be the only electron acceptor available to enterococci for anaerobic respiration. The presence of fumarate reductase in *E. faecalis* has been reported (Deibel and Kvetkas, 1964b), which in addition to its constitutive expression by *E. faecalis* may be present in some strains of *E. faecium* (Aue and Deibel, 1967). Under anaerobic conditions, if fumarate reductase is present, greater amounts of acetate are produced, with concomitantly greater yields of ATP. Excess electrons, present because of the production of acetate rather than lactate, are transferred by NADH dehydrogenase to fumarate by fumarate reductase (Kakinuma, 1998).

Cation Transport

Cations are required for the activities of numerous enzymes, yet these ions are also toxic to cells if present at too high a concentration. Thus, all types of cells contain machinery necessary to regulate intracellular levels of cations, e.g., potassium and sodium, which are maintained at higher and lower levels, respectively, than are generally present in the environment. Many ion transport systems make use of the cell's proton potential for their activity. Since the enterococci lack respiratory chains, they are able to generate proton potential only via the hydrolysis of ATP, a reaction mediated by the proton-translocating enzyme, F_0F_1-ATPase. Consequently, for the enterococci, ATP plays a much more important role in the transport of ions through their membranes than is true of respiring bacterial genera. These microorganisms do synthesize, however, both proton- and ATP-dependent cation transport systems.

Sodium Transport Like the F_0F_1-ATPase of *E. hirae*, the first confirmed presence of a Na^+/H^+ antiporter was also provided through research on this microorganism (Kakinuma, 1987). The gene encoding this antiporter, *napA*, has been cloned (Waser et al., 1992), and its gene product, NapA, was shown to recognize Li^+, as well as Na^+, as a substrate (Strausak and Solioz, 1994). The presence of either cation in the environment will serve as a transcriptional inducer. A second mechanism of sodium extrusion involving a Na^+-ATPase has also been described in *E. hirae* (Heefner and Harold, 1982). This sodium pump belongs to a subcategory (V-ATPases) of ion-translocating ATPases, which do not form enzyme-phosphate intermediates, as do the P-ATPases. This category encompasses the F-ATPases, i.e., F_0F_1-ATPases and the V-ATPases, a term derived from the proton pumps of acidic organelles, such as the vacuoles of fungi and plants, and the endosomes of animal cells. Unlike the F_0F_1-ATPase of *E. hirae*, the Na^+-ATPase exhibits maximal activity at pH 8.5–9.0, but exhibits no detectable activity at pH 6.0. It is stimulated by either Na^+ or Li^+, but catalytic activity absolutely requires Na^+ (Kakinuma, 1998). The V_0V_1-ATPase is encoded by the *ntpFIKECGABDHJ* operon (Murata et al., 1997), and all but *ntpH* and *ntpJ* are required for expression of the ATPase (Kakinuma et al., 1999b). The operon is induced by high intracellular concentrations of Na^+, by high pH, and when NapA is nonfunctional (Murata et al., 1996). A gene, *ntpR*, transcribed in the opposite orientation of the *ntp* operon, was originally thought to be involved in the regulation of that operon. However, interruption of *ntpR* had no effects on expression of the *ntp* operon. On the other hand, results from deletion studies indicated that an AT-rich track of DNA between -198 and -132 of the *ntp* operon is required for operon transcription. It has been speculated that this region is the binding site for a *trans*-activating protein involved in *ntp* transcription (Yasumura et al., 2002).

Potassium Transport Two systems involved in the uptake of potassium by *E. hirai*, KtrI and KtrII, as well as a potassium extrusion system, Kep, have been described. KtrI is the major K^+ uptake system under most conditions and requires both proton motive force and a high energy compound such as ATP. The system appears to be synthesized constitutively, and in addition to K^+, also recognizes Rb^+ (Kakinuma, 1998). First described in 1980 (Bakker and Harold, 1980), the KtrI system probably involves a K^+/H^+ symport, although this has yet to be demonstrated directly and is regulated by an ATP-dependent modification. It is active under neutral and acidic conditions, but inactive at alkaline pHs. The KtrII system requires neither ATP nor a membrane potential (Kobayashi, 1982), nor is it inducible by insufficient K^+ or repressed by excess K^+. It appears to be induced in the presence of excess intracellular Na^+ and requires the integral protein encoded by *ntpJ*, the last gene of the Na^+-ATPase-encoding *ntp* operon (Kakinuma et al., 1999b).

The Kep K^+/H^+ antiporter exports potassium ions against a concentration gradient in exchange for protons. The system is constitutively expressed, but functions only under

alkaline conditions (Kakinuma and Igarashi, 1999a).

More recently, an energy-dependent potassium uptake system with a low affinity for potassium was described that functions at pH 10.0 (Kawano et al., 2001). This system was discovered in a mutant of *E. hirae*, defective in both KtrI and KtrII, that did not grow at pH 10 in the presence of low concentrations of potassium (<1 mM) but grew well in the presence of 10 mM KCl.

Calcium and Iron Transport Calcium is extruded from *E. hirae* by a primary active transport mechanism that can establish a 30:1 out/in gradient (Kobayashi et al., 1978). The system requires ATP, but apparently functions in the absence of proton potential. To date, no calcium-specific uptake system has been described for the enterococci.

Virtually no work has been done on mechanisms of iron transport among the enterococci, although siderophores have been detected in members of this genus. In one study (Lisiecki et al., 2000), 70 strains representing 16 enterococcal species were shown to have linear trihydroxamate or citrate hydroxamate siderophores. In another study, 6 of 6 *E. faecium* and 2 of 6 *E. faecalis* strains were able to utilize siderophores provided by Gram-negative bacterial species, and to some extent, siderophores from other Gram-positive bacterial species (Szarapinska-Kwaszewska and Mikucki, 2001). Enterococci clearly can utilize iron from numerous environmental sources, since strains of several species were shown to grow under conditions of iron depletion in vitro if provided with such sources of iron as bovine hemoglobin, hemin, lactoferrin, transferrin, ovotransferrin, horse myoglobin, ferritin, or cytochrome *c* (Sobis-Glinkowska et al., 2001).

Copper Transport *Enterococcus hirae* (ATCC9790) encodes an operon, *cop*, the products of which maintain copper homeostasis. The operon consists of four genes. The *copA* and *copB* genes encode P-type ATPases, CopA and CopB, whereas *copY* and *copZ* encode a copper-responsive repressor, CopY, and a copper chaperone, CopZ (Wunderli-Ye and Solioz, 1999). Cop A is responsible for copper uptake under conditions of copper limitation. CopB is an extrusion ATPase that exports excess copper from the cytoplasm, rendering the cell copper-resistant. Regulation of the *cop* operon is biphasic, with low-level induction expressed at copper concentrations of 10 μ M and maximum induction at concentrations of 2 mM. Maximum induction is also seen in the presence of μ M concentrations of the fortuitous inducers, Ag^+ and Cd^+. Mutations in *copY* result in constitutive expression of the *cop* operon, suggesting that CopY is a repressor. CopZ is a small (69-aa [amino acid]) protein and a member of a family of copper chaperones that includes MerP (a mercury binding protein), ATX1 (a yeast copper chaperone), and HAH1 (a human copper chaperone).

Oxygen Metabolism by Enterococci

The enterococci produce toxic H_2O_2 and/or superoxide, O_2^- under a variety of environmental and nutritional conditions (Falcioni et al., 1981; Pugh and Knowles, 1983; Huycke et al., 1996; Winters et al., 1998). These bacteria can synthesize several enzymes that remove such toxic metabolic biproducts. *Enterococcus faecalis* may synthesize a flavoprotein NADH peroxidase, as well as a heme-containing catalase, when grown aerobically. The latter also requires the addition of hematin to the growth medium (Pugh and Knowles, 1983). More recently, a chromosomal gene, *katA*, was identified in the sequenced chromosome of *E. faecalis* strain V583, and the purified enzyme synthesized by this strain was shown to belong to a family of monofunctional catalases (Frankenberg et al., 2002). *Enterococcus faecalis* produces an NADH oxidase that catalyzes a four-electron reduction of oxygen to water without formation of any O_2^- or H_2O_2 (Schmidt et al., 1986), as well as an oxygen-inducible superoxide dismutase (Britton et al., 1978).

