Biology and Taxonomy

Chapter 1
The Biology of Staphylococci

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Historical perspective of the isolation and characterization of the staphylococci

The staphylococci make up the family of Gram-positive cocci, Staphylococcaceae, which is in the order Bacillales. The term "staphylococcus" was synthesized from the Greek word *staphyle*, meaning bunch of grapes, for their ability to form microscopic grape-like clusters, and the term "coccus," meaning grain or berry. *Staphylococcus aureus* was one of the first bacterial pathogens identified, and causes a very broad range of infections including impetigo, folliculitis, superficial and deep skin abscesses, wound infections, osteomyelitis, suppurative arthritis, pneumonia, pleural emphysema, meningitis, septicemia and endocarditis, toxic shock syndrome, scalded skin syndrome, and food poisoning [1].

Koch first differentiated Gram-positive cocci in 1878 and recognized that different diseases such as abscesses correlated with the presence of clusters of Gram-positive cocci. Shortly thereafter, in 1884, Rosenbach differentiated species of staphylococci on the basis of colonial pigmentation, whereby the most pathogenic species formed a golden pigment and less pathogenic staphylococci formed white colonies called *S. albus*, now *S. epidermidis*. Also included in the *S. albus* strains were many other coagulase-negative staphylococci that fail to form pigment. Alexander Ogston, in 1880, found "a cluster forming coccus was the cause of certain pyogenous abscesses in man." When Ogston injected the pus from humans containing staphylococci into mice, it produced abscesses; however, when the pus was heated or treated with phenol, it failed to produce abscesses. In 1882, Ogston named the organism staphylococcus. Pasteur had reached similar conclusions at approximately the same time. Coagulase testing later provided a more certain classification of staphylococci than pigment production, wherein a positive coagulase test, which confirmed the identity of *S. aureus*, correlated much better with pathogenicity.

Another common human pathogen is *S. saprophyticus*, which produces urinary tract infections in young women [2]. *Staphylococcus haemolyticus* is somewhat less common, but is important because it can be highly antibiotic resistant even to glycopeptides and linezolid [3]. Many other coagulase-negative strains (41 species identified at present) such as *S. schleiferi* and *S. lugdunesis* have been described, and they can produce a variety of nosocomial infections (reviewed by von Eiff *et al.* [4,5]).

Morphology

Staphylococci have a diameter of 0.7–1.2 μm and a Gram-positive cell wall (Figure 1.1). Division planes occur at right angles and the cocci separate slowly, hence tetrads are frequently found. Clustering of cocci is promoted by growth on solid medium. On occasion, the clusters may be asymmetrical.

Microbiological differentiation of staphylococci

Growth under various conditions

Staphylococci are facultative anaerobes that grow most rapidly under aerobic conditions and in the presence of CO_2. Colonies of *S. aureus* are β-hemolytic due to the production of several hemolysins: α-toxin, β-toxin, γ-toxin, and δ-toxin. Some *S. epidermidis* strains are β-hemolytic due to the production of δ-toxin [6]. Pigmentation is more pronounced after 24 hours and when held at room temperature, or in media enriched with acetate or glycerol monophosphate [7,8]. The pigments are carotenoids, whose biosynthetic pathway has recently been identified in *S. aureus* [9]. Pigment is not produced under anaerobic

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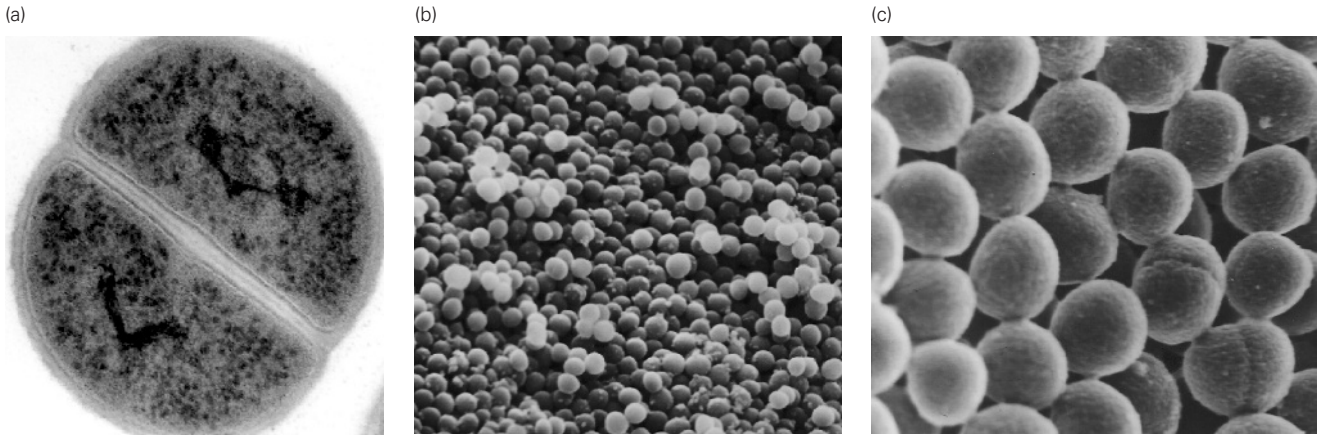

Figure 1.1 (a) Transmission electron microscopy at 126 700× magnification of *S. aureus* cells displaying cell separation by a cross-wall surrounding a highly contrasting splitting system. Scanning electron microscopy of *S. aureus* cells at (b) low (6700×) and (c) high (35 000×) magnification. Reproduced with permission from Kahl BC, Belling G, Reichelt R, Herrmann M, Proctor RA, Peters G. Thymidine-dependent small-colony variants of Staphylococcus aureus exhibit gross morphological and ultrastructural changes consistent with impaired cell separation. *J Clin Microbiol* 2003;41(1):410–3.

conditions or by small colony variants [10]. The formation of pigments is dependent on the stress sigma factor, σ^B [11]. A few *S. aureus* strains produce abundant exopolysaccharide (e.g., Smith strain, which produces a mucoid colony); however, most strains produce only a microcapsule and the colonies appear non-mucoid [12]. When encountered in clinical strains, the most frequent *S. aureus* serotypes are capsule types 5 and 8 [12]. In contrast to *S. aureus*, most clinical *S. epidermidis* isolates produce more exopolysaccharide.

Staphylococci can grow in a wide pH range (4.8–9.4), resist drying, and can survive at temperature extremes as high as 60°C for 30 min. In addition, *S. aureus* grows in high-salt medium due to the production of osmoprotectants [13], and can tolerate 7.5–10% NaCl. The ability of *S. aureus* to ferment mannitol is the basis for differentiating it from *S. epidermidis* and *S. saprophyticus*. When grown on mannitol salt agar, fermentation of mannitol produces a yellow zone around the colony. In addition to mannitol, *S. aureus* can metabolize glucose, xylose, lactose, sucrose, maltose, and glycerol. Further differentiation of staphylococci can be achieved by growth in the presence of novobiocin. *Staphylococcus saprophyticus* [14] and *S. xylosus* [5] are intrinsically resistant to novobiocin because their version of the DNA gyrase B enzyme does not bind novobiocin whereas other coagulase-negative staphylococci such as *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, and *S. schleiferi* are novobiocin susceptible [5].

Special media

Several nonroutine agars are used to study *S. aureus* enzymes. Lipases produce clearing on egg yolk agar, especially when grown under anaerobic conditions [15]. *Staphylococcus aureus* protease activity can be monitored on casein agar plates, where protease-positive strains produce clearing of the agar [16]. When V8 protease (encoded by *sspA*) is present, it produces a zone of white precipitates around the colony [16]. Finally, tellurite is often added to growth media for the selection of *Corynebacterium diphtheriae* in pharyngeal specimens; *S. aureus* may be found in the pharyngeal specimens and can grow in the presence of tellurite, producing gray-black colonies that allow it to be confused with *C. diphtheriae*.

Other methods for differentiation of staphylococci

Staphylococci can be differentiated from micrococcus species based on their susceptibility to lysis by lysostaphin [17]. Lysostaphin is a metalloendopeptidase that targets the pentaglycine bridge of peptidoglycan [18].

Phenol soluble modulins have been associated with more severe staphylococcal infections and require specialized chromatography and mass spectrometry for identification and quantification [19].

While polymerase chain reaction (PCR) testing is not yet routine practice, its use is becoming more widely available in clinical as well as research laboratories. One of the most reliable PCR tests for *S. aureus* [20] detects the presence of the thermonuclease gene *nuc* [20–22]. PCR can also be used to test for the presence of genes encoding Panton-Valentine leukocidin (PVL), which is indicative of strains of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) [23].

Colonization with staphylococci

Nasal carriage of *S. aureus* is persistently present in 30% of people and transiently found in 70% of people; conversely, 30% of people resist nasal colonization [4,24]. A higher incidence rate of nasal carriage of CA-MRSA has also been associated with individuals having frequent contact with

cats, dogs, pigs, and horses, suggesting that animals can be vectors in the spread of CA-MRSA [25–28]. *Staphylococcus aureus* reportedly adheres to nasal mucosa through several surface protein adhesins, including SasG, clumping factor, and fibronectin-binding protein [29–32]. Indeed, the staphylococci have a large array of surface proteins and carbohydrates that enable binding to a broad range of host tissues, including platelets, epithelial cells, endothelial cells, and host intercellular matrix proteins [33]. More detailed information concerning staphylococcal adherence to host tissues is covered in Chapter 7.

CA-MRSA demonstrate distinctive patterns of colonization, as they may be found solely in the throat or on the skin but are culture negative in the nares [34,35]. For this reason, testing individuals for colonization by nasal culture alone may not be sufficient for detection of CA-MRSA colonization.

Several recent studies suggest that streptococci may compete with CA-MRSA for colonization of mucosal surfaces. In a study by Chen *et al*. [36], it was observed that 17% of pregnant women were found to be vaginally colonized with *S. aureus* but only 0.5% were colonized by MRSA. Colonization of the vagina with *S. aureus* was associated with an 11-fold increased risk of postpartum fever. Interestingly, patients vaginally colonized with CA-MRSA were 12.5 times less likely to carry group B streptococci. In contrast, when methicillin-sensitive *S. aureus* (MSSA)-colonized patients were compared with patients who were not *S. aureus* colonized, the MSSA patients were 4.5 times more likely to carry group B streptococci [37]. These data suggest that group B streptococci are especially important for competing with CA-MRSA for colonization of vaginal mucosa. Similarly, on the oropharyngeal mucosal surfaces, *Streptococcus pneumoniae* may be important for preventing oral colonization by CA-MRSA. Children carrying *Streptococcus pneumoniae* are less likely to carry *S. aureus* [38], and pneumococcal vaccination increases *S. aureus* colonization [39,40]. While the heptavalent conjugate vaccine was not licensed for use in children in the USA until 2000 and in Europe until 2001, the first report of oral colonization by CA-MRSA was in 1998 [41]. Nevertheless, vaccination and subsequent eradication of competing bacterial species may be contributing to the rapid spread of CA-MRSA. *Streptococcus pneumoniae* can produce hydrogen peroxide at concentrations capable of killing *S. aureus* [39], but this does not appear to be a major determinant of the patterns of co-colonization [42]. When looking specifically at CA-MRSA colonization, the rate of *S. aureus* colonization is increased in patients receiving the pneumococcal vaccine, but there is no particular increase in CA-MRSA strains [43]. Thus, streptococci on mucosal surfaces compete with *S. aureus*, but this is only one potential factor contributing to the current CA-MRSA outbreak.

Staphylococcus epidermidis resides more permanently on the skin because it is better able to tolerate the acidic pH, lipids, and salt found on skin [44] than is *S. aureus*, which can only persist on the skin for several hours. Recently, USA300, a CA-MRSA strain responsible for a rapidly spreading epidemic of skin and soft tissue infections, was found more frequently on the skin in the absence of nares colonization relative to other strains of *S. aureus* [34,35], suggesting that USA300 strains are particularly well adapted to persist on the skin. Whether skin colonization is permanent or transient, this colonization creates a major problem in hospitals because hands are an excellent means of transmitting MRSA, thus creating a major threat to patient welfare [45].

In addition to the two predominant staphylococcal human pathogens, several other staphylococci are capable of causing disease in humans and animals. *Staphylococcus saprophyticus* is able to colonize the urinary tract and cause infections, showing lower levels of colonization when higher concentrations of Tamm–Horsfall protein are present [46]. Coagulase-negative staphylococci are frequently found on the skin and mucous membranes [5], binding to tissues via teichoic acids, hemagglutinin, fibronectin, and autolysins [47,48]. *Staphylococcus anaerobius* is a pathogen of sheep that causes skin abscess, but only rarely has it produced abscesses or sepsis in humans [49].

Cell structure

The staphylococcal cell envelope is a complex structure that consists of a cell membrane composed of lipids and proteins, a cell wall made from peptidoglycan and teichoic acids, and polysaccharides. As with all cell membranes, its integrity is crucial for maintaining a boundary between the external environment and the cytoplasm. The membrane also contains a large number of proteins that transport solutes across chemical gradients, expending ATP or membrane potential. The electron transport machinery (NADH oxidase, cytochromes, and F_0F_1-ATPase) is also localized to the cell membrane, producing ATP and establishing the electrochemical gradient across the membrane that powers a multitude of activities. The cell wall contains the high osmotic pressure of the cytoplasm of staphylococci.