Genetics

Introduction

The beginnings of enterococcal genetics can be traced to the first report of an association of antibiotic resistance with plasmid DNA in an isolate of *E. faecalis* (Courvalin et al., 1972). This was followed by a description of high frequency conjugative transfer of a hemolysin/bacteriocin trait from one strain of *E. faecalis* to another (Tomura et al., 1973), although no evidence for the involvement of plasmid DNA or any type of transmissible genetic element was presented. By the following year, resistance of a strain of *E. faecalis* to erythromycin and tetracycline was correlated with the presence of two specific plasmids (Courvalin et al., 1974), and the first definitive description of plasmid-mediated antibiotic resistance transfer between strains of *E. faecalis* by a mechanism that resembled conjugation appeared (Jacob and Hobbs, 1974). Prior to these studies, there had been only one unconfirmed report of gene transfer in this genus (Raycroft and Zimmerman, 1964). From the mid-1970s onward, interest in the genetics of the enterococci has continued to intensify, to the extent

that there have been six American Society for Microbiology (ASM)-sponsored international conferences on streptococcal genetics, major sessions of which have been devoted to the enterococci. Papers describing many of the presentations from five of the six conferences have been published (Schlessinger, 1982; Ferretti and Curtiss, 1987; Dunny et al., 1991b; Ferretti et al., 1995; Yother et al., 2002). More recently, four chapters in a book on the genus, *Enterococcus* (Gilmore et al., 2002), were devoted to different aspects of enterococcal genetics.

To date, there still are no reports of transduction in this genus, and natural competence for transformation has never been observed among enterococci. However, numerous plasmids have been shown to occur naturally in members of this genus, and two types of plasmid-mediated conjugation have been described, one of which is unique and apparently specific to enterococci. Plasmid replication and maintenance functions have been examined in some of these plasmids, as have the antibiotic resistance and virulence traits they encode. A number of transposable elements, including conjugative transposons, have been described. Finally, the sequencing of the genomes of two enterococcal species, *E. faecalis* (see The Institute for Genomic Research Web site) and *E. faecium* (see The Department of Energy Joint Genome Institute Web site), make possible the association of individual genes with specific functions, as well as the discovery of new genes whose functions remain to be determined.

Plasmids

A large number of plasmids, ranging in size from <5 to >100 kb, have been detected in and/or isolated from strains of *Enterococcus* spp., mostly *E. faecalis* (Clewell, 1981; Showsh et al., 2001). Enterococcal isolates may harbor as many as five or more distinct plasmid species (Clewell, 1981; Atkinson et al., 1997). Plasmids replicate via two basic mechanisms, rolling circle replication (RCR) and θ-replication (Janniere et al., 1993). On the basis of the ubiquity of RCR plasmids among Gram-positive bacterial species in general (Gruss and Ehrlich, 1989; Khan, 1997), one might expect that many of the smaller plasmids present in members of the genus *Enterococcus* would replicate via a rolling circle mechanism. As yet, no naturally occurring RCR plasmid has been described in enterococci. However, the tetracycline resistance plasmid, pAMα 1, originally isolated from *E. faecalis* strain DS5 (Clewell et al., 1974), was shown to be a composite of two replicons, one of which, pAMα 1Δ 1, is identical to the 4.6-kb RCR plasmid, pBC16, from *Bacillus cereus* (Gruss and Erlich, 1989). The conjugative plasmid, pJH1 from *E. faecalis* strain JH1 (Jacob and Hobbs, 1974), was subsequently shown to be a composite plasmid consisting of at least two replicons, one of which was highly related to pAMα 1Δ 1 (Banai et al., 1985; Perkins and Youngman, 1983). Thus, although RCR plasmids have not been shown to exist as independent replicons in enterococci, they can in fact replicate quite stably in members of this genus, as evidenced by the number of RCR-based vector molecules that have been constructed and used for recombinant DNA studies in enterococci (Weaver et al., 2002). Two types of -replicating plasmids are common among the enterococci, pAMβ 1-like molecules, the so-called "Inc18 plasmids" (Janniere et al., 1993), and the pheromone-responsive plasmids (Clewell, 1999).

INC18 PLASMID REPLICATION pAMβ 1 was first isolated from *E. faecalis* strain DS5, which was also the source of two other well-studied enterococcal plasmids, pAMα 1 and pAD1 (Clewell et al., 1974). pAMβ 1 is closely related to several plasmids originally isolated from various species of *Streptococcus* and a number of plasmids from enterococcal isolates (Janniere et al., 1993). These plasmids range in size from about 25 to 30 kb, with copy numbers that range from 10 to 15 per chromosome equivalent. pAMβ 1 mediates erythromycin resistance (Clewell et al., 1974) due to the presence of an *ermB* determinant (Roberts et al., 1999) encoding resistance to the MLS group of antibiotics (macrolides, lincosamides, and streptogramin B). It was used to demonstrate the transformability by plasmid DNA of naturally competent strains of streptococci (LeBlanc and Hassell, 1976; LeBlanc et al., 1978a), and it was also shown to transfer to, and replicate in, several species of streptococci, if donor and recipient cells were forced together on a solid surface (LeBlanc et al., 1978b). Subsequently, pAMβ 1 and related plasmids have been shown to possess a very broad host range, i.e., the ability to transfer and replicate in a large number of Gram-positive bacterial species (Clewell, 1981; Horaud et al., 1985).

The replication of the Inc18 plasmids has received considerable attention (Bruand et al., 1993; Bruand and Ehrlich, 1998). Early studies narrowed the region of the 26.5-kb plasmid, pAMβ 1, required for replication to less than 3 kb (LeBlanc and Lee, 1984). It has since been shown to replicate via a unique type of mechanism (Bruand et al., 1993; Janniere et al., 1993). A transcript is synthesized that begins at the promoter of the initiation gene, *repE*, and proceeds through the replication origin. The transcript is processed, possibly by an RNase function of the RepE protein, creating a 10-nucleotide primer for the host DNA Pol I at the 3′ end of the

original transcript, and the 5′ end is released. Replication by Pol I continues for approximately 150 bp where an *ssiA* site is exposed for priming of the lagging strand via the product of *priA*. At this point, Pol I is replaced by DNA Pol III for continued synthesis of both the lagging and leading strands in the direction away from *repE*. RepE functions as the replication initiator by binding to double-stranded pAMβ 1 at the origin of replication, which causes bending and melting, resulting in a single-strand bubble at the origin. RepE also possesses strong, nonspecific single-strand binding activity that leads to the binding of additional RepE molecules at the bubble, which in turn results in the extension and stabilization of the single-strand region at the origin (LeChatelier et al., 2001). Also, RepE is both essential and rate-limiting for replication of pAMβ 1. Its synthesis is very tightly regulated by two mechanisms, negatively by a repressor protein, CopF, encoded 5′ of *repE*, and by countertranscript-driven transcriptional attenuation mediated by an antisense RNA molecule transcribed from the opposite strand at the 5' end of *rep*, which causes termination of *rep* transcription (LeChatelier et al., 1996). Inc18 plasmids encode resolvase/invertase proteins (RIs), which appear to play a role in plasmid replication, partition or both. Such a protein, designated "Resβ," is encoded by pAMβ 1, and is involved in plasmid partition through its ability to convert dimers or higher oligomeric forms to monomers. Resβ binds to a target on the plasmid that is located approximately 200 bp 3′ of the origin of replication. When bound to the target, it functions as a roadblock preventing continued elongation by Pol I. Consequently, a replication intermediate is formed consisting of a 200-bp D-loop which, as a single-stranded form, contains a primosome assembly site, *ssiA*, at which the DNA primase, DNA helicase, and Pol III are loaded for continued replication (Alonso et al., 1996).