Membrane

The bacterial membrane is a lipid bilayer where the inner and outer leaflets contain asymmetrically placed lipids [50]. While the basic lipid components of the staphylococcal membrane were defined several decades ago [51] and found to change during different phases of growth [52], the ability of the membrane to respond to environmental, host defense-related, and antimicrobial challenges has only recently been appreciated.

The membrane phospholipids of *S. aureus* include phosphatidylglycerol, lysyl-phosphatidylglycerol, phosphatidic acid, cardiolipin, and traces of phosphatidylethanolamine and phosphatidylglucose [51–53]. These phospholipids,

together with the carotenoids, menaquinone, and the glucolipids (monoglucosyldiglyceride and diglucosyldiglyceride), make up the major components of the staphylococcal membrane [52]. The phospholipids total about 60 μmol, the carotenoids 0.1 μmol, the vitamin K_2 isoprenologues 0.2 μmol, and the glucolipids 10 μmol per gram dry weight in exponentially growing cells [52]. *Staphylococcus aureus* accumulates cardiolipin and loses phosphatidylglycerol during the stationary phase of growth. The minor lipids, phosphatidylethanolamine and phosphatidylglucose, also accumulate, whereas the lysyl-phosphatidylglycerol content of the membrane remains constant during the stationary phase [51].

Because optimal membrane fluidity must be maintained for cell function, staphylococci must adapt to changes in environmental conditions by altering their membrane fluidity [54,55]. Adjustments in membrane fluidity, effected by changes in lipid composition, must occur in response to variations in temperature, pressure, ion concentrations, pH, nutrient availability, and xenobiotics. In many bacterial species membrane fluidity is greatly increased by introducing unsaturated fatty acids into the lipid bilayer, which disrupts packing of the lipids. However, this strategy is not utilized by *S. aureus*, which employs anteiso-branching of the fatty acids to increase fluidity in response to conditions such as high salt concentrations [44], and increases iso-branched fatty acids and carotenoids to decrease fluidity. The production of branched-chain fatty acids is regulated in part by the two-component regulatory system YycFG [56].

Some examples of changes in membrane lipids in response to environmental challenges are described here. Cardiolipin content in the membrane increases when *S. aureus* is challenged with cell wall-active antibiotics [57]. Cardiolipin is synthesized from two phosphatidylglycerol molecules [51], and it helps to place a cap on the extracellular face of the membrane to prevent protons from slipping through the bilayer [58]. Cardiolipin concentrates around the F_0F_1-ATPase, which may help to direct protons into this ATP-forming complex [58,59]. The carotenoid pigments of *S. aureus* are optimally produced when the bacteria are held at room temperature rather than 37°C [60]. In *S. aureus*, these pigments are triterpenoid carotenoids possessing a C_{30} chain, whereas most other bacterial carotenoids are C_{40} [61,62]. These pigments have multiple functions, including protection from ultraviolet light [63], resistance to detergents [54,64], stabilization of more fluid membranes [54,64,65], increased tolerance to thermal challenge [66], and protection from oxidant challenge [67]. The apparently contradictory responses, i.e., increased production of carotenoids at room temperature, yet increased protection from thermal stress probably relates to their role in stabilizing the membrane. Under cold conditions, carotenoids stabilize the membrane that has become much more fluid due to changes in lipid composition. In contrast, heating disorganizes the membrane, so stabilization will again be advantageous. Carotenoid pigment biosynthesis is part of the σ^B regulon [63], and it probably accounts for many of the protective stress responses that are induced by σ^B. The function of carotenoids and membrane fluidity extends beyond stress survival; decreased pigmentation is also associated with increased toxic shock syndrome toxin-1 production [68].

Altering the chemical composition of the membrane not only changes membrane fluidity but also the surface charge of the membrane. For example, addition of L-lysine to phosphatidylglycerol can confer a positive surface charge, e.g., lysyl-dipalmitoylphosphatidylglycerol (lysyl-DPPG) [69]. Similarly, modifying teichoic acid by adding D-alanine can decrease negative charge [69]. Each of these changes decreases the negative charge on the surface of the bacteria, which reduces the ability of cationic peptides and positively charged antimicrobial agents such as gentamicin and calcium carrying daptomycin to bind to *S. aureus*, making it more resistant to these challenges [70].

For bacteria to maintain the chemical (ΔpH) and electrical (ΔΨ) components of the proton motive force, the membrane of bacteria must act as a barrier to the movement of hydrogen ions [71]. Therefore, increased membrane fluidity, as caused by warmer temperatures, decreases packing of the lipids in the membrane [72], which allows protons to flow through the disordered lipids of the membrane [73–75]. Cardiolipin acts as a barrier to proton flux across the membrane and it often surrounds the F_0F_1-ATPase, which enhances ATP production since protons do not leak back into the cytoplasm without producing ATP [76,77].

Cell wall

Peptidoglycan: structure, synthesis, autolysis, and cell division

Peptidoglycan, also called murein, is a large macromolecule that is cross-linked into a three-dimensional structure forming a rigid cell wall. It is composed of alternating β-1,4-linked amino sugars composed of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) that are cross-linked by short peptide chains usually composed of tetrapeptides of L-alanine, D-isoglutamine, L-lysine, and D-alanine [78,79]. These short peptides are linked via an amide bond to the D-lactyl moiety of MurNAc [80–82]. The peptides are then cross-linked in a second dimension by interpeptide bridges consisting of penta- or hexa-glycine peptides extending from the carboxy-terminal D-alanine to the ε-amino group of the L-lysine in position 3 of the adjacent peptide. The glycan strands and the cross-linked peptides create a three-dimensional macromolecule with various cross-links that give the cell wall rigid structure [82,83]. The biosynthetic pathway is shown in Figure 1.2.