Pheromone-Responsive Plasmid Replication

The pheromone-responsive plasmids are a group of enterococcal replicons that are self-transmissible at high frequency in broth only to other enterococci (Wirth, 1994; Dunny and Leonard, 1997; Clewell, 1999). The pheromone-responsive plasmids described to date range in size from 37 to 91 kb, are maintained at very low copy numbers (2–4 per chromosome equivalent), and with one exception, pHKK100 from *E. faecium* (Handwerger et al., 1990), have all been detected in isolates of *E. faecalis*. The first of these plasmids to be described was pAD1 (Dunny et al., 1978), and it, together with pCF10 (Dunny et al., 1981), has been studied in the greatest detail. The basic replicons of pAD1 (Weaver et al., 1993), pCF10 (Hedberg et al., 1996), pPD1 (Fujimoto et al., 1995), and pAM373 (De Boever et al., 2000) have been identified. Equivalent genes have been identified in the basic replicons of all four plasmids with the designations for pAD1 being *repA*, *repB*, *repC* and *par* (different designations have been assigned to the corresponding homologs of pCF10; Hedberg et al., 1996), with iterons located on either side of the *repBC* operon. The *repA* gene encodes a replication initiator protein that falls within a family of Gram-positive bacterial plasmid initiator proteins (Hirt et al., 1996; Berg et al., 1998; Kearney et al., 2000). Within the central region of *repA* is a repeat structure that serves as the replication origin of pAD1 (Weaver et al., 2002), as well as other plasmids of Gram-positive bacteria (Gering et al., 1996; Tanaka and Ogura, 1998). This same region of pAD1 also functions as the origin of pheromone-mediated transfer of pAD1 (An and Clewell, 1997).

Transposon insertions in *repB* resulted in elevated copy numbers of pAD1 (Ike and Clewell, 1984), suggesting that this gene may be involved in maintenance of plasmid copy numbers. However, the likelihood that *repBC* is an operon would suggest that this result could also be due to a polar effect on *repC*. The general structure of *repBC* and its location between repeat sequences would suggest a relationship to plasmid *par* loci associated with plasmid partitioning (Gerdes et al., 2000).

The function of *par* in pAD1, and to a lesser extent pCF110, has been studied in some detail (Weaver et al., 1994; Weaver et al., 1996; Weaver et al., 1998). The *par* locus is not required for plasmid replication, but functions to insure stable inheritance of the plasmid via a post-segregational killing (PSK) mechanism. The system consists of two RNA transcripts that are synthesized convergently from opposite ends of the *par* locus and terminate at the same bidirectional transcriptional terminator, so that they have at their respective 3′ ends, complementary stem-loops. Each transcript also has the same repeat sequence at its 5′ end, such that the shorter transcript, RNA II (~145 nucleotides), is totally complementary to the longer transcript, RNA I (~250 nucleotides), but only at the 5′ and 3′ ends of the latter. Within RNA I is a gene, *fst*, that encodes a 33-aa peptide, Fst. Translation of *fst* from the RNA I transcript is normally inhibited in a pAD1-containing cell owing to the binding of the antisense molecule, RNA II, to RNA I at its 5′ end, which results in an inhibition of ribosome binding. Both RNA I and RNA II are transcribed by pAD1-containing cells of *E. faecalis*, such that Fst is never present. However, if cells in a population lose the plasmid, *fst* can be translated in plasmid-free cells because RNA II has a much shorter half-life than RNA I. Synthesis of Fst, which is an enterococcal toxin,

results in cell killing. Normally, in plasmid-containing cells, RNA II functions as an antitoxin by preventing the synthesis of the toxin. Recent data suggest that Fst compromises the integrity of the *E. faecalis* cell membrane, which is accompanied by cessation of all macromolecular synthesis and cell growth (Weaver et al., 2003).

Transposons

Tn3 Family of Transposons The first transposon identified in any enterococcal isolate was Tn*917*, a 5.4-kb erythromycin resistance element present on pAD2, one of two plasmids harbored by *E. faecalis* DS16 (Tomich et al., 1980). Both transposition and resistance were shown to be inducible by erythromycin, with relocation to pAD1 being increased by an order of magnitude by the presence of the antibiotic. A second transposon, Tn*3871*, very similar to Tn*917*, was identified on pJH1 from *E. faecalis* strain JH1 (Banai and LeBlanc, 1984), and Tn*917*-like elements were subsequently shown to have been disseminated among enterococcal isolates of both human and animal origin (Rollins et al., 1985). Results of studies of Tn*917* in *Bacillus subtilis* (Perkins and Youngman, 1984), as well as analyses of its nucleotide base sequence, and the predicted amino acid sequences of six identified open reading frames (ORFs; Shaw and Clewell, 1985), placed this enterococcal transposon in the Tn*3* family of transposons (Lett, 1988). Another enterococcal transposon belonging to the Tn*3* family is Tn*1546*, a 10.9-kb element encoding resistance to vancomycin, isolated from a strain of *E. faecium* (Arthur et al., 1993). Results from a recent study (Willems et al., 1999) have shown that Tn*1546* has undergone a large number of changes in vancomycin resistant enterococci (VRE). In this study, the *vanA*-encoding transposons present in 97 enterococcal isolates of human and animal origin were examined. A total of 22 different Tn*1546*-like elements were identified containing point mutations, insertions of IS elements, and deletions. Most of the transposons (76%) contained 1–3 copies of IS*1216V*. Insertions of IS*1251*, and of an IS*1216V*-IS*3*-like element were also detected.

Composite Transposons A type of transposon commonly found in enterococci is the composite transposon (Snyder and Champness, 1997). These transposons are characterized by the bracketing of genes on the host chromosome, a plasmid, or a pre-existing transposon, by two IS elements of the same type, thereby forming a larger transposon. The first composite transposon to be described in an enterococcal isolate was Tn*5281*, present on a conjugative plasmid, pBEM10, from a gentamicin-resistant strain of *E. faecalis* (Hodel-Christian and Murray, 1991). Tn*5281* is very similar, if not identical, to the 4.7-kb staphylococcal gentamicin-resistance transposons, Tn*4001* and Tn*4031*, and contains at its ends the IS element, IS*256*. All of these transposons carry the bifunctional *aacA-aphD* gene (Ferretti et al., 1986) that mediates resistance to gentamicin, as well as other aminoglycoside antibiotics. Not all gentamicin resistant enterococci that encode *aacA-aphD* carry this gene on Tn*5281* (Hodel-Christian and Murray, 1992). Although three isolates from two different countries were shown to contain Tn*5281*-like transposons, two other isolates were shown to contain a transposon more like a Tn*4001* hybrid transposon with three copies of IS*257*, and which is no longer mobile. The *aacA-aphD* determinant of yet a fifth strain could not be associated with either type of transposon. A much larger gentamicin-resistance composite transposon (27 kb) designated "Tn*924*," was detected in an *E. faecalis* clinical isolate from Canada (Thal et al., 1994). This transposon was flanked by IS*257*-like elements.

IS*256* appears to be common among enterococci, even strains that are susceptible to gentamicin. In a study of more than 100 clinical enterococcal isolates (85 *E. faecium* and 15 *E. faecalis*), 85% were shown to contain copies of IS*256* (Rice and Thorisdottir, 1994). Nearly half of the isolates examined were susceptible to gentamicin, and these strains contained between one and six copies of Tn*256*. All but two of the Tn*256*-containing isolates were resistant to one or more of the antibiotics tested, which included, in addition to gentamicin, ampicillin, streptomycin and vancomycin. The presence of multiple copies of an IS element in enterococci has been demonstrated, and this situation could result in the formation of composite transposons encoding multiple antibiotic resistance (Rice et al., 1995). A strain of *E. faecalis* was shown to carry a 26-kb composite transposon comprised of a Tn*4001*-like element flanked by IS*256*, as well as an erythromycin-resistance determinant flanked by one of the former IS*256* sequences, plus a third copy of this IS element. Even larger multiple antibiotic-resistance composite transposons have been described. For instance, Tn*5385* is a 65-kb genetic element detected in the chromosome of an *E. faecalis* clinical isolate (Rice and Carias, 1998a). It contains genes encoding resistance to gentamicin, erythromycin, streptomycin, tetracycline/minocycline, penicillin (due to β-lactamase activity), and resistance to mercury, and is comprised of at least four previously described transposons, two of enterococcal origin (Tn*5381* and Tn*5385*) and two of staphylococcal origin (Tn*4001* and Tn*552*). These transposons are linked by copies of IS*256*, 20019 IS*257* and

IS*1216*; copies of the latter have been shown to be present at the ends of Tn*5385*.

Tn1*547* is a somewhat unique 64-kb element identified in a strain of *E. faecalis*, which encodes the entire *vanB* gene cluster, and has two different though distantly related IS elements at its ends (IS*256* and IS*16*; Quintiliani and Courvalin, 1996). Despite the presence of different IS elements, Tn*1547* was mobile.