Peptidoglycan biosynthesis occurs in five stages. The first stage involves the biosynthesis of MurNAc and the addition of a pentapeptide to the MurNAc, which uses

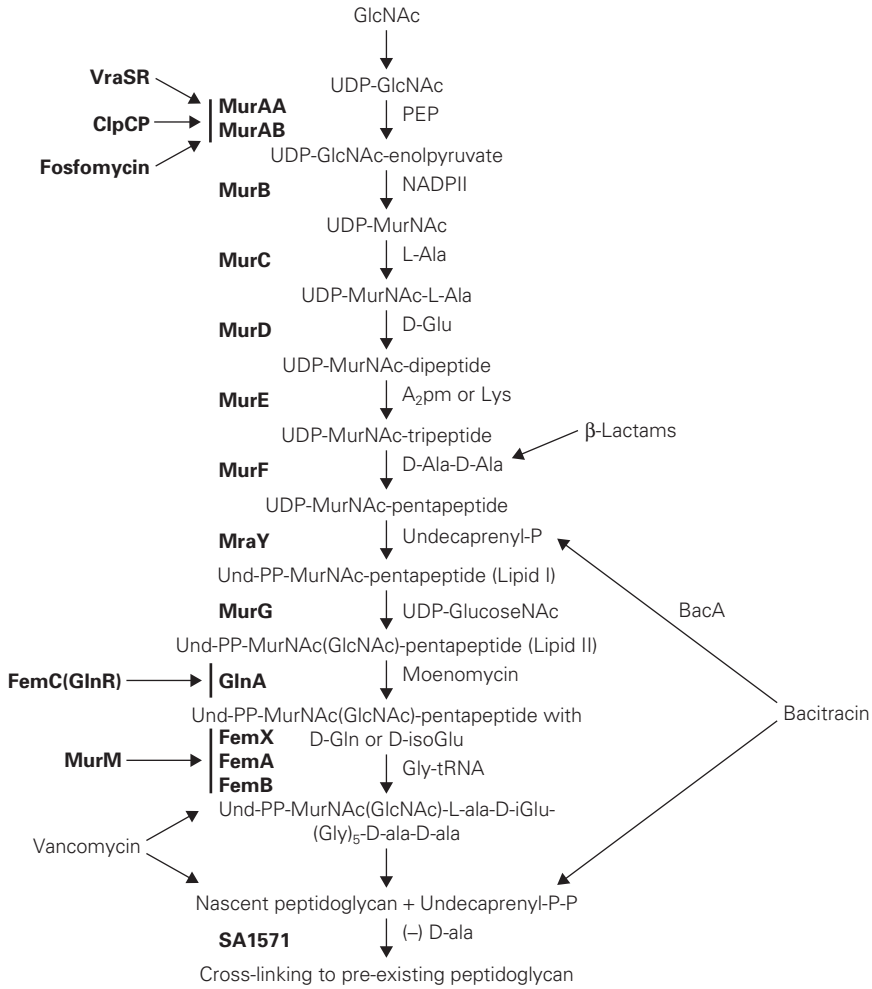

Figure 1.2 The biochemical pathway leading to peptidoglycan synthesis. Enzymes that catalyze each step are shown in bold. Antimicrobial agents that inhibit different steps are indicated.

the enzymes MurAA, MurAB, MurB, MurC, MurD, MurE, and MurF. In the second stage, an undecaprenyl acyl chain is added by MraY forming Lipid I, as the macromolecule is localized to the membrane. Next, GlcNAc is added by MurG to form the disaccharide MurNAc-GlcNAc, called lipid II. In the fourth stage, a pentaglycine peptide is added to the ε-amino group of the L-lysine by FemX, FemA, and FemB [84]. The fifth step involves release of the undecaprenyl group, movement of the nascent peptidoglycan to the outside of the cell, removal of the terminal D-alanine, and cross-linking to the preexisting peptidoglycan via penicillin-binding proteins (PBPs). Variations can occur in the initial tetrapeptide attached to the MurNAc such that the isoglutamate residue can be a glutamine or a meso-2,6-diaminopimeloyl (DAP). Of note, DAP is added with very low efficiency and does not allow the Fem factors to work properly. This results in increased susceptibility of MRSA strains to methicillin.

Lipids I and II

Lipid I (undecaprenylphosphate-MurNAc-pentapeptide) is formed in the first lipid-linked step of cell wall synthesis by MraY, which transfers the soluble UDP-MurNAc-pentapeptide to the lipid carrier undecaprenylphosphate (C55-P). The translocase MurG subsequently links UDP-activated *N*-acetylglucosamine (UDP-GlcNAc) to the muramoyl moiety of lipid I, thus yielding lipid II (undecaprenylphosphate-GlcNAc-MurNAc-pentapeptide) [85]. Recently, farnesol has been reported to increase β-lactam susceptibility of MRSA by inhibition of cell wall biosynthesis through reduction of free C55 lipid carrier with subsequent retardation of murein monomer precursor transport across the cell membrane [86] but it had no effect on PBP2′, which is the altered PBP in MRSA. In addition, it completely suppressed staphyloxanthin production [86]. The D-glutamyl residue in lipid II is converted to D-iso-Glu or D-Gln by GlnA (FemC), which is also needed for methicillin resistance [87].

Pentaglycine bridge

The pentaglycine bridge is synthesized by three enzymes, FemX (*fmhB*), FemA, and FemB, that add the first, second and third, and fourth and fifth glycines, respectively [88]. The pentaglycine interpeptide bridge links the ε-amino group of the L-lysine of one peptidoglycan chain to the D-alanine in position 4 of a neighboring chain [88].

Penicillin-binding proteins

PBPs are DD-transpeptidases that catalyze the last step of peptidoglycan biosynthesis [89]. This occurs on the external surface of the cytoplasmic membrane [89]. PBPs catalyze both the transglycosylation and the transpeptidation reactions required for assembly from the disaccharide oligopeptide precursors, leading to incorporation of the lipid-linked peptidoglycan precursors into the growing cell wall. PBPs probably function in multienzyme complexes that combine peptidoglycan synthetases and hydrolases into a macromolecular peptidoglycan synthesizing complex [90,91]. *S. aureus* has only one mode of cell wall synthesis, which takes place at the septum [92].

The PBPs of *S. aureus* are of particular importance because MRSA strains have acquired resistance to all β-lactam antibiotics due to a modified PBP2 called PBP2a (see Chapter 8 for a more detailed discussion), which is capable of transpeptidation even in the presence of high concentrations of antibiotic because of its low affinity for β-lactam antibiotics [93,94]. Nevertheless, PBP2 is also required for the full expression of methicillin resistance [95]. In the presence of high concentrations of methicillin, PBP2a is responsible for the transpeptidation of peptidoglycan, but the transglycosylase function of PBP2 is still required for the expression of resistance, suggesting cooperation between these PBPs [96,97]. The β-lactam antibiotics bind and inactivate the transpeptidase domain of PBPs in an irreversible manner [98] by preventing the acylated protein from binding to its substrate, the D-Ala-D-Ala terminus of peptidoglycan muropeptides. PBP2 is normally localized at the division septum, the primary location of cell wall synthesis in *S. aureus*; however, oxacillin causes PBP2 to become dispersed over the entire surface of the cell [92].

Once the cell wall has been formed, it must be broken to allow cell division. The cell division plane is formed by FtsZ, a protein that directs autolysins to a localized site in the cell wall as well as PBP2 for new cell wall formation [95]. Atl in *S. aureus* and AtlE in *S. epidermidis* are the major autolysins in these organisms and possess both amidase activity (targets the amide bond between MurNAc and L-alanine) and glucosaminidase activities [99]. How these activities are regulated in staphylococci is an area of active research.

Teichoic acids

Low guanosine plus cytosine (GC) organisms like staphylococci produce lipoglycans with phosphodiester bonds in repeating units called teichoic acids [100]. Teichoic acids are polyanionic molecules [80,100,101] that can be subdivided into wall teichoic acids (WTAs), which are covalently linked to the peptidoglycan, and lipoteichoic acids (LTAs), which are anchored in the outer leaflet of the cytoplasmic membrane via a glycolipid [100,101]. Both LTAs and WTAs extend to the bacterial surface and alter the surface properties of the organisms. Most staphylococci produce both types of teichoic acids (Figure 1.3).

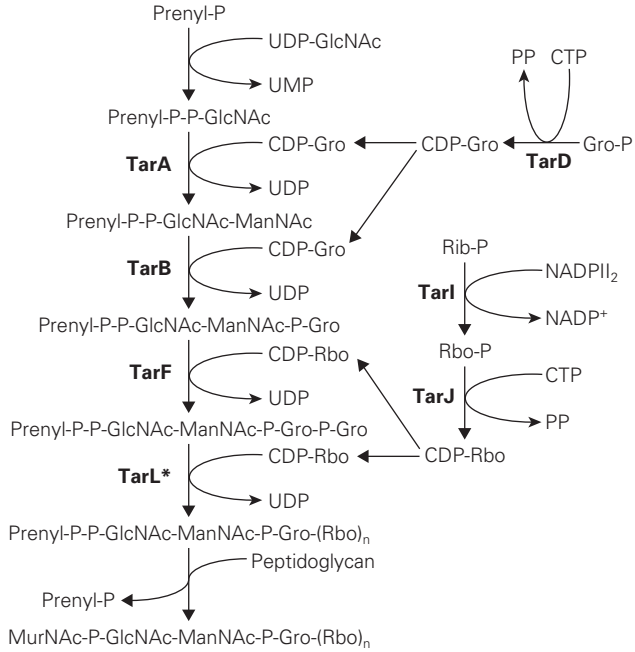

Figure 1.3 The biochemical pathway leading to teichoic acid synthesis. Enzymes that catalyze each step are shown in bold. * Details of the last step are still under investigation, but *S. aureus* appears to lack TarK and use TarL instead.

WTA mutants have been generated [32,102,103]; however, LTA mutations have proven to be lethal [104]. One reason why mutations in LTA may be lethal is that LTA affects cell division [104] and autolysin activity [105].

In staphylococci and many bacilli, LTA is composed of poly(glycerolphosphate) ($[Gro\text{-}P]_n$) attached to the glycolipid anchor β-D-$Glcp^{II}$-(1→6)-β-D-$Glcp^{I}$-(1→3)-diacylglycerol (DGlcDAG) [106]. Recently, several genes in the biosynthetic pathway of LTA have been identified. YpfP is a glycolipid synthase that mediates DGlcDAG in *S. aureus* [107]. Translocation of DGlcDAG from the inner to the outer leaflet is probably accomplished by LtaA [104]. The LTA is polymerized by LtaS [108]. A deletion in YpfP markedly reduced LTA content in *S. aureus*, but there was no change in WTA content [109].

LTA has a function in the regulation of autolysin activity, surface hydrophobicity, and biofilm formation [107]. (–)-Epicatechin gallate (ECg), a component of green tea, sensitizes MRSA to β-lactam antibiotics, promotes staphylococcal cell aggregation, and increases cell wall thickness by reducing peptidoglycan cross-linking [110]. ECg promotes LTA release from the cytoplasmic membrane, but it does not alter PBP2a expression, even though it reduces Triton X-100 autolysis, suggesting that ECg effects focus on loss of the LTA-inhibitory activity against the autolysin.

Exopolysaccharides

Biosynthesis and regulation of polysaccharide intercellular adhesin

In *S. epidermidis*, and to a lesser degree in *S. aureus*, the synthesis of polysaccharide intercellular adhesin (PIA) is an important process for medical device-associated infections [111,112], is required for the maturation of biofilms (for further review see Chapter 5) [113], and is important for evasion of the host innate immune system [114,115]. PIA is a β-(1,6)-linked GlcNAc polymer [116] of at least 130 residues [117] whose synthesis requires the enzymes encoded within the intercellular adhesin (*ica*) operon (*icaADBC*) [113]. IcaA and IcaD are thought to form a heterodimeric, cell membrane-associated, UDP-GlcNAc transferase [118]. IcaC is a membrane-associated protein hypothesized to be involved in the formation of long polymers and the subsequent export of the growing PIA chain [118]. IcaB catalyzes the deacetylation of poly-GlcNAc [114]. Of importance in the synthesis of PIA is the availability of GlcNAc.

Regulation of the *icaADBC* operon is complex, involving at least two DNA-binding proteins (IcaR and SarA), the alternative sigma factor σ^B, and the *luxS* quorum-sensing system. IcaR is a transcriptional repressor that binds immediately 5′ to the *icaA* transcriptional start site of the *ica* operon [119,120]. SarA is a positive regulator of *ica* transcription that binds to the *icaA* promoter region [121,122]. σ^B affects PIA synthesis indirectly by regulating the expression of *icaR* [123–125]. The *luxS* quorum-sensing system negatively regulates PIA synthesis and biofilm formation; however, the mechanism of regulation remains to be determined [126]. In addition to these regulatory elements, staphylococci regulate the synthesis of PIA and the formation of biofilms in response to nutrient availability, oxygen tension, and a variety of stress factors [127–131]. Interestingly, many of the same environmental and stress factors that affect PIA synthesis also alter tricarboxylic acid (TCA) cycle activity, leading to the hypothesis that PIA synthesis is linked to TCA cycle activity and that regulatory proteins capable of responding to TCA cycle-associated signals control this process [132].