Conjugative Transposons The transfer of tetracycline resistance from one strain of *E. faecalis* to another via a mechanism that required cell-to-cell contact and could occur in the absence of plasmid DNA was described for the first time in 1981. Transfer of the tetracycline-resistance element, as well as its integration into chromosomal or plasmid DNA was *recA*-independent. The conjugative transposon responsible for both transfer and transposition was designated "Tn*916*" (Franke and Clewell, 1981). Subsequently, conjugative transposons have been discovered in both Gram-positive and Gram-negative bacterial genera, and numerous reviews have appeared in the literature. A partial list is included here (Clewell and Flannagan, 1993c; Scott, 1993; Salyers et al., 1995; Scott and Churchward, 1995; Lyras and Rood, 1997; Smith et al., 1998; Churchward, 2002). Transfer-specific aspects of Tn*916* will be covered elsewhere in this chapter, and only those functions associated with transposition will be discussed here. Tn*5* insertional mutagenesis was used to decipher the genetic organization of the transposon, permitted a separation of conjugative transposition from intracellular transposition, and identified regions of the element associated with excision, which is required for both conjugation and transposition (Senghas et al., 1988). It was discovered that Tn*916* forms a covalently closed intermediate structure upon excision from a host genome or plasmid (Scott et al., 1988). Excision and covalently closed circular intermediate formation occurred in *E. coli* following its cloning on an *E. coli* plasmid vector. Intermediate structures purified from *E. coli* could be used to transform *Bacillus subtilis*, and these transformants were able to donate Tn*916* to a strain of *Streptococcus pyogenes*. Excision of Tn*916* includes noncomplementary base pairs at each end of the transposon that form a heteroduplex at the joint of the circular intermediate. When the transposon inserts into a new target, those base pairs are inserted into the target DNA by a reversal of the excision process (Caparon and Scott, 1989). The complete sequence of Tn*916* was published in 1994, which confirmed a size of just over 18 kb and permitted the identification of 24 ORFs (Flannagan et al., 1994). Previously identified excision, *xis*, and integration, *int*, loci were assigned to specific ORFs, and one ORF was shown to be required for conjugation.

The Int protein of Tn*916* contains two DNA binding domains, one at its C-terminus, which binds to the ends of the transposon, as well as to the target DNA, and one at its N-terminus, which binds to sequences internal to the transposon, but close to each end (Lu and Churchward, 1994). It was postulated that the existence of the two DNA binding sites on Int provided a mechanism for insuring that only correctly aligned transposons and target molecules would undergo recombination. Results of additional studies showed that the C-terminal DNA binding domain of Int exhibited different binding affinities for different target sites, and that these affinities correlated with observed frequencies of transposon insertion at those sites (Lu and Churchward, 1995). Xis protein also binds near the ends of Tn*916*, close to direct repeat sequences associated with Int binding (Rudy et al., 1997). Both Int and Xis are required for Tn*916* excision (Marra and Scott, 1999). The Int protein is involved in both strand cleavage and joining reactions and is also essential for transposon integration (Jaworski et al., 1996).

Conjugation

The transfer of chloramphenicol resistance between strains of *E. faecalis*, by a mechanism that required cell-to-cell contact, was first reported in 1964 (Raycroft and Zimmerman, 1964). However, the donor strain was a chloramphenicol-resistant mutant of *E. faecalis* and was thought to transfer this mutant phenotype to recipient cells. No conjugative genetic element was ever identified, and the results were never confirmed in the literature. Approximately nine years later, a paper was published that reported the high frequency conjugative transfer between enterococci and the ability to produce bacteriocin (Tomura et al., 1973). Although the genetic element responsible for transfer was not identified, the transfer of this type of lytic activity from strains of *E. faecalis* has been confirmed numerous times since. The following year, plasmid-mediated, multiple antibiotic-resistance transfer between strains of *E. faecalis* at high frequency in broth culture was clearly documented (Jacob and Hobbs, 1974), as was the high frequency transfer of plasmid-mediated bacteriocin and hemolysin production as well as bacteriocin resistance, a year later (Jacob et al., 1975). Jacob and coworkers clearly demonstrated that two different large plasmids from the same strain of *E. faecalis* strain JH1 were transmissible: pJH1 encoded resistance to four different antibiotics, and pJH2 encoded hemolysin production and bacteriocin resistance. They also identified

another transmissible hemolysin/bacteriocin plasmid from a second donor strain.

Pheromone-responsive Broth Mating It was shown within three years that the conjugative transfer described by Jacob and colleagues in 1975 was mediated by plasmids that respond to a substance, termed "sex pheromone," which was produced by recipient strains that stimulated the formation of mating cell aggregates (Dunny et al., 1978). To date, at least 20 different pheromone responsive plasmids from 14 different enterococcal isolates have been isolated and characterized to some extent (Clewell and Dunny, 2002). All but one of these plasmids, pHKK100, from a strain of *E. faecium* (Handwerger et al., 1990) has been from *E. faecalis*. The pheromone-response mechanism of plasmid transfer has been studied in considerable detail, and several reviews describing it have been published (Clewell and Weaver, 1989; Dunny, 1990; Dunny, 1991a; Clewell, 1993a; Clewell, 1993b; Clewell, 1999; Wirth, 1994; Dunny et al., 1995; Wirth et al., 1996; Dunny and Leonard, 1997; Maqueda et al., 1997). This mechanism will be described briefly below.

Plasmid-free strains of *E. faecalis* excrete several chromosome-encoded small peptides (pheromones) that induce a clumping response by strains harboring one or more pheromone-responding plasmids. A potential donor cell responds to a specific pheromone by synthesizing plasmid-encoded aggregation substance (AS), which in turn binds to enterococcal binding substance (EBS) on the surfaces of both donor and recipient cells. Binding substance is at least in part composed of lipoteichoic acid. It has been suggested (Dunny et al., 1995) that the donor-recipient cell contacts formed as a result of AS-EBS binding in some manner allows for the formation of a channel through which plasmid DNA can transfer from the donor cell to the recipient cell. Nearly all pheromone-responsive plasmids encode surface exclusion determinants that prevent, or at least drastically lower the frequency of donor-to-donor plasmid transfer. Pheromone-responsive plasmids also encode inhibitors of the pheromones that induce their response. Thus, once a recipient cell receives a specific pheromone-responsive plasmid, it no longer excretes that pheromone, although it will continue to synthesize and excrete pheromones specific for other pheromone-responsive plasmids. Pheromones and pheromone inhibitors are small peptides composed of 7–8 hydrophobic amino acids. Both are synthesized as precursor molecules requiring processing.

Four pheromone-responsive plasmids, pAD1, pCF10, pAM373 and pPD1, have received considerable attention. The entire nucleotide base sequences of pAD1 (Francia et al., 2001) and pAM373 (De Boever et al., 2000) have been published, and the sequences of large segments of pCF10 have also been published (Kao et al., 1991; Hedberg et al., 1996), with the remainder soon to follow (Clewell and Dunny, 2002). Determinants that share the same or similar functions also share a good deal of sequence homology, and the arrangement on each of the four plasmids of groups of homologous functions, such as replication, pheromone response, transfer, etc., is also similar. Regulation of the pheromone response of plasmids pAD1 (Clewell, 1999) and pCF10 (Dunny and Leonard, 1997) has been examined in considerable detail. Basically, the synthesis of transfer-specific genes by each plasmid is under the regulation of two promoters. One, P_0, regulates the synthesis of an inhibitor of plasmid-specific pheromone and is negatively regulated by the product of a consititutively synthesized protein, TraA of pAD1 and PrgX of pCF10. A second promoter, P_a, from which antisense transcripts are synthesized, further insure that expression from P_0 does not proceed through two transcriptional terminators just 5' of two ORFs that encode two positive regulators of transcription and/or translation of conjugation-specific functions. Basically, pAD1-specific or pCF10-specific pheromone produced by a potential recipient interacts with TraA or PrgX, respectively, inactivating negative regulator. The resulting release in the negative control of P_0 allows read-through past both transcriptional terminators into the genes that encode positive regulation of transfer-specific genes.

Plasmid-mediated Solid-surface Mating The erythromycin resistance plasmid, pAMβ 1, from *E. faecalis* strain DS5 (Clewell et al., 1974) was used to demonstrate the transformability of competent strains of streptococci by plasmid DNA (LeBlanc and Hassell, 1976; LeBlanc et al., 1978a). Subsequently, the ability of streptococcal transformants containing pAMβ 1 to transfer the plasmid to other species of *Streptococcus* by a mechanism that required cell-to-cell contact was demonstrated (LeBlanc et al., 1978b). Unlike the pheromone-responsive plasmids, pAMβ 1 is transmissible only if donor and recipient cells are forced together on a solid surface, i.e., either directly on an agar-based medium, or by collection of donor and recipient cells on a filter membrane followed by incubation of the filter on the surface of such media. The transmissibility of pAMβ 1 from its strain of origin, *E. faecalis* strain DS5, could not be demonstrated, likely because of the presence of pheromone-responsive plasmids in this strain and because another such plasmid, pAD1, was shown to inhibit its transfer (Clewell et al., 1982). Numerous plasmids from enterococci and various streptococcal species, many exhibiting considerable

homology to pAMβ 1, have been shown to mediate their own transfer by solid surface mating to a broad spectrum of Gram-positive bacteria, including most species of streptococci (Clewell, 1981; Horaud et al., 1985). However, although a region of pAMβ 1 (LeBlanc and Lee, 1984) and several regions of the closely related plasmid, pIP501 (Evans et al., 1985; Krah and Macrina, 1989), required for conjugation have been identified, the actual mechanism of transfer has never been elucidated.

Transposon-mediated Solid-surface Mating

Conjugative transfer in the absence of any plasmid DNA was first demonstrated in enterococci (Franke and Clewell, 1981) and chromosome-borne transposon, Tn*916*, was shown to mediate this transfer. As in the transfer mediated by pAMβ 1-like plasmids, transposon-mediated conjugation also occurs at detectable frequencies only on solid surfaces. Although a large number of conjugative transposons have now been described in both Gram-positive (Clewell and Flannagan, 1993c; Lyras and Rood, 1997) and Gram-negative (Salyers et al., 1995; Smith et al., 1998) bacteria, the most thoroughly studied have been Tn*916* and the multiple antibiotic-resistance transposon from *Streptococcus pneumoniae*, Tn*1545* (Courvalin and Carlier, 1986). On the basis of results obtained from studies on both Tn*916* and *Tn1545*, the conjugative transposition process is initiated by the excision of the transposon from DNA of the donor, either the donor chromosome or a resident plasmid. The excision of the transposon is similar to excision of prophage λ in *E. coli* in that staggered cleavages occur at both ends of the transposon (Poyart-Salmeron et al., 1990). These cleavages result in 6-bp protruding 5' hydroxyl ends that differ in sequence (Manganelli et al., 1996; Rudy and Scott, 1996). The transposon then circularizes, followed by ligation of the ends, which results in a 6-bp heteroduplex where the ends were ligated (Caparon and Scott, 1989). If at this point the transposon-containing cell is in contact with a potential recipient, one strand of the ligated element is transferred to the recipient, in which a second, complementary strand is synthesized (Scott et al., 1994). The transposon may then insert into the recipient chromosome or a resident plasmid at a site determined not by any homology to the element, but rather by an AT-rich region in the target DNA, usually shown to contain a static bend (Lu and Churchward, 1995). A 6-bp heteroduplex is formed at each end of the newly integrated transposon via ligation to the site on the element from which it was originally excised from the donor DNA. The mismatched bases at each end of the insertion site are thought to be resolved during normal replication or by mismatch repair (Marra and Scott, 1999). As in intracellular transposition, the only transposon-encoded functions known to be involved in conjugative transposition are Int and Xis.

Antibiotic Resistance

Most enterococcal species are intrinsically resistant to β-lactam antibiotics and to clindamycin, aminoglycosides, and the folate pathway inhibitors, trimethoprim and sulfamethoxazole. Resistance to β-lactams is due to the low affinities of the penicillin-binding proteins produced by *Enterococcus* species (Fontana et al., 1996). Minimum inhibitory concentrations (MICs) of penicillins can range from 2 to greater than 64 μg/ml, depending on specific antibiotic, enterococcal species, or strain. MICs tend to be even higher for the semisynthetic penicillins, and the cephalosporins are clinically ineffective (Murray, 1990). The enterococci are resistant to low levels of aminoglycoside antibiotics, with MICs that may range between 8 and 250 μg/ml, again depending on the aminoglycoside, enterococcal species, or strain, owing to limited transport across the cell membrane. Classically, the intrinsic resistance of this genus to the β-lactam antibiotics and the aminoglycosides has been overcome by the use of antibiotic combinations. The presence of a cell wall inhibitor, such as a β-lactam, appears to increase the uptake of the aminoglycoside by the *Enterococcus*, resulting in a synergistic killing (Moellering and Weinberg, 1971). Acquired high resistance to cell wall inhibitors and to all clinically useful aminoglycosides has eliminated the usefulness of a synergistic approach in the treatment of many enterococcal infections. The enterococci are also intrinsically resistant to the lincosamides, such as lincomycin and clindamycin, with MICs reaching as high as 100 μg/ml (Karchmer et al., 1975; Murray, 1990). The intrinsic resistance of the enterococci to folate pathway inhibitors is due to their ability to utilize exogenous thymidine, thymine, or folates, such as folinic acid, dihydrofolate, and tetrahydrofolate (Hamilton-Miller, 1988).

High Level Aminoglycoside Resistance

One method by which the enterococci resist synergistic killing by a cell wall inhibitor and an aminoglycoside is to acquire genes that encode enzymes that modify one or more aminoglycosides. Three types of aminoglycoside-modifying enzymes that have been described include phosphotransferases (APHs), acetyltransferases (AACs) and nucleotidyltransferases (ANTs), which, respectively, mediate the phosphorylation of a hydroxyl group on the aminoglycoside at the expense of ATP, the acetylation of an amino

group using acetyl coenzyme A as a donor, and the adenylylation of a hydroxyl group with the adenyl group of ATP. Acquisition of these enzymes, which are usually plasmid-encoded, results in high level aminoglycoside resistance, i.e., MICs of the antibiotics affected are greater than 2000 μg/ml and can reach levels greater than 40,000 μg/ml.

High level resistance to streptomycin has been observed in greater than 50% of enterococcal strains isolated as early as the mid-1950s (Jones and Finland, 1957; Atkinson et al., 1997). Two types of streptomycin-modifying enzymes, nucleotidyltransferase, ANT(6)-Ia and ANT(3″)-Ia, encoded by *ant(6)-Ia* and *ant(3″)-Ia*, respectively, have been described (Krogstad et al., 1978; Clark et al., 1999). Mutations in the 30S ribosomal subunit, which result in decreased binding of streptomycin to the ribosome, can also result in streptomycin MICs that may be as high as 128,000 μg/ml (Eliopoulos et al., 1984). One of the most common plasmid-mediated aminoglycoside modifying genes found in enterococci is *aph(3′)-IIIa*, which by encoding the aminoglycoside phosphotransferase, APH(3′)-IIIA, causes high level resistance to kanamycin (Trieu-Cuot and Courvalin, 1983). This gene was identified on kanamycin resistance plasmids harbored by 70, 52 and 79% of enterococci isolated from chickens, pigs, and humans, respectively (LeBlanc et al., 1987). An *ant(9)-Ib* gene, encoding resistance to spectinomycin, via adenylylation at the 9 position, has been isolated from a single *E. faecalis* isolate (LeBlanc et al., 1991).

The most clinically relevant enterococcal gene resulting in aminoglycoside modifying activity is *aac(6′)-Ie-aph(2″)-1a*, which encodes a bifunctional enzyme, AAC(6')-APH(2″) that mediates resistance to gentamicin, tobramycin, amikacin, kanamycin, netilmicin, and dibekacin (Ferretti et al., 1986; Azucena et al., 1997). This gene was present in the first high-level gentamicin resistant *E. faecalis* isolates described (Horodniceanu et al., 1979), which were also resistant to kanamycin and tobramycin, but not streptomycin, and in the first enterococci to be described as resistant to all aminoglycosides, including gentamicin and streptomycin (Mederski-Samoraj and Murray, 1983; Murray et al., 1983b). Although greater than 90% of gentamicin resistant enterococcal isolates encode the bifunctional enzyme, single aminoglycoside modifying enzymes that mediate resistance to gentamicin, as well as other aminoglycosides, have also been described. For example, *aph(2″)-Ic*, from a strain of *Enterococcus gallinarum*, encodes an aminoglycoside phosphotransferase that results in resistance to gentamicin, tobramycin, kanamycin and dibekacin (Chow et al., 1997). The aminoglycoside phosphotransferase genes, *aph(2″)-Ib*, from a strain of *E. faecium* (Kao et al., 2000), and *aph(2″)-Id*, from a strain of *Enterococcus casseliflavus* (Tsai et al., 1998), encode aminoglycoside phosphotransferase activity leading to resistance to gentamicin, tobramycin, kanamycin, netilmicin and dibekacin. The *aph(2″)-Ic* and *aph(2″)-1d* have also been detected in *E. faecium*, and the former has been found in *E. faecalis* as well (Kak and Chow, 2002).