Capsule biosynthesis and regulation

As stated earlier in this chapter, the most commonly encountered *S. aureus* capsule types are 5 and 8 [12]. Both capsule types 5 and 8 are polymers of *N*-acetyl-D-fucosamine, *N*-acetyl-L-fucosamine and *N*-acetyl-D-mannosaminuronic acid (discussed in a later chapter of this book). The biosynthetic precursor of the three capsule sugars is UDP-GlcNAc, the same amino sugar used in synthesizing PIA. GlcNAc is synthesized from the glycolytic intermediate fructose 6-phosphate, and in a rich medium containing glucose abundant levels of fructose 6-phosphate will be generated by the glycolytic (Embden–Meyerhof–Parnas) pathway; however, capsule is most abundantly synthesized in the postexponential phase of growth when glucose is growth limiting [133]. In the absence of glucose, fructose 6-phosphate can be synthesized by gluconeogenesis from the TCA cycle intermediate oxaloacetate. The first step of gluconeogenesis is ATP-dependent decarboxylation and phosphorylation of oxaloacetate by phosphoenolpyruvate carboxykinase (*pckA*) to generate phosphoenolpyruvate (PEP) [134]. Gluconeogenesis can then generate fructose 6-phosphate from PEP, which can be used for UDP-activated GlcNAc biosynthesis. Support for the idea that postexponential capsule biosynthesis requires TCA cycle activity and phosphoenolpyruvate carboxykinase can be found in the observation that capsule is made during aerobic growth [135]. In addition, inactivation of phosphoenolpyruvate carboxykinase (*pckA*), the TCA cycle (*citB*, *citC*, *citG*, or *odhA*), or GlcNAc biosynthesis (*glm*M) reduces killing in a *Caenorhabditis elegans* killing assay to the same extent as do mutations in capsule genes [136]. These data suggest that regulators of TCA cycle activity, such as CodY, are likely to regulate capsule biosynthesis.

Metabolism

Amino acid requirements for staphylococcal growth

Numerous phenotypic studies of *S. aureus* have attempted to determine the amino acid requirements for growth [137–142]. These studied revealed that *S. aureus* required between three and twelve amino acids for growth, with proline, arginine, valine, and cysteine being most frequently required; hence, these studies concluded that *S. aureus* frequently possessed multiple amino acid auxotrophies. Interestingly, it was also observed that these auxotrophies would revert to a prototrophic state at a high frequency, suggesting the auxotrophies were not due to the absence, or genetic inactivation, of biosynthetic pathways [137,143]. This suggestion was subsequently confirmed by whole-genome sequencing of *S. aureus*, where it was determined that biosynthetic pathways exist for all amino acids [144–149]. If the amino acid auxotrophies are not due to inactive or absent biosynthetic pathways, then the two more likely explanations are feedback inhibition of the biosynthetic enzymes or repression of enzyme synthesis. Although the former possibility is the more transient phenomenon, both possibilities should be quickly reversed by growth in culture medium lacking the inhibitor/amino acid; however, it was observed that reversal of *S. aureus* auxotrophies could require several passages of the bacteria in medium lacking the specific amino acid [137,143]. These observations imply that metabolic regulation in staphylococci can be unconventional, or at least divergent from that of *Escherichia coli* and *Bacillus subtilis*, and that we need to look elsewhere for the cause of the amino acid auxotrophies. One possibility is that feedback inhibition or repression of

Embden–Meyerhof–Parnas	Pentose phosphate pathway	Tricarboxylic acid cycle
Glucose 6-phosphate	Ribose 5-phosphate	Oxaloacetate
Fructose 6-phosphate	Sedoheptulose 7-phosphate	α-Ketoglutarate
Dihydroxyacetone phosphate	Erythrose 4-phosphate	Succinate
Glyceraldehyde 3-phosphate		
Phosphoenolpyruvate		
Pyruvate		
Acetyl-Coenzyme A		

Table 1.1 Metabolic pathways and biosynthetic intermediates.

enzyme synthesis could occur at an earlier step in amino acid biosynthesis; specifically, decreasing the availability of amino acid biosynthetic precursors.

Bacteria live in environments subject to rapid changes in the availability of the nutrients necessary to provide energy and biosynthetic intermediates for the synthesis of macromolecules; hence, a large percentage of the bacterial genome codes for proteins involved in metabolism and macromolecular synthesis [144,145,148–150]. Despite the large percentage of the genome dedicated to metabolism, bacteria require only 13 biosynthetic intermediates (Table 1.1) to synthesize all macromolecules in the cell. These 13 biosynthetic intermediates are derived from three metabolic pathways: the glycolytic pathway (Embden–Meyerhof–Parnas pathway), the pentose phosphate pathway (hexose monophosphate shunt), and the TCA cycle (Krebs cycle). While the glycolytic and pentose phosphate pathways are highly conserved throughout nature, the TCA cycle pathway is often found to be incomplete [151,152]. Both *S. aureus* and *S. epidermidis* possess complete glycolytic, pentose phosphate, and TCA cycle pathways; however, the TCA cycle lacks the glyoxylate shunt (discussed later in this chapter). The presence of these three pathways indicates that staphylococci can synthesize all the biosynthetic precursors necessary to make all macromolecules. In addition to being present in staphylococci, many of the genes encoding enzymes for the glycolytic and pentose phosphate pathways are essential for viability [153]; hence these data suggest that the amino acid auxotrophies are unlikely to be due to the absence of biosynthetic precursors generated by the glycolytic and pentose phosphate pathways. In contrast, the TCA cycle, which supplies the biosynthetic intermediates oxaloacetate, succinate, and α-ketoglutarate, can be genetically inactivated [136,148, 154,155] and "naturally occurring" TCA cycle mutants have been reported in *S. aureus* [152,156].

Biosynthetic intermediates and carbon catabolism

In staphylococci, the primary pathways for carbohydrate catabolism are the glycolytic pathway and pentose phosphate pathway [157,158]. The processing of glucose, in the form of glucose 6-phosphate, by the pentose phosphate pathway produces the biosynthetic intermediates ribose 5-phosphate, sedoheptulose 7-phosphate, and erythrose 4-phosphate and can also generate the glycolytic intermediates fructose 6-phosphate and glyceraldehyde 3-phosphate. Glycolysis produces two molecules of pyruvate for every molecule of glucose consumed and in the process reduces two molecules of NAD^+ to NADH. The catabolic fate of pyruvate is determined by the growth conditions; specifically, the availability of oxygen. During anaerobic growth, pyruvate is primarily reduced to lactic acid [159,160] with the concomitant reoxidation of NADH, thus allowing the continuation of glycolysis. During aerobic growth, pyruvate is enzymatically oxidized to acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex [161]. Acetyl-CoA can be further oxidized by the TCA cycle when grown in the presence of certain citric acid cycle intermediates [162]; however, the amount of acetyl-CoA that enters the TCA cycle is low during the exponential growth phase [157,158,163]. In the exponential phase of growth, acetyl-CoA is usually converted into acetyl-phosphate by phosphotransacetylase, which is then used as a substrate for acetate kinase in substrate-level phosphorylation to generate ATP and acetate. The exponential phase of growth continues until the concentration of an essential nutrient (e.g., glucose, as shown in Figure 1.4) decreases to a level where it can no longer sustain rapid growth. Alternatively, entry into the postexponential growth phase can be caused by the accumulation of growth inhibitory molecules (e.g., lactic acid). If conditions are favorable, then entry into postexponential growth corresponds with the catabolism of nonpreferred carbon sources such as acetate [155].