High β-Lactam Resistance

Isolated in 1981, *Enterococcus faecalis* strain HH22 was the first enterococcal strain shown to produce β-lactamase activity (Murray and Mederski-Samoraj, 1983a) and the first high-level gentamicin-resistant *Enterococcus* to be isolated in the United States (Mederski-Samoraj and Murray, 1983). Both the β-lactamase gene and the *aac(6′)-Ie-aph(2″)-1a* gene are encoded by a pheromone-responsive plasmid (Murray et al., 1988). β-Lactamase-producing *E. faecalis* strains have been isolated from patients in at least four countries, many of which have been disseminated by inter- and intra-hospital clonal spread (Murray et al., 1991; Murray et al., 1992b). Even among those isolates that are not clonal, β-lactamase activity is almost always associated with high level gentamicin resistance (Murray, 1992a), and the single β-lactamase producing *E. faecium* isolate described thus far also expresses high level aminoglycoside resistance (Coudron et al., 1992). The gene encoding β-lactamase activity in enterococci was likely acquired from *Staphylococcus aureus* and is nearly identical to the *S. aureus blaZ* gene (Murray, 1992a). Despite the reports describing several isolates in the United States and elsewhere, the incidence of β-lactamase-producing enterococci remains rare (Gordon et al., 1992; Murray, 1998).

High resistance of enterococci to β-lactam antibiotics, particularly penicillins, may also be due to either the overexpression of a low-affinity penicillin-binding protein (PBP) and/or to mutations in a low-affinity PBP structural gene, which lowers its affinity for penicillins to an even greater degree (Fontana et al., 1996). Spontaneous mutants of *E. hirae* strain 9790 (previously designated "*E. faecium* strain 9790") with higher resistance to penicillins were shown to produce higher levels of the low affinity PBP5 (Fontana et al., 1983). Spontaneous mutants of *E. faecalis* and *E. faecium* strains also were shown to produce higher levels of this low affinity PBP. Subsequently, a gene designated "*psr*," located upstream of the PBP5 gene in *E. hirae* 9790 and encoding a 19-kDa protein, was shown to be a negative regulator of PBP5 expression (Ligozzi et al., 1993). Penicillin-resistant mutants had suffered deletions in this gene. Spontaneous mutants of eight different species of *Enterococ-*

cus, selected for resistance to penicillin G, were resistant to all β-lactam antibiotics tested and produced increased levels of a low affinity PBP (al-Obeid et al., 1990). In some clinical isolates of *E. faecalis* against which ampicillin MICs were high (i.e., 32 to 64 μg/ml), higher levels of PBP5 were produced and two other PBPs, 1 and 6, with decreased levels of binding to penicillin were expressed (Cercenado et al., 1996). Penicillin-resistant clinical isolates of *E. faecium* were shown to be resistant via one of the two mechanisms described for the clinical isolates of *E. faecalis* (Zorzi et al., 1996). In these instances, however, strains of *E. faecium* with intermediate levels of resistance (i.e., penicillin MICs between 16 and 64 μg/ml) expressed higher than normal levels of PBP5fm, whereas strains with higher resistance (i.e., MIC = 90 μg/ml) had amino acid substitutions in PBP5fm that resulted in even lower affinities for penicillin.

Recently, a new mechanism of β-lactam resistance was described that involved neither altered expression, binding properties of PBPs, nor production of β-lactamase activity (Mainardi et al., 2000). The resistant mutant strain of *E. faecium* studied contained β-lactam-insensitive L-Lys → D-Asx-L-Lys crosslinks, rather than the normal wild type β-lactam sensitive D-Ala → D-Asn (or D-Asp)-L-Lys crosslinks. Whereas the latter crosslinks are due to PBP-associated DD-transpeptidation activity, the former is due to an LD-transpeptidation that is not associated with any PBP.

Glycopeptide Resistance

The first enterococcal clinical isolate shown to be resistant to glycopeptide antibiotics was a strain of *E. faecium* isolated in 1986, which harbored a plasmid that mediated resistance to both vancomycin and teicoplanin (Leclercq et al., 1988). Subsequently, this resistance was shown to be due to genes encoded on a transposon, Tn*1546*, and the products of these genes was shown to result in the synthesis of a depsipeptide peptidoglycan precursor, rather than the normal pentapeptide ending in D-Ala-D-Ala (Arthur et al., 1993). This original glycopeptide resistance phenotype subsequently became known as "the VanA type," and an additional five phenotypes (VanB to VanE) plus an unclassified type have since been described. The mechanisms of resistance in all types come down to the replacement of D-Ala-D-Ala, the target of glycopeptides, by either D-Ala-D-lactate or D-Ala-D-Ser, either of which has a much lower affinity for glycopeptides than D-Ala-D-Ala. These mechanisms and the genetics of glycopeptide resistance in enterococci have been reviewed recently (Evers et al., 1996; Malathum and Murray, 1999) and will be described only briefly here.

VanA enterococcal isolates express high resistance to both vancomycin and teicoplanin, a phenotype mediated by the *vanA* gene cluster encoded by Tn*1546* (Arthur et al., 1993) or a related transposable element (Handwerger et al., 1995; Palepou et al., 1998; Woodford et al., 1998b). Genetic elements encoding the *vanA* gene cluster have been described in eight different enterococcal species. Tn*1546* and related transposable elements encode nine genes. Two of these, *orf1* and *orf2*, encode proteins related to transposases and resolvases (Arthur et al., 1993). The genes *vanA*, *H*, *X*, *Y* and *Z*, encode proteins directly associated with glycopeptide resistance. VanA is an enzyme that catalyzes the formation of an ester bond between D-Ala and D-lactate (Bugg et al., 1991a; Arthur et al., 1992a). VanH catalyzes the reduction of pyruvate to D-lactate, one of the two substrates of VanA (Bugg et al., 1991b). VanX catalyzes the hydrolysis of D-Ala-D-Ala synthesized by the normal chromosome encoded D-Ala:D-Ala ligase, which helps to insure that D-Ala-D-lactate is incorporated into the pentapeptide (Reynolds et al., 1994a). Some D-Ala-D-Ala synthesized by the chromosomal ligase may still be incorporated into the pentapeptide precursor in VanA strains despite the predominance of D-Ala-D-lactate and the accompanying activity of VanX. However, VanY can catalyze the cleavage of the terminal D-Ala in this pentapeptide, resulting in tetrapeptide-containing precursors that bind less effectively to glycopeptides (Gutmann et al., 1992; Arthur et al., 1994). No enzymatic role has been identified for VanZ, although it may affect resistance of VanA enterococcal strains to teicoplanin (Arthur et al., 1995). The *vanR*/*vanS* genes encode a two-component regulatory system in which VanS recognizes an external signal, likely vancomycin, which results in self-phosphorylation. Then VanR is phosphorylated, activating its own promoter as well as that of the vancomycin resistance structural genes (Arthur et al., 1992b; Silva et al., 1998).

VanB enterococcal isolates express variable levels of resistance to vancomycin but are susceptible to teicoplanin, and resistance is mediated by the *vanB* gene cluster, most often chromosomally located (Malathum and Murray, 1999), but it may also be present on a plasmid (Woodford et al., 1995; Rice et al., 1998b). The *vanB* gene cluster has also been located on the composite transposon, Tn*1547*, within a large chromosomal conjugative element (Quintiliani et al., 1994; Quintiliani and Courvalin, 1996). The VanB phenotype has been associated primarily with strains of *E. faecalis* and *E. faecium* (Malathum and Murray, 1999), and occasionally with *E. casseliflavus* (Woodford, 1998a) and *E. gallinarum* (Liassine et al., 1998). There are six genes in the *vanB* cluster that are homologs of

vanA genes, $vanS_B$, $vanR_B$, $vanH_B$, $vanX_B$, $vanY_B$ and *vanB* (Evers and Courvalin, 1996). There is no *vanZ* homolog, but there is an additional gene, *vanW*, of unknown function. The gene cluster is induced by vancomycin, but not by teicoplanin (Evers and Courvalin, 1994). Induction by vancomycin results in resistance to teicoplanin.