On depletion of a preferred carbon source such as glucose, the postexponential growth of staphylococci will depend on the growth conditions, principally the availability of oxygen. Microaerobic or anaerobic growth will derepress the arginine deiminase pathway (*arcABDC*), generating energy via substrate-level phosphorylation using carbamoyl-phosphate as the phospho-donor [164]. Additionally, derepression of the histidine utilization pathway (*hutUI*, *hutG*, and *hutH*) will increase histidine catabolism, providing a source of glutamate [165]. If the culture is sufficiently anoxic and nitrate is present, then

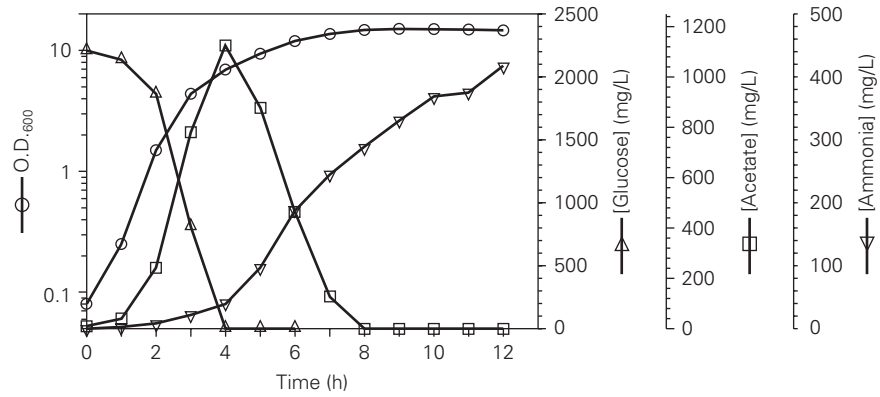

Figure 1.4 Temporal depletion and accumulation of ammonia, glucose, and acetate in *S. aureus* supernatants grown under aerobic conditions.

nitrate reductase-catalyzed nitrate reduction will maintain the proton motive force and generate ATP via anaerobic respiration [166,167]. Maintaining the proton motive force will also facilitate the oxidation of lactic acid into pyruvate by the NAD-independent lactate dehydrogenase, thus providing an important biosynthetic intermediate [168]. As stated, during aerobic growth, pyruvate undergoes oxidative decarboxylation to produce acetyl-CoA [161], which is converted into acetyl-phosphate and used for substrate-level phosphorylation, resulting in the accumulation of acetate in the culture medium (Figure 1.4). If oxygen is present, then the transition from exponential growth to postexponential growth is accompanied by a diauxic shift from glucose catabolism, or other rapidly catabolized carbohydrate, to acetate catabolism.

The first step in the catabolism of acetate is the ATP-dependent formation of a thioester bond between acetate and coenzyme A catalyzed by acetyl-CoA synthetase. The second step in acetate catabolism is the citrate synthase-catalyzed condensation of acetyl-CoA and oxaloacetate. Citrate synthase uses the energy of thioester hydrolysis to drive carbon–carbon bond formation to generate citric acid and nonesterified coenzyme A (CoASH). Citric acid is then catabolized by the TCA cycle; hence acetate catabolism is mediated by the TCA cycle [160,162,169]. In some bacteria, the glyoxylate shunt acts as a carbon salvage pathway that bypasses the two oxidative decarboxylation reactions catalyzed by isocitrate dehydrogenase and the α-ketoglutarate dehydrogenase complex. Because staphylococci lack a glyoxylate shunt, two carbons are lost as CO_2 for every two carbons (i.e., acetate) that enter the TCA cycle; for this reason, if carbons are withdrawn from the TCA cycle for biosynthesis, then anaplerotic reactions are required to maintain TCA cycle function. The substrates used in the anaplerotic reactions are most often amino acids (e.g., aspartate → oxaloacetate; glutamate → α-ketoglutarate; alanine → pyruvate → oxaloacetate), which require deamination prior to entry into the TCA cycle. The deamination of amino acids will result in the accumulation of ammonia in the culture medium; consequently, as TCA cycle activity increases during the postexponential growth phase, the rate of ammonia accumulation increases (Figure 1.4) [155]. In total, catabolism of acetate through the TCA cycle provides biosynthetic intermediates (α-ketoglutarate, succinyl-CoA, and oxaloacetate), ATP, and reducing potential, but consumes amino acids in the process.

The majority of ATP arising from TCA cycle activity is derived from the oxidation of NADH via the electron transport chain. During aerobic growth, electrons enter into the electron transport chain from NADH and are transferred to menaquinone by the NADH dehydrogenase complex [170]. Menaquinone transfers the electrons to oxidized cytochrome *c*, generating the reduced form of cytochrome *c*. The electrons are then transferred to oxygen by cytochrome *c* oxidase, generating water and driving protons across the membrane to produce a pH and electrochemical gradient. Protons return to the cytoplasm by way of the F_0 subunit of the F_0F_1-ATP synthase complex and drive the formation of ATP from inorganic phosphate, ADP, and free energy.

Amino acid auxotrophies and the TCA cycle

As previously stated, the biosynthesis of amino acids requires one or more precursors of pyruvate, acetyl-CoA, 3-phosphoglycerate, oxaloacetate, α-ketoglutarate, or succinyl-CoA. Pyruvate, acetyl-CoA, and 3-phosphoglycerate are derived from the glycolytic pathway, while the latter three molecules are intermediates of the TCA cycle (see Table 1.1). The glycolytic pathway is highly conserved and essential in staphylococci [153]; therefore, it is very unlikely that amino acid auxotrophies could be due to the absence of pyruvate, acetyl-CoA, or 3-phosphoglycerate. The TCA cycle is not essential in staphylococci and mutants can be readily identified based on their inability to catabolize acetate [152,156]. In addition to being unable to catabolize acetate, TCA cycle defective strains display multiple amino acid auxotrophies [163,171]; however, these auxotrophies can often be masked by growth in complex media. Although it is likely that TCA cycle mutants account for some of the stable amino acid auxotrophic staphylococci [152], TCA cycle mutants would be an

unlikely cause of the transient amino acid auxotrophies [137,143] because TCA cycle mutants would require a suppressor mutation to restore prototrophy. An alternative to auxotrophy by mutation is prolonged repression of enzyme synthesis that limits biosynthetic intermediates. To understand this possibility, it is necessary to understand a little about the regulation of TCA cycle genes.