VanC resistance is not an acquired trait but rather is intrinsic to *E. gallinarum* and *E. casseliflavus* (Malathum and Murray, 1999). VanC is a D-Ala:D-Ser ligase which results in a pentapeptide precursor with a D-Ala-D-Ser terminal dipeptide (Reynolds et al., 1994b). The VanD phenotype, detected in strains of *E. faecium*, is characterized by a constitutive resistance to both vancomycin and teicoplanin (Perichon et al., 1997; Ostrowsky et al., 1999). It has been chromosomally located only, has not been transmissible, and the pentapeptide, as in VanA strains, ends in D-lactate. The *vanD* gene cluster contains the *vanA*/*vanB* gene cluster homologs ($vanS_D$, $vanR_D$, $vanH_D$, $vanX_D$ and $vanY_D$) but no homologs of *vanW* or *vanZ* (Casadewall and Courvalin, 1999). The VanE phenotype, chromosomally determined in a strain of *E. faecalis*, is represented by low resistance to vancomycin and susceptibility to teicoplanin; this phenotype is due to vancomycin-induced synthesis of D-Ala-D-Ser as the terminal dipeptide (Fines et al., 1999).

Resistance to Miscellaneous Antibiotics

Resistance by enterococci to certain antibiotics has been observed in strains isolated as early as the mid-1950s. Greater than 50% of enterococcal isolates obtained from patients in Boston City Hospital between 1953 and 1954 (Jones and Finland, 1957) and in two Washington, DC, area hospitals (Atkinson et al., 1997) were resistant to streptomycin and tetracycline. Whereas 96 and 25% of the Boston isolates were resistant to chloramphenicol and erythromycin, respectively, only 1 and 3%, respectively, of the Washington, DC, isolates were resistant to these two antibiotics. All but 8 of 126 tetracycline-resistant enterococcal isolates from the Washington, DC, area hospitals were also resistant to minocycline, and half of the erythromycin-resistant isolates from these hospitals were also resistant to high levels of lincomycin. None of the strains examined in the latter study (Atkinson et al., 1997) were resistant to ampicillin, gentamicin, or vancomycin, but for 5% of the Boston City Hospital isolates, the MICs of penicillin were >8 μg/ml (Jones and Finland, 1957).

The most common mechanism for resistance to erythromycin among enterococci and Gram-positive bacteria, in general, involves a dimethylation of adenine residues in 23S rRNA within the 50S ribosomal subunit (Horaud et al., 1985; Jensen et al., 1999). These alterations result in the MLS resistance phenotype (Weisblum, 1995), i.e., highly reduced binding to the ribosome by macrolides, such as erythromycin, as well as by newer macrolides (such as azithromycin and clarithromycin; Kak and Chow, 2002), lincosamides (e.g., lincomycin and clindamycin), and streptogramin B antibiotics. MLS resistance among enterococci is almost always encoded by an *erm* (B) gene, which now includes genes previously designated "*erm* (AM)" or "*erm* (AMR)" (Roberts et al., 1999). Occasionally, an MLS-resistant enterococcal isolate will carry an *erm* (A) gene (Roberts et al., 1999; Portillo et al., 2000). MLS-resistance genes are usually plasmid-mediated (Horaud et al., 1985) and may also be located within a transposon, such as Tn*917* (Tomich et al., 1980), or one closely related to it (Banai and LeBlanc, 1984; Rollins et al., 1985). Plasmids encoding MLS resistance often encode one or more additional resistance traits as well (Horaud et al., 1985; LeBlanc et al., 1986).

Among erythromycin resistant isolates obtained from patients in Washington, DC, hospitals in the mid-1950s, half expressed the MLS-resistance phenotype and half were resistant to erythromycin but not lincomycin. While half of the lincomycin resistant isolates expressed the MLS phenotype, the other half was not erythromycin resistant (Atkinson et al., 1997). Those isolates resistant to erythromycin (owing to the expression of an efflux pump) exhibited only the M phenotype and were likely carrying a *mef* (E) gene (Sutcliffe et al., 1996; Tait-Kamradt et al., 1997), currently designated "*mef* (A)" (Clancy et al., 1996; Kak and Chow, 2002). The *mef* (A) gene of many enterococci is located on a large transmissible plasmid (Luna et al., 1999). A *lin* (B) gene (Bozdogan et al., 1999), currently designated "*lnu* (B)" (Roberts et al., 1999), encoding a nucleotidyltransferase that adenylylates both clindamycin and lincomycin, may account for those Washington, DC, enterococcal isolates resistant to lincomycin but not erythromycin.

The reported incidence of chloramphenicol resistance among enterococcal isolates has varied considerably, between <1 and > 50%, dependent to a large extent on the geographical origin, time of isolation or both of the particular strains studied (Jones and Finland, 1957; Toala et al., 1969; Atkinson and Lorian, 1984; Pepper et al., 1986; Atkinson et al., 1997; Jones et al., 2001). Only one mechanism for enterococcal chloramphenicol resistance has been described (i.e., acetylation of a hydroxyl group on chloramphenicol, which inactivates the antibiotic and eliminates ribosomal binding; Shaw, 1983) and is mediated via a plasmid- or chromosome-borne *cat* gene (Pepper et al., 1986; Pepper et al., 1987; Trieu-Cuot et al., 1993).

The incidence of resistance to tetracycline among enterococci appears to vary between 50 and 80%, and as with chloramphenicol resistance, is somewhat dependent on geographical origin and time of isolation (Jones and Finland, 1957; Atkinson and Lorian, 1984; Acar and Buu-Hoi, 1988; Atkinson et al., 1997; Jones et al., 1998). Two mechanisms of tetracycline resistance have been identified in enterococci, active efflux and ribosomal protection (McMurry and Levy, 2000). Determinants encoding these two mechanisms were first classified on the basis of phenotype, i.e., *tet* (L), initially associated with small nonconjugative plasmids, mediated resistance to tetracycline, whereas *tet* (M), usually not found on plasmids, mediated resistance to tetracycline and minocycline (Burdett et al., 1982). Subsequently, TetL was shown to mediate resistance to tetracycline via an energy-dependent efflux mechanism (McMurry et al., 1987). TetM was shown to have no effect on the cellular accumulation of tetracycline, nor did it modify the antibiotic. Rather, cell-free protein biosynthetic machinery from cells containing TetM was resistant to tetracycline (Burdett, 1986), hence TetM was thought to protect ribosomes. From determination of the sequence of *tet* (M) from Tn*916* (Burdett, 1990), TetM protein was found to be homologous to the translation elongation factors EF-G and EF-Tu. Most of the identity between TetM and EF-G lies in the respective GTP-binding domains (Burdett, 1991). Although TetM can protect cellular translation from inhibition by tetracycline, it cannot substitute for either EF-G or EF-Tu (Burdett, 1996).

The *tet* (M) gene is the predominant tetracycline-resistance determinant found in enterococci, and in general, in both Gram-positive and Gram-negative bacteria (Roberts and Hillier, 1990; Roberts, 1997). Other ribosomal protection-mediating genes, such as the *tet* (O) gene (Zilhao et al., 1988; Bentorcha et al., 1991) and the *tet* (S) gene (Charpentier et al., 1994), also have been detected in enterococci. The *tet* (O) gene was originally detected in members of the Gram-negative bacterial genus, *Campylobacter* (Sougakoff et al., 1987), and *tet* (S), originally detected in the Gram-positive bacterial pathogen, *Listeria monocytogenes* (Charpentier et al., 1993). Besides *tet* (L), *tet* (K) is the only other tetracycline-resistance determinant encoding an efflux mechanism detected in enterococci (Roberts, 1997). Although in the enterococci, *tet* (M) is usually chromosome-borne and often on a transposon (many closely related to Tn*916*), it may also be present on a plasmid (Pepper et al., 1987; Zilhao et al., 1988; Bentorcha et al., 1991). The genes, *tet* (L) and *tet* (O), although usually plasmid-borne in enterococci, may also be chromosome-borne (Pepper et al., 1987; Zilhao et al., 1988; Bentorcha et al., 1991), and *tet* (S), which is plasmid-borne in *Listeria*, was detected in the chromosome of *E. faecalis* (Charpentier et al., 1994). Several resistant enterococcal isolates have been shown to carry more than one tetracycline resistance determinant (LeBlanc and Lee, 1982; Roberts, 1997; Zilhao et al., 1988). A novel tetracycline resistance determinant, *tet* (U) from a strain of *E. faecium*, has been cloned and characterized (Ridenhour et al., 1996). On the basis of its mediation of resistance to both tetracycline and minocycline and its limited homology to *tet* (M) and *tet* (O), a ribosomal protection mechanism was proposed for the gene product Tet(U).