Synthesis of the first three enzymes of the TCA cycle is regulated by the availability of amino acids and carbohydrate(s) [161–163,172,173]. When amino acids and carbohydrates are exogenously available to staphylococci, TCA cycle activity is repressed [163]. Derepression of the TCA cycle occurs when the availability of exogenous amino acids and/or carbohydrates becomes growth limiting. In low-GC Gram-positive organisms like *S. aureus*, amino acid- and carbohydrate-dependent regulation of TCA cycle gene expression is largely mediated by the transcriptional regulators CodY and CcpA [174–180]. In addition to being regulated by the availability of amino acids and carbohydrates, TCA cycle genes are repressed by the two-component regulatory system SrhSR during anoxic growth [181,182]. In general, TCA cycle genes are repressed by a combination of amino acids, carbohydrates, and microaerobic/anaerobic conditions. Interestingly, carbohydrate-rich, amino acid replete, and microaerobic conditions were used to determine the length of time it took amino acid auxotrophic staphylococci to revert to a prototrophic state [137,138,143]. In other words, the staphylococci were grown under conditions that repress the major source of amino acid biosynthetic precursors at the same time researchers were trying to select for amino acid prototrophs. Although this explanation is unlikely to account for all the transient amino acid auxotrophies, it is a very plausible explanation for the prolonged repression of enzyme synthesis necessary to make biosynthetic intermediates.

Staphylococcal transport

In bacteria, there are four broad means by which molecules enter the cytoplasm: simple diffusion, secondary active transport (facilitated diffusion), ATP-dependent active transport, and group translocation.

Diffusion

Diffusion is the Brownian motion-driven movement of molecules from a higher concentration to a lower concentration. Bacteria gain oxygen and displace CO_2 by diffusion; however, a more important molecule that bacteria acquire or lose by diffusion is water. Sudden increases in the salt concentration of the culture medium or changes in the host environment can result in the rapid movement of water out through the cell membrane by osmosis, resulting in osmotic stress. Similarly, if the intracellular concentration of salt(s) dramatically increases relative to the external environment, then the diffusion of water into the bacterium can increase intracellular pressure causing the bacteria to burst. Diffusion-induced osmotic stress in *S. aureus* can alter the transport of other compounds, in particular the osmoprotectants glycine betaine, proline, and choline [13].

Secondary active transporters

Secondary active transporters allow transport of a molecule against a gradient by coupling it with the transport of another compound (e.g., Na, P_i, H^+, and ornithine) that flows with its gradient. One of the most commonly encountered members of the secondary active transporter family is the major facilitator superfamily transporters, which includes uniporters, antiporters, and symporters [183]. To continue the example of osmotic shock-induced transport of proline, proline enters the staphylococcal cytoplasm by one of two means: via a high-affinity major facilitator superfamily transporter known as the proline permease (PutP) [13,184,185] or via a low-affinity proline/betaine transporter like ProP [186]. In the case of the proline permease, PutP is a sodium/proline symporter, meaning that proline and sodium are transported together into the cytoplasm; thus for proline to be transported into the cytoplasm, a high extracellular sodium concentration is required [13,184]. From a clinical perspective, staphylococcal antiporters may have a greater impact on patient outcomes than do the symporters because of their involvement in antibiotic efflux from the cytoplasm [187]. Although the staphylococci contain several major facilitator superfamily antiporters, two well-studied examples are QacA, which is involved in multidrug resistance [188,189], and NorA, which mediates fluoroquinolone resistance [190]. As drug efflux-mediated antibiotic resistance mechanisms are covered more extensively in Chapter 9, they are not discussed further in this chapter. In addition to antibiotic efflux, staphylococcal antiporters have critical roles in nutrient acquisition.

Glycerophosphodiester phosphodiesterase (GlpQ) is a secreted protein [191] that can generate glycerol 3-phosphate by hydrolysis of deacylated phospholipids. This is important because glycerol 3-phosphate can be used to generate the biosynthetic intermediate dihydroxyacetone phosphate (see Table 1.1), via glycerol 3-phosphate dehydrogenase, which can be further metabolized to glyceraldehyde 3-phosphate by triosephosphate isomerase. Glycerol 3-phosphate dehydrogenase and triosephosphate isomerase are cytoplasmic proteins, meaning that glycerol 3-phosphate must be transported into the cytoplasm. To accomplish this, staphylococci utilize the GlpT antiporter [192]. GlpT uses the inorganic phosphate (P_i) gradient to drive the importation of glycerol 3-phosphate. In addition to glycerol 3-phosphate, antiporters import a wide array of solutes, including PEP, sugar-phosphates, and amino acids [183,193].

ATP-dependent active transport

ATP-dependent active transport utilizes the energy of ATP hydrolysis to overcome the electrochemical gradient and drive solute transport. ATP-dependent translocation is most often mediated by transporters belonging to the ATP-binding cassette (ABC) transporter superfamily. The basic structural unit of ABC transporters consists of two proteins containing transmembrane domains, which form the membrane channel, and a pair of cytoplasmic modules that bind and hydrolyze ATP to drive solute transport. In *S. aureus*, ABC transporters have been demonstrated to drive translocation of a wide array of solutes such as metal ions [194], heme [195], and oligopeptides [196] and are predicted to transport polyamines (e.g., spermine), maltose, and amino acids [197].

Group translocation

Group translocation, also known as the phosphotransferase system (PTS), is the process by which bacteria import different sugars into the cytoplasm using energy transferred from PEP [198]. PTS uses three enzymes to transfer the high-energy phosphate from PEP to the sugar being translocated: enzyme I (EI), enzyme II (EII), and a histidine-containing phosphocarrier protein (HPr) [198]. EI and HPr are soluble cytoplasmic proteins involved in the transport of all PTS transported sugars. In contrast to EI and HPr, EII components (domains EIIA, EIIB, and EIIC) are sugar-specific permeases; hence staphylococci contain multiple genes coding for EII components [147]. Transfer of the high-energy phosphate from PEP to a sugar starts with PEP transferring its phosphate to a histidine residue on EI, which is then transferred to a histidine residue on HPr. The phosphorylated HPr transfers the high-energy phosphate to EIIA, which then transfers it to the sugar. Transfer of phosphate from EIIA to the sugar has two primary consequences: first, the phosphorylated sugar is no longer recognized by the EII permease, preventing efflux of the sugar out of the cytoplasm; and second, the phosphorylated sugar is activated for catabolism. The exact number of sugars, sugar alcohols, and amino sugars transported by PTSs in staphylococci is not known; however, based on DNA sequence homology, the number is probably greater than 15 [147].

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