Mechanisms of fluoroquinolone resistance in clinical isolates of *E. faecalis* (Kanematsu et al., 1998) and *E. faecium* (El Amin et al., 1999) have been examined. For both species, high resistance was associated with amino acid substitutions in the quinolone resistance determinant regions (QRDRs) of the A subunit (GyrA) of DNA gyrase and of the C subunit (ParC) of topoisomerase IV. In neither study were the B subunits (GyrB) of DNA gyrase, the E subunits (ParE) of topoisomerase IV, or homologs of known efflux pumps, examined for mutations. Thus, although mutations in *gyrA* and *parC* were clearly associated with high fluoroquinolone resistance in the isolates examined in both studies, additional contributions to resistance of mutations in *gyrB* and *parE*, or in the genes or regulatory elements of efflux systems, cannot be excluded. In the *E. faecalis* study (Kanematsu et al., 1998), 17 isolates exhibiting high resistance had a single amino acid change in the QRDR of GyrA, Ser83Arg, Ser83Ile, Glu87Lys, or Glu87Gly, plus a single change in the QRDR of ParC, Ser80Arg or Ser80Ile. One such isolate had, in addition to a Ser83Ile change in GyrA and a Ser80Ile change in ParC, an additional change in Par C, Glu84Ala. One strain, exhibiting intermediate fluoroquinolone resistance had only a Ser80Arg change in ParC, and no alterations in GyrA. The MICs of ciprofloxacin against 10 isolates in the *E. faecium* study (El Amin et al., 1999) ranged from 32 to >256 μg/ml, and each isolate had a single change in the QRDR of GyrA (Ser83Arg, Ser83Ile, Ser83Tyr or Glu87Lys) and a single change in ParC (Ser80Ile). No specific genotype could be correlated with a specific MIC, leaving open the possibility of mutations in *gyrB*, *parE*, or a gene encoding or regulating an efflux pump.

Linezolid is the only member of a new class of antimicrobial agents, the oxazolidinones, currently in clinical use for the treatment of certain Gram-positive bacterial infections, including infections of vancomycin-resistant enterococci (VRE). The mechanism of action of linezolid is unique in that it inhibits protein synthesis by interfering with the complexing of 70S ribo-

somes, mRNA, IF2, IF3, and fmet-tRNA (Swaney et al., 1998). Although the results of in vitro studies had indicated that the development of resistance to linezolid occurred at a very low frequency, linezolid-resistant VRE were isolated from patients shortly after its introduction into clinical practice. Two of these isolates, both strains of *E. faecium*, were each shown to have undergone a G2576U conversion in the peptidyl transferase region of 23S rRNA (Zurenko et al., 1999). The patients from whom the resistant strains were isolated had received prolonged courses of linezolid for VRE bacteremia. Subsequently, a collection of clinical VRE isolates susceptible to linezolid were subjected to serial passages in doubling concentrations of the antibiotic, and the emergence of resistance was monitored (Prystowsky et al., 2001). *Enterococcus faecalis* isolates began to develop resistance in three passages. After the final passages, the MICs of linezolid ranged from 16 to 128 μg/ml, whereas resistant *E. faecium* strains did not begin to develop their resistance until the tenth passage, and the linezolid MICs reached were only between 8 and 16 μg/ml. The resistant *E. faecalis* mutants were shown to have suffered the same G2576U conversions as the previously studied resistant *E. faecium* clinical isolates (Zurenko et al., 1999), whereas the single in vitro-derived resistant *E. faecium* mutant from which 23S rRNA was examined had a single mutation, G2505A. A recent study has confirmed the role of a G2576U conversion in 23S rRNA in the resistance to linezolid of clinical isolates of *E. faecium* and one *E. faecalis* isolate (Marshall et al., 2002). However, the results of this latter study also showed that the level of resistance to linezolid could be correlated with the number of 23S rRNA genes that contained the mutation, G2576T. The number of 23S rRNA genes with the G2576T mutation and the corresponding MICs of linezolid against the *E. faecium* isolates examined were 0/6 = 2 μg/ml, 1/6 = 8 μg/ml, 2/6–3/6 = 32 μg/ml, and 4/6–5/6 = 64 μg/ml. For the single *E. faecalis* isolate examined, 4/4 23S rRNA genes had the mutation, and the linezolid MIC was 128 μg/ml.

Plasmid-mediated Virulence Traits

Two plasmid-mediated traits, aggregation substance (AS; reviewed in Muscholl-Silberhorn et al., 2000) and cytolysin (reviewed in Haas and Gilmore [1999] and Gilmore et al. [2002]), have been linked to the virulence of *E. faecalis*. AS is encoded by all pheromone-responsive plasmids, and cytolysin may be encoded by pheromone-responsive and non-pheromone-responsive plasmids. A role of AS in host cell adherence was predicted on the basis of the presence of two Arg-Gly-Asp (RGD) motifs in the AS sequence (Galli et al., 1990). Subsequently, a strain of *E. faecalis* that expressed AS constitutively was shown to bind to pig renal tubular cells considerably better than a plasmid-free strain or a plasmid-containing strain in which AS expression was uninduced (Kreft et al., 1992). The adherence could be blocked by the addition of synthetic RGD peptide. The expression of AS has also been correlated with the uptake of *E. faecalis* by intestinal epithelial cells (Olmsted et al., 1994), and the ability of AS to bind host integrins has been associated with the interaction of these bacteria with polymorphonuclear neutrophils (PMNs; Vanek et al., 1999) and with macrophages (Sussmuth et al., 2000), which in turn stimulates enterococcal phagocytosis by these host cells. Survival of enterococci in PMNs and macrophages apparently is enhanced by AS-inhibited phagolysosome acidification (Rakita et al., 1999) and respiratory burst (Sussmuth et al., 2000), respectively. Although AS has been shown to lead to increased vegetation size in a rabbit endocarditis model (Chow et al., 1993; Schlievert et al., 1998), no such effect was observed in a rat endocarditis model (Berti et al., 1998).

The cytolysins produced by *E. faecalis* are able to lyse a variety of eukaryote cell types, including erythrocytes, as well as several species of Gram-positive bacteria (Haas and Gilmore, 1999). Thus these molecules have been designated "bacteriocins" as well as "cytolysins." The cytolysins have been shown to enhance virulence in a variety of infection models (Gilmore et al., 2002). The cytolysin operon, as well as its regulation, has received considerable attention (Haas and Gilmore, 1999; Gilmore et al., 2002). Briefly, the cytolysin molecule is comprised of two peptides, $CylL_L$ and $CylL_S$, which are subjected to post-translational cytoplasmic modification by CylM. The modified products, Cyl_L* and Cyl_S*, are then secreted from the cell by the ATP-binding transporter, CylB, with the secretion of Cyl_L*, but not Cyl_S*, dependent on ATP hydrolysis. Leader sequences on both, Cyl_L* and Cyl_S* are removed during secretion by a CylB-associated serine protease activity. The extracellular products (Cyl_L' and Cyl_S') are again cleaved by another serine protease, CylA, which is also extracellular, yielding the active cytolysin subunits, Cyl_L" and Cyl_S". The regulation of cytotoxin expression has been partially elucidated (Haas et al., 2002). Synthesis of the cytolysin appears to be dependent on cell density, with $CylL_S$" serving as an autoinduction signal. By a mechanism yet to be elucidated, the products of two additional genes (*cylR1* and *cylR2*) apparently function as represssors of the operon in the absence of $CylL_S$".

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